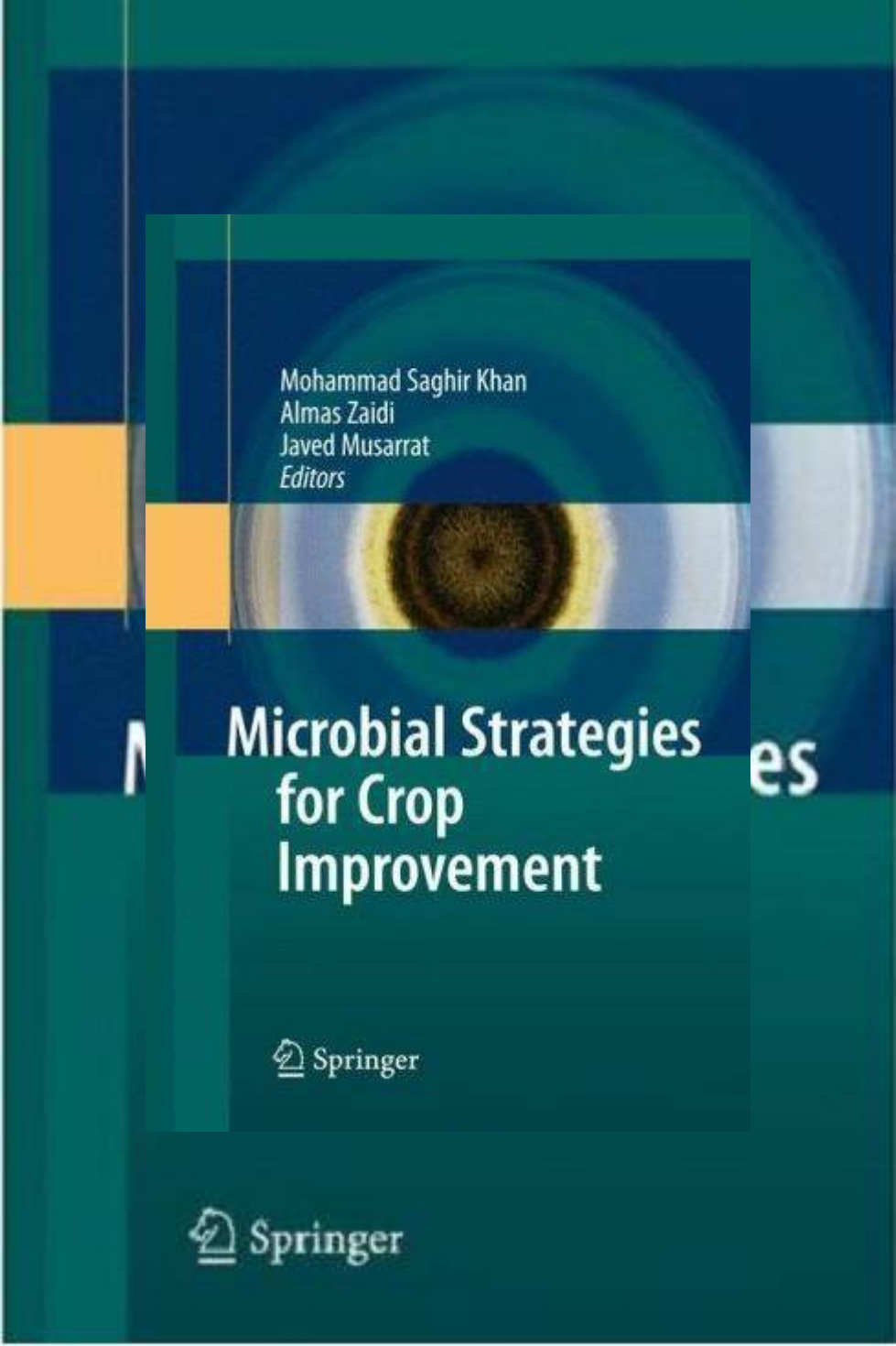

Mohammad Saghir Khan
Almas Zaidi
Javed Musarrat
Editors
Microbial Strategies for Crop Improvement
Springer
Springer

# Microbial Strategies for Crop Improvement

Mohammad Saghir Khan • Almas Zaidi •
Javed Musarrat
Editors

# Microbial Strategies for Crop Improvement

Springer

*Editors*
Dr. Mohammad Saghir Khan
Aligarh Muslim University
Faculty Agricultural Sciences
Dept. Agricultural Microbiology
Aligarh-202002
India
khanms17@rediffmail.com

Dr. Almas Zaidi
Aligarh Muslim University
Faculty Agricultural Sciences
Dept. Agricultural Microbiology
Aligarh-202002
India

Prof. Javed Musarrat
Aligarh Muslim University
Faculty Agricultural Sciences
Dept. Agricultural Microbiology
Aligarh-202002
India

ISBN 978-3-642-01978-4 e-ISBN 978-3-642-01979-1
DOI: 10.1007/978-3-642-01979-1
Springer Dordrecht Heidelberg London New York

Library of Congress Control Number: 2009928629



*Cover design:* WMXDesign GmbH, Heidelberg, Germany

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

# Foreword

With an ever-increasing human population, the demand placed upon the agriculture sector to supply more food is one of the greatest challenges for the agrarian community. In order to meet this challenge, environmentally unfriendly agrochemicals have played a key role in the green revolution and are even today commonly recommended to circumvent nutrient deficiencies of the soils. The use of agrochemicals is, though, a major factor for improvement of plant production; it causes a profound deteriorating effect on soil health (soil fertility) and in turn negatively affects the productivity and sustainability of crops. Concern over disturbance to the microbial diversity and consequently soil fertility (as these microbes are involved in biogeochemical processes), as well as economic constraints, have prompted fundamental and applied research to look for new agro-biotechnologies that can ensure competitive yields by providing sufficiently not only essential nutrients to the plants but also help to protect the health of soils by mitigating the toxic effects of certain pollutants. In this regard, the role of naturally abundant yet functionally fully unexplored microorganisms such as biofertilizers assume a special significance in the context of supplementing plant nutrients, cost and environmental impact under both conventional practices and derelict environments. Therefore, current developments in sustainability involve a rational exploitation of soil microbial communities and the use of inexpensive, though less bio-available, sources of plant nutrients, which may be made available to plants by microbially-mediated processes. These organisms affect plant growth directly by fixation of atmospheric nitrogen, solubilization of minerals such as phosphorus, or by production of plant growth regulators (phytohormones), or indirectly by improving growth-restricting conditions either via production of antagonistic substances, by inducing resistance against pathogens, and by improving soil properties by leaving organic residues. In addition, the rhizobacterial strains can improve plant stress tolerance to drought, salinity, and metal toxicity leading thereby to increased plant growth. Plant growth-promoting rhizobacteria also increase the growth of plants through the synthesis of specific enzymes, which induce physiological changes in plants. For example, ethylene plays a critical role in various developmental processes and regulates nod factor signaling and nodule formation. At higher concentrations, ethylene inhibits growth

and development of plants. However, bacterial enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase alleviates the stress induced by an ethylene-mediated impact on plants. Other organism of great practical significance to plant system is arbuscular mycorrhizal (AM) fungi, which play a major role in plant resource capture and nutrient cycling. Thus, microbial populations could participate in many key ecosystem processes such as those involved in the biogeochemical cycling of essential nutrients and biological control of plant pathogens, and, consequently, may affect plant development under different agro-ecosystems. The alternative approach to traditional biofertilizers could be the development of bacterial biofilms that may be used effectively as biofertilizers for various crops. Soil microbial communities play an important role in crop improvement in different agro-ecosystems by decomposing various organic matters, but due in part to the scarcity of convenient methods for exploration, our understanding of the different degrees and dynamics of microbial community including structure and functional diversity is so far limited. In addition, soil microbial communities are constantly under environmental pressure. Therefore, we need a better understanding of how different factors including plant genotypes, soil management practices, and agro-chemicals influence the microbial variations in soil environments. Furthermore, there is also a need to examine the rate of these responses to environmental changes and the factors that influence the rate of change and how these responses are related to community composition. Pathogenic microorganisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. To address such problems, pesticides are considered as a relatively reliable method of crop protection, but may instead cause several negative effects including development of pathogen resistance to the applied agents and their nontarget environmental impacts. Furthermore, there are also a number of fastidious diseases for which chemical solutions are few, ineffective, or nonexistent. Control of soil-borne pathogens by applying microbes or microbial products (biopesticides) provides a viable alternative way of reducing the use of chemicals in crop improvement.

Worldwide, salinity is one of the most important abiotic stresses that limit crop growth and productivity. Ion imbalance and hyper-osmotic stress in plants caused by high concentrations of salt often lead to oxidative stress conditions for plants. Soil salinization may be due to natural causes, and is common in the hot and dry regions of the world, or it may be a consequence of inadequate irrigation management practices. In this context, the use of microorganisms offers an attractive alternative to facilitate the growth of plants in otherwise inhibitory levels of salt. An understanding of these mechanisms is likely to lead to the development of simple and practical approaches to dealing with this problem in the field. Furthermore, in the Mediterranean basin, a millenarian history of overexploitation has led to the loss of most primeval forests and an increase of the surface area covered by shrublands that represent stages of degradation of mature forests. In this situation, environmental characteristics act as barriers to succession and, hence, human intervention by applying reforestation practice is usually necessary to improve recovery of woodlands. To address such problems, some native plant species could act as “nurse plants” through their positive impacts on soil abiotic

characteristics and also through positive influence on soil microbiota, especially on symbiotic microorganisms. The beneficial effects of these plant nurses on the growth of Mediterranean tree species, the soil bio-functioning, and the underlining of the benefits of using native plant species to rehabilitate degraded areas especially in stressful conditions are suggested. Release of heavy metals without proper treatment poses a serious threat to soil health because of their nonbiodegradability and persistence. The remediation of heavily contaminated soils involving microbes offers a promising and inexpensive approach to design strategies for crop improvement in soils contaminated with heavy metals. In some instances, indeed, the availability of metal-tolerant microbes with very specific mechanisms of action has contributed substantially to our understanding of plant improvement under stressed environments.

Despite the significant scientific developments, the role of microbial communities in productivity of crops and sustainability of environment is not fully exploited for the benefit of crop improvement under different agro-ecosystems around the world. The beneficial microbial systems involved in plant growth promotion are very often crop- and region-specific besides soil-specific in natural ecosystems. The advent of molecular and biotechnological tools has, however, made it possible to tailor the genetic traits of such microbes to make them suitable for a specific environment.

In recent years, the volume of research published on the contribution of microbes in crop improvement has increased dramatically. This can be attributed to several factors: in general, there has been an increasing interest in the application of inexpensive natural bio-resource, microbes, within the scientific and agrarian communities; there has been the advent of molecular engineering techniques which help to identify and design microbes with specific activities; and finally this has prompted industries to become much more involved in the exploitation of beneficial microbes at a commercial scale. Progress in this exciting and applied field of microbiology has been so pronounced that there has been a strong need for a book on this subject. Although the research in this area has been published in various scientific media, they are unlikely to provide sufficient information on the themes covered in this book. Given the large and growing body of information available on microbial effects in crop improvement, it is becoming difficult to maintain familiarity with the subject. University students and teachers, agronomists in particular and scientists in the private sectors in general, require access to this information. In this book "Microbial Strategies for Crop Improvement", we have attempted to meet such needs, and to integrate the relevant information on microbial strategies employed to improve crop productivity in different agro-ecological niches into a single volume. Broadly, this book addresses the multidisciplinary nature and many fascinating aspects of microbiological approaches for crop improvement in both conventional and stressed soils where quality and safety are the key concerns. The major goal of microbial strategies for crop improvement is intended to provide a cross-section of latest accomplishments and envisaged future directions in these areas. Furthermore, the information provided here gives a holistic view of the basic concepts and practical utility of microbes. We hope that

the reader will find this book a useful source of information on many aspects of crop improvement through microbial applications as well as a starting point for more advanced reading and research in this area. This compilation is expected to help students, teachers, industry regulators, and nonspecialist readers to get balanced and up-to-date information on potential application of microbes in crop improvement. This book is thus likely to benefit the people working in the area of agronomy, biotechnology, environmental biology, microbiology, plant physiology, plant protection, and soil science.

The contributions by eminent academicians and professionals for this volume have ensured a good equilibrium between theory and practice without compromising the basic conceptual framework of the concerned subject. Qualified editors and authors from different countries have tried to bring in quality and the most attractive presentation. This book will thus present an all-inclusive contemporary treatise on strategic aspects of the diverse microbial communities providing solutions to many of common agronomic problems and indeed some bizarre ones facing the agrarian communities. The book also provides an opportunity to the readers to understand the complexity of microbes and exploit these natural resources for the benefit of crops. This book will serve as an important and comprehensive source material because it includes recent data focusing mainly on the microbial strategies for improvement and sustainability of crops leading thereby to achieve global food security.

# Preface

The dramatic increase in the use of chemical fertilizers for achieving optimum yields has become an integral component of present day agricultural practices. The frequent and imbalanced application of such fertilizers is not only expensive but also pollutes the environment at a faster rate and makes soils unsuitable for cultivation. In addition, soil deterioration (soil salinization), disturbances in composition and functional properties of soil microbial communities and, consequently, loss of soil fertility following various soil management practices has further compounded the agronomic problems. To overcome such environmentally undesirable activities, we need to develop a viable substitute that could address those problems more effectively in a sustained manner. In this context, the microbiological strategies involving the use of functionally diverse groups of microbes as vital components of soil ecosystems provides an inexpensive alternative to chemical fertilizers. In recent times, much interest has been generated in exploiting microbial strategies to facilitate plant growth and development, and in some cases they have been commercialized for different crops. The majority of organic growers adopt these microbial technologies without proper knowledge and understanding of it, and use them to inoculate seeds/soils to provide nutrients like phosphate, nitrogen, and other phyto-compounds. In addition, microbes have also attracted worldwide attention due to their role in disease management and remediation of salinized/polluted soils. Thus, the microbial communities in general are the potential tools for sustainable production of crops and the trend for the future. Scientific researchers, however, involve multidisciplinary approaches to understand the complexity and practical utility of the wide spectrum of microbes for the benefits of crops. The success of crop improvement, however, depends largely on the performance of microbes and the willingness and acceptance by the growers. Substantial amounts of research work has been done to highlight the role of microbes in the improvement of crops, but very little attempt is made to organize such findings in a way that could substantially help students/teachers/scientists and to progressive farmers.

"Microbial Strategies for Crop Improvement" written by experts in the field provides a comprehensive source of information on strategies and concepts of microbial technology for the improvement of crops in different agro-ecosystems.

The book presents strategies for the management of salinized soils and crop diseases, and explores means of integrating various approaches to achieve desired levels of crop yield under both conventional and derelict soils. Traditional applications for molecular plant–microbe interactions in crop improvement (e.g., nitrogen fixation in legume–rhizobia symbioses and the improvement of plant P nutrition by arbuscular mycorrhizae) are broadly covered in the book. This book also presents a broad and updated view of the phosphate-solubilising microbes and role of metal-tolerant microbes in providing protection to plants grown in metal-contaminated soils. Thus, it provides a reference point that will be an invaluable resource for crop improvement. The preparation and application of microbial biofilm inoculants, the role of bacterial ACC deaminase in the development of functional symbiosis between rhizobia and legumes, and functional diversity among plant growth-promoting rhizobacteria are also discussed. The book further describes how the plant growth-promoting rhizobacteria facilitate plant growth and how advanced information strategies can be used to manipulate and modify the soil environment. Plant growth-promoting diazotrophs and productivity of wheat on the Canadian Prairies is discussed separately. The common mechanisms regulating symbiosis and development in biopesticides to control pathogenic microbes are discussed, making it possible to further examine their role in the crop improvement. The other factors affecting the crop productivity include the health of soil and how soil management practices affects the fertility of soils. Special attention is paid to highlight the role of a fertile soil in crop improvement and to understand the impact of various management practices on the variability of microbes both in terms of community structure and their function. This book is useful for a wide audience of students/researchers/practitioners specializing in different areas of microbiology and plant science, agrochemistry, soil biology, molecular biology, and other related disciplines.

We very sincerely wish to acknowledge our colleague authors who participated in this endeavor from different countries and who have assisted in the development of "Microbial Strategies for Crop Improvement" by providing the recent information and comprehensive scripts without which it would have been extremely difficult to complete this herculean task. Also, we want to thank our scientific colleagues who generously spared their valuable time to respond to our queries regarding the preparation of this book and who have informally made suggestions to us to improve the overall presentation of the book. We also wish to thank research scholars working with us, who have helped immensely in making this dream a reality. We also gratefully thank the publisher of this book.

We are grateful to our families for their support during the compilation of this book.

Finally, we believe this book may have some conceptual or printing mistakes arising unintentionally during preparation for which we apologise in anticipation and, if pointed out to us, will certainly correct in subsequent printings/editions. We also invite suggestions and healthy criticism from the readers of this book so that the information on the subject it contains can be improved in future.

India, March 2009

*Mohammad Saghir Khan*
*Almas Zaidi*
*Javed Musarrat*

# Contents

**1 The Use of Microorganisms to Facilitate the Growth of Plants in Saline Soils** ..... 1
Elisa Gamalero, Graziella Berta, and Bernard R. Glick

**2 Recent Advances in Plant Growth Promotion by Phosphate-Solubilizing Microbes** ..... 23
Almas Zaidi, Mohammad Saghir Khan, Munees Ahemad, Mohd Oves, and P.A. Wani

**3 Developing Beneficial Microbial Biofilms on Roots of Non legumes: A Novel Biofertilizing Technique** ..... 51
Gamini Seneviratne, RMMS Thilakaratne, APDA Jayasekara, KACN Seneviratne, KRE Padmathilake, and MSDL De Silva

**4 Role of 1-Aminocyclopropane-1-carboxylate deaminase in *Rhizobium*–Legume Symbiosis** ..... 63
Javed Musarrat, Abdulaziz A AlKhedhairy, Saud Al-Arifi, and Mohammad Saghir Khan

**5 Strategies for Crop Improvement in Contaminated Soils Using Metal-Tolerant Bioinoculants** ..... 85
Anju Rani and Reeta Goele

**6 Functional Diversity Among Plant Growth-Promoting Rhizobacteria: Current Status** ..... 105
Mohammad Saghir Khan, Almas Zaidi, P. A. Wani, Munees Ahemad, and Mohammad Oves

**7 Plant Growth Promoting Rhizobacteria and Sustainable Agriculture** ..... 133
Azeem Khalid, Muhammad Arshad, Baby Shaharoona, and Tariq Mahmood

**8 Soil Health – A Precondition for Crop Production** ..... 161
Niharendu Saha and Biswapati Mandal

**9 Recent Advances in Biopesticides** ..... 185
Parvez Qamar Rizvi, Rummana A. Choudhury, and Arshad Ali

**10 Benefits of Arbuscular Mycorrhizal Fungi to Sustainable Crop Production** ..... 205
M. Vosátka and J. Albrechtová

**11 Enhancement of Rhizobia–Legumes Symbioses and Nitrogen Fixation for Crops Productivity Improvement** ..... 227
Hamdi Hussein Zahran

**12 Monitoring the Development of Nurse Plant Species to Improve the Performances of Reforestation Programs in Mediterranean Areas** ..... 255
R. Duponn gois, M. Hafidi, J. Thioulouse, A. Galiana, L. Ouahmane, B. Dreyfus, and Y. Prin

**13 Pea Cultivation in Saline Soils: Influence of Nitrogen Nutrition** .... 267
Etelvina Figueira

**14 Plant Growth-Promoting Diazotrophs and Productivity of Wheat on the Canadian Prairies** ..... 287
Anthony O. Anyia, Daniel J. Archambault, Carols J. Bécquer, and Jan J. Slaski

**15 Factors Affecting the Variation of Microbial Communities in Different Agro-Ecosystems** ..... 301
Munees Ahemad, Almas Zaidi, Md Saghir Khan, and Mohammad Oves

**16 Strategies for Utilizing Arbuscular Mycorrhizal Fungi and Phosphate-Solubilizing Microorganisms for Enhanced Phosphate Uptake and Growth of Plants in the Soils of the Tropics** ..... 325
Nelson Walter Osorio and Mitiku Habte

**Index** ..... 353

# The Editors

*Mohammad Saghir Khan, Ph.D.* is an Associate Professor at the Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, India. Dr. Khan *received his* M.Sc. from Aligarh Muslim University, Aligarh, India and his Ph.D. (Microbiology) from Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, India. He has been teaching microbiology to postgraduate students for the last 13 years and has research experience of 17 years. In addition to teaching, Dr. Khan is engaged in guiding students for their doctoral degree in microbiology. He has published over 50 scientific papers including, original research articles, review articles, and book chapters in various national and international publication media. Dr. Khan has also edited a book published by one of the leading publishers. His interest in the various aspects and applications of microbiology coupled with his classroom and laboratory experience makes him uniquely qualified to author this book. He is deeply involved in research activities focusing mainly on rhizobiology, microbiology, environmental microbiology, especially, heavy metals–microbes–legume interaction, bioremediation, pesticide–PGPR–plant interaction, biofertilizers and rhizo-immunology.

*Almas Zaidi, received her* M.Sc. and Ph.D. (Agricultural Microbiology) from Aligarh Muslim University, Aligarh, India, and currently serving as Guest faculty/Assistant Professor at the Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh. She has been teaching microbiology at postgraduate level for the last 5 years and has research experience of 13 years. She has published about 40 research papers and reviews in journals of national and international repute. She has also contributed chapters to different books. Dr. Zaidi has also edited a book published by one of the leading publishers. Her main focus of research is to address problems related with rhizo-microbiology, microbiology, environmental microbiology, and biofertilizer technology.

*Javed Musarrat,* M.Sc., Ph.D. (Biochemistry), former Chairman of the Department of Agricultural Microbiology and Ex-Dean, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, India is presently working as a Professor, DNA Research Chair at the King Saud University, Riyadh. He has been teaching

biochemistry, microbiology, and molecular biology to postgraduate students for the last 21 years and has research experience of about 25 years. He has contributed more than 50 national and international scientific publications. Recently, Dr. Musarrat has edited a book published by one of the leading publishers. He is associated with several scientific bodies such as DBT, CSIR, UGC, ICAR, and UPCST, in various capacities. His major area of interest includes the molecular microbiology, microbial ecology, and genetic toxicology.

# Contributors

**Munees Ahemad**, Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, U.P., India

**Pervaze Ahmad Wani**, Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, U.P., India

**Abdulaziz A Al Khedhairy**, DNA Research Chair, College of Science, King Saud University, Riyadh, SA

**Saud Al-Arifi**, DNA Research Chair, College of Science, King Saud University, Riyadh, SA

**J. Albrechtová**, Charles University in Prague, Faculty of Science, Department of Plant Physiology, Vinicna 5, 128 44 Prague, Czech Republic

**Arshad Ali**, Department of Plant Protection, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh; Uttar Pradesh, India

**Anthony O. Anyia**, Alberta Research Council, Bioresource Technologies, P.O. Bag 4000, Vegreville, Alberta, Canada, T9C 1T4

**Daniel J. Archambault**, Laurentian University, 935 Ramsey Lake Road, Sudbury, Ontario, Canada, P3E 2C6

**Muhammad Arshad**, Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad 38040, Pakistan

**Carols J. Bécquer**, Ministerio de la Agricultura, Instituto de Ganadería Tropical, Estación Experimental Sancti-Spiritus, Cuba

**Graziella Berta**, Dipartimento di Scienze dell'Ambiente e della Vita, Università del Piemonte Orientale, "A. Avogadro", Alessandria, Italy

**Rummana A. Choudhury**, Department of Plant Protection, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh; Uttar Pradesh, India

**M.S.D.L. De Silva**, Tea Research Institute of Sri Lanka, Talawakele, Sri Lanka

**B. Dreyfus**, IRD. UMR 113. CIRAD/INRA/IRD/SUPAGRO/UM2. Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM). Montpellier, France

**R. Duponnois**, IRD. UMR 113. CIRAD/INRA/IRD/SUPAGRO/UM2. Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM). Montpellier. France

**Etelvina Figueira**, Centre for Cell Biology, Departamento de Biologia, Universidade e Aveiro, Campus, de Santiago, 3810-193 Aveiro, Portugal

**A. Galiana**, CIRAD. UMR 113 CIRAD/INRA/IRD/AGRO-M/UM2. Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM). Montpellier. France

**Elisa Gamalero**, Dipartimento di Scienze dell'Ambiente e della Vita, Università del Piemonte Orientale, "A. Avogadro", Alessandria, Italy

**Bernard R. Glick**, Department of Biology, University of Waterloo, Waterloo, Ontario, Canada

**Mitiku Habte**, University of Hawai'i at Manoa, College of Tropical Agriculture and Human Resources, St. John Room 102, 3190 Maile Way, Honolulu, HI, USA, 96822

**M. Hafidi**, Equipe Ecologie et Environnement, Faculté des Sciences Semlalia, Université Cadi Ayyad. Marrakech. Maroc

**Hamdi Hussein Zahran**, Botany Department, Faculty of Science, Beni-Suef University, Beni-Suef 62511, Egypt

**A.P.D.A. Jayasekara**, Tea Research Institute of Sri Lanka, Talawakele, Sri Lanka

**Azeem Khalid**, Department of Environmental Sciences, PMAS Arid Agriculture University, Rawalpindi 46300, Pakistan

**Tariq Mahmood**, Department of Environmental Sciences, PMAS Arid Agriculture University, Rawalpindi 46300, Pakistan

**Biswapati Mandal**, AICRP on Soil Test Crop Response Correlation, Directorate of Research, Bidhan Chandra Krishi Viswavidyalaya, Kalyani- 741235 Nadia, West Bengal, India

**Javed Musarrat**, Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, U.P., India; DNA Research Chair, College of Science, King Saud University, Riyadh, SA

**Nelson-Walter Osorio**, Universidad Nacional de Colombia at Medellin, College of Sciences, Soil Microbiology Research Group, Calle 59A No. 63-20, Medellin, Colombia

**L. Ouahmane**, Equipe Ecologie et Environnement, Faculté des Sciences Semlalia, Université Cadi Ayyad. Marrakech. Maroc

**Mohammad Oves**, Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, U.P., India

**K.R.E. Padmathilake**, Biological Nitrogen Fixation Project, Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka

**Y. Prin**, CIRAD. UMR 113 CIRAD/INRA/IRD/AGRO-M/UM2. Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM). Montpellier. France

**Parvez Qamar Rizvi**, Department of Plant Protection, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh; Uttar Pradesh, India

**Anju Rani**, Department of Microbiology, Govind Ballabh Pant University of Agriculture & Technology, Pantnagar, Uttaranchal – 263145, India

**Goel Reeta**, Department of Microbiology, Govind Ballabh Pant University of Agriculture & Technology, Pantnagar, Uttaranchal – 263145, India

**Mohammad Saghir Khan**, Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, U.P., India

**Niharendu Saha**, AICRP on Soil Test Crop Response Correlation, Directorate of Research, Bidhan Chandra Krishi Viswavidyalaya, Kalyani- 741235 Nadia, West Bengal, India

**Gamini Seneviratne**, Biological Nitrogen Fixation Project, Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka

**K.A.C.N. Seneviratne**, Royal Botanic Gardens, Peradeniya, Sri Lanka

**Baby Shaharoona**, Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad 38040, Pakistan

**Jan J. Slaski**, Alberta Research Council, Bioresource Technologies, P.O. Bag 4000, Vegreville, Alberta, Canada, T9C 1T4

**R.M.M.S. Thilakaratne**, Biological Nitrogen Fixation Project, Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka

**J. Thioulouse**, Université Lyon 1; CNRS; UMR 5558, Laboratoire de Biométrie et Biologie Evolutive, 43 boulevard du 11 novembre 1918, Villeurbanne F-69622, France

**M. Vosátka**, Institute of Botany, Academy of Sciences of the Czech Republic, 252 43 Pruhonice, Czech Republic

**Almas Zaidi**, Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, U.P., India

# Chapter 1
# The Use of Microorganisms to Facilitate the Growth of Plants in Saline Soils

**Elisa Gamalero, Graziella Berta, and Bernard R. Glick**

**Abstract** Worldwide, salinity is one of the most important abiotic stresses that limits crop growth and productivity. Ion imbalance and hyperosmotic stress in plants caused by high concentrations of salt often lead to oxidative stress conditions for plants. Soil salinization may be due to natural causes, and is common in the hot and dry regions of the world, or it may be a consequence of inadequate irrigation management practices. It has been estimated that around 20% of the world's cultivated lands and up to half of all irrigated lands are affected by high salinity. Moreover, at the present time, there is more arable land being lost through salinity than is gained through the clearing of forests. In this chapter, the ability of plant beneficial microorganisms, notably plant growth-promoting (PGP) bacteria and mycorrhizae, to facilitate plant growth in the presence of salt is reviewed and discussed. Particular attention is paid to the development of a fundamental understanding of precisely how these microorganisms enable plants to proliferate in the presence of otherwise inhibitory levels of salt. A better understanding of these mechanisms is likely to lead to the development of simple and practical approaches for dealing with this problem in the field.

## 1.1 Introduction

Salinity is an enormous worldwide problem for agriculture, especially for crops that are grown under irrigation. This is because salt is inhibitory to the growth of a large number of different plants (Cuartero and Fernandez-Munoz 1999). Moreover, the

E. Gamalero (✉) and G. Berta
Dipartimento di Scienze dell'Ambiente e della Vita, Università del Piemonte Orientale "A. Avogadro", Alessandria, Italy
e-mail: elisa.gamalero@unipmn.it
B.R. Glick
Department of Biology, University of Waterloo, Waterloo, ON Canada

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_1, 

amount of salt-affected land worldwide is estimated to be >900 million hectares which is approximately 6% of the total global land mass, or about 20% of the world's cultivated area (Flowers 2004). Importantly, around half of the land devoted to the growth of irrigated crops is adversely affected by salt.

Soil salinity inhibits plant growth and development with adverse effects such as osmotic stress, $Na^+$ and $Cl^-$ toxicity, ethylene production, plasmolysis, nutrient imbalance, production of reactive oxygen species (ROS), and interference with photosynthesis (Sairam and Tyagi 2004). The consequence of these physiological changes is an inhibition of seed germination, seedling growth and vigor, flowering and fruit set. Early responses of plants to drought and salinity are very similar; both are attributed to water stress. Water stress-induced metabolic processes include a decrease in photosynthesis, the production of ROS and generation of the plant hormone abscisic acid (Bartels and Sunkar 2005). When plants are exposed to high salinity, a decrease in the growth rate followed by a gradual recovery to a new reduced growth rate is the plant's first response to the decrease in water potential caused by salt, rather than to any salt-specific toxicity (Verslues et al. 2006). Once taken up, $Na^+$ may be translocated to shoots in the rapidly moving transpiration stream in the xylem (Smith et al. 1980). Although $Na^+$ can return to roots via the phloem, this flow is minimal so that leaves or shoots typically accumulate higher concentrations of $Na^+$ than roots (Tester and Davenport 2003). Moreover, most crops translocate little $Na^+$ to reproductive or storage structures such as seeds as these organs are fed mainly through the phloem. On the other hand, vegetative tissues are supplied mainly by the xylem flow and therefore tend to be higher in $Na^+$ levels. The metabolic toxicity of $Na^+$ is mainly attributed to the $Na^+$ competition with $K^+$ for binding sites essential for cellular functioning (Blaha et al. 2000; Tester and Davenport 2003).

Chlorine ion, an essential micronutrient for higher plants, is involved in the oxygen-evolving reactions of photosynthesis, maintaining electrical neutrality across membranes, and adjusting the vacuolative osmotic condition. Root cells take up $Cl^-$ from soil through anion channels; the $Cl^-$ then traverses the root by a symplastic pathway to reach the xylem. Like $Na^+$, floral tissues generally have lower $Cl^-$ levels than other shoot parts, and tissues that are fed predominantly through the phloem (e.g., fruits and seeds) tend to have the lowest $Cl^-$ concentrations (White and Broadley 2001). Salinity-induced stress in plants is partly the result of ethylene production (O'Donnell et al. 1996; Cuartero and Fernandez-Munoz 1999; Blumwald 2000; Mayak et al. 2004; Shibli et al. 2007). For instance, ethylene production was stimulated from two- to about tenfold in tomato (*Lycopersicon esculentum*) and *Arabidopsis* plants that were exposed to salinity stress (Richard and El-Abd 1989; Hall and Smith 1995). In this regard, in chickpea (*Cicer arietinum*), Kukreja et al. (2005) not only observed a salinity-induced increase in ethylene evolution, but also increases both in 1-aminocyclopropane-1-carboxylate (ACC) content, the immediate precursor of ethylene, and in the activity of the enzyme ACC oxidase. Furthermore, the relationship between salinity stress and ethylene production was consistent with the observation that aminoethoxyvinylglycine (AVG), a chemical inhibitor of ethylene biosynthesis, alleviated salinity-induced

plant responses such as increased hook closure and thickness of seedlings (El Beltagy et al. 1979).

Salt-tolerant plants may be grouped into two categories: salt-excluders and salt-includers. The former group of plants adapt to a saline environment by avoiding salt, whereas the includers take up salt and sequester it. Biochemical strategies to cope with salt stress include (1) selective accumulation or exclusion of ions, (2) control of ion uptake by roots and transport into leaves, (3) compartmentalization of ions at the cellular and whole-plant levels, (4) synthesis of compatible solutes, (5) alteration of membrane structure, (6) induction of antioxidative enzymes, and (7) induction of plant hormones (Khan and Rizvi 1994; Parida and Das 2005). However, it is important to bear in mind that the salt tolerance of any particular species is likely to vary with the growth phase of the plant, the ionic composition of the soil, and the overall health of the plant.

## 1.2 Mechanisms Used by Plant Growth-Promoting Bacteria

Plant beneficial bacteria are of two general types: those that form a symbiotic relationship, which involves formation of specialized structures or nodules on host plant roots, and those that are free-living in the soil; the latter are often found near, on, or even within the roots of plants (Kloepper et al. 1989; van Peer and Schippers 1989 Frommel et al. 1991). Beneficial free-living soil bacteria are often referred to as plant growth-promoting rhizobacteria (PGPR) and are found in association with the root surfaces of many different plants. However, to be inclusive of the many different types of bacteria that facilitate plant growth, the term plant growth-promoting bacteria (PGPB), is preferred (Bashan and Holguin 1998). Moreover, while numerous free-living soil bacteria are considered to be PGPB, not all bacterial strains of a particular genus and species have identical metabolic capabilities. Thus, for example, some *Pseudomonas putida* strains may actively promote plant growth while others have no measurable effect on plants.

PGPB can function either indirectly or directly (Glick 1995). Indirect promotion of plant growth occurs when bacteria decrease or prevent some of the deleterious effects of a phytopathogenic organism (a fungus or a bacterium) by any one or more of several different mechanisms. On the other hand, direct promotion of plant growth by PGPB generally entails providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment.

Direct promotion of plant growth can occur in several different ways. PGPB may fix atmospheric nitrogen and supply it to plants; synthesize and secrete siderophores which can solubilize and sequester iron from the soil and provide it to plant cells; synthesize different phytohormones, including auxins, cytokinins and gibberelins; solubilize minerals such as phosphorus which then become more readily available for plant growth; and they may synthesize an enzyme that can modulate plant ethylene levels (Brown 1974; Kloepper et al. 1986, 1989; Davison 1988; Lambert and Joos 1989; Glick 1995; Patten and Glick 1996, 2002). In addition, a particular

bacterium may affect plant growth and development using any one, or more, of these mechanisms. Moreover, since many PGPB possess several traits that enable them to facilitate plant growth, a bacterium may utilize different traits at various times during the life cycle of the plant, and may vary considerably in its effectiveness depending upon the plant host and the soil composition. Interestingly, PGPB generally have little or no measurable effect on plant growth when the plants are cultivated in nutrient-rich soil and grown under optimal conditions in the absence of stress.

One of the major mechanisms that many PGPB use to facilitate plant growth is the lowering of plant ethylene levels through the action of the enzyme ACC deaminase (Glick 1995, 2004; Glick et al. 1998, 2007a, b). A model was previously developed to explain the role of ACC deaminase in the promotion of plant growth (Glick et al. 1998). In this model, the ACC deaminase-expressing bacteria bind to the surface of either plant seeds or roots and, in response to exuded tryptophan (or other small molecules), the bacteria synthesize and secrete indole acetic acid (IAA) (Fallik et al. 1994; Patten and Glick 2002), some of which is taken up by the plant. Together with the endogenous pool of plant IAA, the IAA taken up by the plant can stimulate plant cell proliferation and elongation and/or it can induce synthesis of the plant enzyme ACC synthase which converts *S*-adenosylmethionine to ACC (Kende 1993). Some of the newly synthesized ACC is exuded by the plant, along with other small molecules, and taken up by the bacteria where it is cleaved by ACC deaminase to form α-ketobutyrate and ammonia, both of which are readily metabolized by the bacteria (Penrose and Glick 2001; Whipps 1990). Some of the ACC that remains within the plant root or seed may be converted to ethylene when the enzyme ACC oxidase is present (Kende 1993). In essence, bacteria that express ACC deaminase, and are bound to the surface of roots or seeds, act as a sink for ACC, thereby decreasing the amount of ethylene that can form when the plant is stressed (Glick et al. 1998; Glick 2004). In this way, ACC deaminase-expressing PGPR protect plants against the growth inhibition that might otherwise result following flooding, extremes of temperature, the presence of organic or inorganic toxicants, phytopathogens, drought, or high salt (Glick 2004).

Given that the overwhelming majority of rhizobacteria are able to synthesize and secrete IAA (Patten and Glick 1996), the question arises why the IAA produced by these bacteria does not eventually result in inhibitory levels of ethylene in response to the IAA (activating ACC synthase transcription). In fact, as the level of ethylene rises, it progressively blocks IAA signal transduction (Dharmasiri and Estell 2004; Glick et al. 2007a). Thus, ACC deaminase works synergistically with IAA so that when a bacterium expresses ACC deaminase, the enzyme lowers the ethylene repression of auxin response factor synthesis, and allows IAA to "do its job" and increase plant growth without producing excessive amounts of ethylene (Glick et al. 2007a).

## 1.3 Mechanisms Used by Plant Growth-Promoting Fungi

There is an enormous amount of literature regarding the use of fungi to protect agricultural plants from salt-induced damage. However, microorganisms promoting salt tolerance in trees have received relatively little attention. Thus, while numerous

reports have highlighted the beneficial role of arbuscular mycorrhizal (AM) fungi, only a few of them have focused on the positive effects induced by ectomycorrhizal (ECM) fungi which are typically found in association with many trees in nature. Surprisingly, to our knowledge, only one report (Waller et al. 2005) deals with the alleviation of salt stress by the employment of a nonmycorrhizal soil fungus. The mechanisms by which PGP fungi (both mycorrhizal and nonmycorrhizal ones) enhance plant tolerance to salinity are described in the following section.

The main mechanisms thought to be involved in salt stress alleviation by AM fungi may be summarized as follows: (1) improvement of mineral nutrition leading to plant growth promotion; (2) variation in the plant accumulation of Na and K as well as soluble sugars and electrolytes; (3) modification of some physiological processes and enzymatic activities involved in plant antioxidative reactions; and (4) alteration of the root architecture facilitating water uptake by the plant. Obviously, differential gene expression/protein synthesis induced by the establishment of the symbiosis between the fungi and plant under salt stress is at the base of these strategies. Early studies demonstrated that improved P nutrition increases crop yields under saline conditions (Hirrel and Gerdemann 1978; Champagnol 1979). In addition, several papers (Poss et al. 1985; Awad et al. 1990; Azcón and Atrash 1997; Ruiz-Lozano et al. 1996; Cantrell and Linderman 2001; Feng et al. 2002; Colla et al. 2008) have compared the effect of AM fungi with P applications on plant growth in saline conditions. It is now evident that the increased salinity tolerance in mycorrhizal plants is based on AM fungi-catalyzed effects, such as the improvement of the plant's water status, and the enhancement of nutrient uptake from the soil (Smith and Read 1997). Moreover, the improvement of plant nutrition and water uptake may be attributed to mycorrhizal-induced modifications of the root architecture, which result in more numerous, thicker and/or more branched roots in herbaceous plants, and in a strong increase in the number of laterals of highest order in woody plants (Berta et al. 1993, 2002, 2005; Gamalero et al. 2002, 2004). Furthermore, studies on the effect of salinity on mycorrhizal plants have shown that AM roots maintain a high $K^+/Na^+$ ratio (Allen and Cunningham 1983; Pfeiffer and Bloss 1988; Sannazzaro et al. 2006; Giri et al. 2004, 2007).

Some physiological processes, such as increased carbon dioxide exchange rate, stomatal conductance and water use efficiency, all involved in osmoregulation, are facilitated by AM fungi (Ruiz-Lozano et al. 1996). Depending on the particular fungal species involved, AM plants under water deficit conditions are more efficient in taking up water than non-AM plants under the same conditions (Marulanda et al. 2003; Khalvati et al. 2005). An improved osmoregulation capacity in AM maize (*Zea mays* L. cv. Yedan 13) plants is indicated by the higher soluble sugar and electrolyte concentrations recorded (Feng et al. 2002). Furthermore, salt is known to reduce the activity or abundance of Hg-sensitive water channels, termed aquaporins, in plants (Carvajal et al. 1999, 2000). In salt-stressed tomato plants preinoculated with a mixture of *Glomus geosporum* and *G. intraradices*, the content of aquaporins in roots is reduced, while the content of these proteins in leaves is significantly increased, suggesting a major impact of AM fungi on the distribution of water throughout the plant. In addition, it is likely that AM fungi facilitate the mobilization of water in the presence of salt by mediating its transfer from the root to the shoot (Ouziad et al. 2006).

A higher relative water content, leading to the prevention of leaf dehydration caused by drought or salinity, has been measured in the leaves of common beans (*Phaseolus vulgaris*) inoculated with the AM fungus *G. intraradices* (Aroca et al. 2007). In agreement with the previously mentioned beneficial effect of AM fungi on the host plant water status, the variation in the relative water content of AM plants is associated with a reduction of the transpiration stream and an increase of free exuded sap flow (Aroca et al. 2007).

Damage to plants that are induced by salt stress may also be a consequence of the production of ROS (Hernandez et al. 1995). In this regard, plants with high concentrations of antioxidants or antioxidative enzymes are typically more resistant to damage by ROS (Spychalla and Desbough 1990; Dionisio-Sese and Tobita 1998; Jiang and Zhang 2002). It has been well established that AM fungi affect the expression of various antioxidative enzymes. For example, in soybean (*Glycine max*) plants that were grown in the presence of salt and were inoculated with a salt-adapted isolate of the AM fungus *G. etunicatum*, the amount of peroxidase and polyphenoloxidase activity was found to increase (Ghorbanli et al. 2004).

Clear positive effects of AM fungi on the formation of nodules and the expression of antioxidative activity by leguminous plants have recently been described (Garg and Manchanda 2008). Thus, although pigeonpea (*Cajanus cajan*) plants exposed to salt had a higher number of nodules compared to untreated plants, these nodules were characterized by reduced biomass, relative permeability, lipid peroxidation, acetylene-reducing activity (ARA), and leghemoglobin content. Inoculation with *G. mosseae* increased nodulation, leghemoglobin content, nitrogenase activity, and the activity of several antioxidant enzymes (superoxide dismutase, catalase, ascorbate peroxidase, peroxidise, and glutathione reductase), suggesting that these factors may be responsible for the positive effects of mycorrhiza to stress-induced premature nodule senescence. The synthesis of the plant polyamines putrescine, spermidine and spermine is often affected by stressful environmental conditions such high salt concentrations (Krishnamurthy and Bhagwat 1989; Aziz et al. 1998; Simon-Sarkadi et al. 2002). Recently, Sannazzaro et al. (2007) found higher amounts of total free polyamines in AM plants (*Lotus glaber*) compared to non-AM ones suggesting that the modulation of polyamine content could be one of the mechanisms involved in the adaptation of plant by AM fungi to saline soils.

AM fungi may influence the level of the plant hormone, abscisic acid (Danneberg et al. 1992). This hormone plays a key role in mediating plant responses to several stresses (Zhang et al. 2006) For instance, it promotes stomatal closure to reduce transpirational water loss and activates a number of genes involved in stress responses. As observed by Jahromi et al. (2008), the abscisic acid content in lettuce (*Lactuca sativa*) roots colonized by the AM fungus *G. intraradices* was significantly higher than in non-AM lettuce roots. Furthermore, in the presence of salt, non-AM roots accumulated more abscissic acid than roots colonized by the AM fungus. Thus, AM fungi may alter the expression of abscisic acid and

thereby modulate gene expression that results in protection of the plant against salt stress.

In addition to the other mechanisms used by AM fungi, it has recently been demonstrated that modifications of plant morphogenetic parameters and photosynthetic efficiency induced by these organisms may be involved in supporting the plant's development in salt conditions (Gamalero et al. 2009). Thus, leaves of cucumber plants inoculated with the AM fungus *Gigaspora rosea* and grown under salt stress were more abundant and had a higher average and total leaf projected area than uninoculated plants. Roots of AM plants under salt stress were more developed than those of uninoculated plants grown in presence or absence of salt. It is likely that the higher total root length, surface area and tip number found in the roots of AM plants may be more efficient at supporting plant growth in saline soils. In addition, while the presence of salt caused a fivefold reduction in leaf photosynthetic efficiency (PI), AM plants exposed to salt showed PI values comparable to plants grown in absence of salt (Gamalero et al. 2009). It is likely that the observed improvement of photosynthetic activity in the presence of AM fungi is related to the increase of chlorophyll content in the leaves of AM plants (Ruiz-Lozano et al. 1996; Giri and Mukerji 2004; Sannazzaro et al. 2006; Zuccarini 2007).

Although several in vitro studies (Hutchison 1990; Chen et al. 2001; Kernaghan et al. 2002; Bois et al. 2006; Langenfeld-Heyser et al. 2007) have demonstrated the potential of salt-tolerant ECM fungi to enhance the tolerance of trees to salinity, little is known about the mechanisms involved. However, variations in the plant water status and in the $Na^+$ and $K^+$ content induced by the interaction of the plant with ECM have been reported. For example, Muhsin and Zwiazek (2002a, b) found that the ECM fungus *Hebeloma crustuliniforme* enhanced the root hydraulic conductance of white spruce (*Picea glauca*) trees in the presence of salt. Reduction of $Na^+$ and $Cl^-$, with a concomitant increase of $PO_4^{-2}$ and $K^+$, in the tissues of seagrape (*Coccoloba uvifera* L.) seedlings colonized by the ECM *Scleroderma bermudense* indicate a higher osmoregulating capacity of ECM plants in saline soils (Bandou et al. 2006). Similar results were obtained by Nguyen et al. (2006) in ECM spruce and pine. Nevertheless, this salt exclusion mechanism seems to be host plant-specific (Yi et al. 2008). In fact, while salt-treated aspen (*Populus tremuloides* Michx.) colonized by the ECM fungus *H. crustuliniforme* had higher $Na^+$ and $Cl^-$ than non-ECM plants, $Na^+$ and $Cl^-$ concentrations in ECM and non-ECM birch (*Betula papyrifera* Marsh.) did not differ. Nevertheless, no information exists about the possibility of salt tolerance in these trees when colonized by AM fungi.

Finally, it has been found that the PGP-endophytic fungus *Piriformospora indica* protects barley (*Hordeum vulgare*) seedlings from the stress induced by moderate concentration of salt as well as biotic stress, such as infection with the necrotrophic fungus *Fusarium culmorum*. This beneficial activity appears to be a consequence of an increase of antioxidant activity in the plant and the activation of systemic resistance induced by the colonization of the plant by *P. indica* (Waller et al. 2005). From this brief overview, it is apparent that the mechanisms employed by PGP fungi are quite different from those used by PGPB. Moreover, these two types

of microorganisms colonize plant roots (and possibly other tissues) very differently. Thus, it should be possible, by better understanding of how plants, bacteria, and mycorrhizae interact with one another, to eventually design/engineer this tripartite relationship so that it is most beneficial for the promotion of plant growth in the presence of salt and other environmental stresses.

## 1.4 Amelioration of Salt Stress by Plant Growth-Promoting Bacteria

Numerous reports on the role of soil bacteria in mitigating the inhibitory effects of salt on growth and development of plants are available. For the most part, the mechanistic basis of this protection is unknown. However, several studies have suggested a variety of possible explanations for this behavior.

It is well known that a variety of environmental stresses, including the presence of salt, induce the production of ethylene in a number of different plants (Jones and El-Abd 1989; Abeles et al. 1992; Hall and Smith 1995; Kukreja et al. 2005; Parida and Das 2005). While the physiology of salt-stressed plants is often dramatically altered compared to plants grown in the absence of salt (Munns 2002; Sairam and Tyagi 2004; Parker et al. 2006), many of the changes observed (including increased production of osmolytes and antioxidants) may be secondary effects of increased ethylene levels within the plant. In this regard, it has been demonstrated that the *NTHK1* gene from tobacco, which encodes an ethylene receptor, is specifically induced by salt (Zhang et al. 2001; Cao et al. 2006). If ethylene does indeed act as a stress signal activating the synthesis of a range of salt-stress response genes in plants then it should be possible, by attenuating plant ethylene levels, to significantly modify the stress response of the plant to salt. Based on the idea that bacterial strains that contain the enzyme ACC deaminase can lower ethylene levels throughout the plants where these bacteria are bound to the roots, Mayak et al. (2004) reasoned that these bacteria should not only lower the level of stress ethylene following exposure of plants to salt, they should also prevent much of the growth inhibition that might otherwise ensue. Mayak et al. (2004) collected soil samples from dried river beds in the arid and salty Arava region in the southern part of the Negev desert in Israel. PGPB isolated from these soil samples were selected to assess their ability to produce ACC deaminase and, on the basis of preliminary testing, one bacterium, *Achrobacter piechaudii* ARV8, was selected for further characterization. This strain not only significantly lowered the ethylene produced by tomato plants following the addition of salt, but also dramatically increased both root and shoot growth in the presence of salt concentrations up to 200 mM. This pioneering work served as the basis for a spate of experimental work on several different plants by scientists from all over the world. Since then, laboratories from India, Pakistan, China and Canada have reported successfully employing ACC deaminase-expressing bacteria to promote plant growth in the presence

of high levels of salt (Saravanakumar and Samiyappan 2006; Cheng et al. 2007; Nadeem et al. 2007; Yue et al. 2007). These studies have included groundnut (Saravanakumar and Samiyappan 2006), maize (Nadeem et al. 2007), cotton (Yue et al. 2007), and canola (Cheng et al. 2007). Importantly, Saravanakumar and Samiyappan (2006) demonstrated that the protection against growth inhibition that ACC deaminase-expressing bacteria provide to plants grown in salt-impacted soil is evident in the field as well as in a laboratory or greenhouse setting. In the experiments reported by Cheng et al. (2007), only wild-type *P. putida* UW4 and not an ACC deaminase minus mutant of this bacterium protected canola plants against growth inhibition by salt. This result provides a clear demonstration of the role of ethylene in growth inhibition by high salt. In addition to the work mentioned above, we are aware of the unpublished results of researchers in Uzbekistan and Iran which are also consistent with the successful use of ACC deaminase-expressing bacteria to promote plant growth in the presence of high levels of salt, both in the laboratory and in the field.

It was found, in a recent study of tomatoes treated with salt, that while the chemical ethylene inhibitors $CoCl_2$ and $NiCl_2$ significantly reduced ethylene accumulation in the headspace of the reaction vessel, thereby reducing epinasty, the negative effects of the salt on growth and other physiological parameters were essentially unchanged (Shibli et al. 2007). These results were interpreted as indicating that ethylene does not make a major contribution to the inhibition of plant growth under saline conditions (Shibli et al. 2007). The differences between the results with chemical inhibitors of ethylene production and those observed with bacteria that express ACC deaminase may reflect artifacts that are part of the closed system including tomato microshoots that were utilized to study the effects of salt plus chemical inhibitors. On the other hand, several studies that employed PGPB that contained ACC deaminase were conducted with whole plants in various types of soil (including in the field) reflecting a more natural situation. Moreover, in addition to the studies reported using ACC deaminase-expressing bacteria, transgenic canola plants that express a bacterial ACC deaminase gene under the control of a root-specific promoter were significantly protected against the growth inhibitory effects of 50, 100 and 200 mM salt compared to nontransformed plants (Sergeeva et al. 2006), consistent with the fact that ethylene is a major contributor to growth inhibition in the presence of salt.

A number of researchers have reported that treatment of plants with either *Azospirillum lipoferum* or *A. brasilense* can mitigate some of the inhibitory effects of salt stress on wheat, maize, beans or lettuce (Bacilio et al. 2004; Hamdia et al. 2004; Rabie and Almadini 2005; Barassi et al. 2006). Since at least some of these strains do not possess ACC deaminase activity (Holguin and Glick 2001), those bacteria must utilize mechanisms other than lowering ethylene with ACC deaminase to protect plants. In this regard, it is possible (but not proven) that bacterial IAA, synthesized by these *Azospirillum* spp. Strains, is responsible for the promotion of plant growth in the presence of salt.

A number of other bacterial mechanisms have been implicated, but not proven, to be involved in ameliorating some of the inhibitory effects of high salt concentrations on plant growth and development. In one study, several different commercial

seed treatments, primarily consisting of various *Bacillus* spp. strains, were tested for the ability to protect squash plants against salt stress (Yildrim et al. 2006). These workers concluded that these seed treatments worked by altering the uptake of minerals into the plant thereby increasing the $K^+/Na^+$ ratio which is positively correlated with plant growth. Other workers have suggested that *N*-acyl-homoserine lactone quorum-sensing signaling molecules can mediate the ability of two different strains of the PGPB *Burkholderia graminis* to promote tomato plant growth and to induce protection against salt stress (Barriuso et al. 2008). While the data presented clearly indicate the involvement of quorum sensing as a component of the overall functioning of these bacteria, quorum sensing per se is unlikely to fully explain the mechanistic basis behind the ability of these strains to protect plants against salt stress.

In a novel approach to developing an understanding of some of the mechanisms used by PGPB to facilitate plant growth in the presence of salt, protein changes that occur in the bacterium *Pseudomonas fluorescens* MSP-393 as a consequence of salt stress were elaborated using a proteomics approach (Paul et al. 2006). Thus, when *P. fluorescens* MSP-393 was incubated in the presence of high salt, the synthesis of a number of unique proteins was induced. Not surprisingly, the majority of the proteins that could be identified were homologous to stress proteins from other prokaryotes. It was therefore suggested that, by maintaining its normal metabolic capabilities in the presence of salt, a salt-stressed PGPB is still able to facilitate plant growth (by whatever, unspecified, mechanism(s) it normally employs).

Finally, two separate laboratories have reported the isolation and characterization of different bacterial strains, all of which confer some measure of salt tolerance on treated plants, specifically maize and wheat (Príncipe et al. 2007; Egamberdieva et al. 2008). These studies evaluated various biological activities of these bacteria such as IAA production, biocontrol activity, siderophore production, phosphate solubilization, plant growth promotion with and without salt, and bacterial genus and species. Unfortunately, from the data presented, no correlation between salt stress amelioration activity and any one or two biological traits measured was found. Thus, while it may be straightforward to select soil bacteria that can ameliorate some of the inhibitory effects of salt stress, these studies do not provide any clear indication as to the mechanism (s) that are employed by these bacteria.

## 1.5 Amelioration of Salt Stress by Plant Growth-Promoting Fungi

Despite the fact that some AM fungi naturally occur in saline environments (Pond et al. 1984; Rozema et al. 1986; Ho 1987; van Duin et al. 1989; Cooke and Lefor 1990; Sengupta and Chaudhuri 1990; Hoefnagels et al. 1993; Johnson-Green et al. 1995), the reports on the effects of salts on AM fungi are contradictory. For instance, some researchers observed inhibition of AM colonization by salt

(Ojala et al. 1983; Duke et al. 1986; Rozema et al. 1986; Dixon et al. 1993; Johnson-Green et al. 1995; Juniper 1996; McMillen et al. 1998), while others noted that the development of the symbiosis remained unaffected by salt application (Levy et al. 1983; Hartmond et al. 1987). It is likely that AM fungi isolated from saline soils are better adapted to this environmental condition and, therefore, are more efficient at plant growth promotion under salt stress. Surprisingly, in one instance, it was found that AM fungi from nonsaline soil performed better in the amelioration of plant growth under salt stress than AM fungi from saline soils (Cantrell and Linderman 2001). On the other hand, in these experiments, AM fungi from saline soils colonized roots more intensively than AM fungi from nonsaline soils, indicating that, in this case, the plant beneficial effects of AM fungi were independent of the root colonization efficiency and the hypothetical adaptation of the microorganism to salt. Similar results were obtained for two strains of *Glomus mosseae*, one isolated from a saline soil and the other from a nonsaline soil (Tian et al. 2004). The fungus from the saline soil induced a high accumulation of sodium and chloride and had very little effect on the alleviation of salt stress in cotton (*Gossypium hirsutum*) plants suggesting that the main mechanism involved in the protection of these plants from the detrimental effects of salinity by AM fungi is related to the capability of the fungi to influence the uptake of sodium and chloride. This notwithstanding, performing salt-acclimatization of AM fungi, by exposing the fungal isolate to gradual increases of NaCl concentration, has been reported to increase their efficiency in supporting plant growth in saline conditions (Sharifi et al. 2007). In addition, it is important to stress that a preferential association exists between plants and AM fungi (Bever et al. 2002; Gollotte et al. 2004; Copetta et al. 2006; Pivato et al. 2007) that could help to select the appropriate/specific fungus to better tolerate salt stress.

Since AM fungi are obligate symbionts, differentiation between the AM fungal response to environmental stress and the AM fungal response to plant-mediated factors is not straightforward. In an effort to sort it out, several workers have attempted to understand how AM fungi react to environmental stimuli by focusing on the precolonization phase and spore germination (Daniels and Trappe 1980; Elias and Safir 1987; Gianinazzi-Pearson et al. 1989). Following this approach, Juniper and Abbott (2006) observed that the main effect of soil salinity is a delay of spore germination and inhibition of hyphal growth from propagules of AM fungal isolates. In addition, in the presence of salt, the number of branched absorbing structures and the rate of sporulation both significantly declined (Jahromi et al. 2008).

Despite numerous contradictory results, especially in the earlier literature, the more recent literature that is our main focus document a significant extent of plant protection by AM fungi. As pointed out by Cantrell and Linderman (2001), it may be important to preinoculate plants with AM fungi in order to firmly establish their symbiotic relationship which might otherwise be inhibited/prevented by salt in the soil. In fact, AM lettuce and onion (*Allium cepa* L.) transplants grown in saline soil had higher shoot biomass than noninoculated plants indicating that preinoculated AM plants grow better than non-AM plants under saline conditions (Cantrell and Linderman 2001). The composite application of AM fungi has been reported to be

more efficient than single inoculation in alleviating plant salt stress. As an example, the growth of *Acacia auriculiformis* under salinity stress varied with the fungal species used, but the mixed inoculum (*Glomus fasciculatus* and *macrocarpum*) resulted in the highest root colonization, biomass production, $K^+$ concentration, $PO_4^{2-}$ level, and chlorophyll content at all salinity levels (Giri et al. 2003). The contribution of this mixed inoculum to plant growth is not limited to improved nutrition, but also to variation in root development so that plants treated with the two fungi had very branched roots. This AM-mediated modification of root architecture is probably caused by the decreased meristematic activity of root apices as shown in leek (Berta et al. 2002). AM fungi also increase the number of adventitious roots, thereby improving nutrient uptake and water balance in the host plant under salinity stress. The root modifications previously described occur in herbaceous plants, where AM fungi also delay root senescence (Lingua et al. 1999). Different modifications are described in AM woody plants where root turnover may be facilitated. These two strategies (delayed senescence and root turnover) enable AM fungi to accomplish the same result, namely a more vital and efficient root system, that could allow plants to better tolerate abiotic stresses, including salt stress. The positive effects of AM fungi on plants under salt conditions may also lead to increases in fruit yield. As an example, in tomato, fruit fresh yield was enhanced by 29% under nonsaline and by 60% under saline water conditions following preinoculation of transplants with *G. mosseae* (Al-Karaki 2006). It would be interesting to ascertain whether the same occurs in other fruit plants, whose root systems are strongly influenced by AM fungi (Schellenbaum et al. 1991; Berta et al. 1995; Manschke et al. 1995).

AM fungi can also stimulate the rhizobial symbiosis efficiency of leguminous plants exposed to salt. As observed by Giri et al. (2004), in saline soil, plants of *Sesbania aegyptiaca* and *S. grandiflora* inoculated with *Glomus macrocarpum* had higher root and shoot dry biomass production, chlorophyll content and $PO_4^{-2}$, N and $Mg^{+2}$ concentrations, than non-AM seedlings. Moreover, the number of nodules was significantly higher in AM than non-AM plants. Interestingly, the mycorrhizal dependency of both *Sesbania* species in salt conditions increased with plant age suggesting that, under salinity, plants need AM fungi not only for acclimatization but also for continued nutrient uptake for their development (Giri et al. 2004). In addition, the mycorrhizal dependency is higher for salt-sensitive than for salt-tolerant plant varieties (Sannazzaro et al. 2006).

Although Basiodiomycetes are generally considered to be relatively salt-sensitive (Tresner and Hayes 1971), several isolates of *Laccaria*, *Hebeloma*, and *Pisolithus* have been characterized as relatively salt tolerant (Chen et al. 2001; Kernaghan et al. 2002). In addition, several in vitro studies highlight the salt tolerance of ECM fungi, generally ascribed to salt exclusion and osmoregulation, and their potential exploitation under field conditions (Chen et al. 2001; Kernaghan et al. 2002; Bois et al. 2006). Experiments performed under greenhouse conditions confirmed the beneficial effects of ECM fungi on plant growth in the presence of salt. Thus, seagrape plants colonized by *S. bermudense* were exposed to a wide range of NaCl levels. The beneficial effects of the fungus on plant growth and

mineral nutrition occurred both under salt stress and nonstress conditions, suggesting that the ECM effect in improving plant growth is not a specific process induced by salinity. In addition, as already reported for AM fungi, the mycorrhizal dependency of seagrape increased with increasing NaCl levels (Bandou et al. 2006).

It has recently been shown that the fungus *Paxillus involutus* can tolerate high NaCl concentrations (Langenfeld-Heyser et al. 2007). The establishment of symbiosis between this fungus and the salt-sensitive poplar hybrid *Populus canescens* positively affected plant biomass and reduced $Na^+$ uptake. The plant's symptoms of salt stress (e.g., leaf degradation) were delayed but not fully prevented by the symbiosis (Langenfeld-Heyser et al. 2007). It has been suggested that the fungus might provide a physical barrier against salt uptake into the root (Muhsin and Zwiazek 2002b). However, microscopical analysis performed by energy-dispersive X-ray microanalysis showed that tissues of both mycorrhizal and nonmycorrhizal roots contained comparable amounts of $Na^+$ and $Cl^-$. In addition, outer cell walls adjacent to mycorrhizal hyphae contained higher sodium concentrations than those of nonmycorrhizal plants indicating that mycorrhizal roots have access to more NaCl than nonmycorrhizal roots and that the hyphal mantle does not provide a physical barrier against NaCl influx.

Under saline conditions, the effect of ECM fungi may vary according to the plant. In their work, Yi et al. (2008) compared the impact of the ECM *H. crustuliniforme* on trembling aspen (*P. tremuloides* Michx.) and paper birch (*B. papyrifera*) in the presence of salt. Despite the fact that both tree species are relatively tolerant to NaCl, ECM inoculation in NaCl-treated plants increased the biomass. However, roots of NaCl-treated aspen inoculated with *H. crustuliniforme* had over twofold higher concentrations of sodium compared with nonmycorrhizal ones. On the contrary, $Na^+$ and $Cl^-$ concentrations in mycorrhizal and nonmycorrhizal birch did not differ. What is clear from the bulk of the data examining the interaction of mycorrhizae and plants in the presence of salt is that these fungi typically protect plants from growth inhibition by salt. On the other hand, notwithstanding many years of experimentation with different plants and mycorrhizal fungi in different soils, the detailed mechanisms used by these PGP fungi remain elusive. However, it is likely that through the use of microarray technology, proteomics and metabolomics to study the changes in both plants and mycorrhizae that occur when these two organisms interact will provide greater insight into these fundamental mechanisms.

## 1.6 Examples of Generating Salt Tolerance with Bacteria and Mycorrhizae

Many of the manuscripts mentioned earlier in this chapter summarize the host of beneficial effects of either PGPB or fungi on salt-stressed plants. In addition, several pieces of evidence suggest the possibility of additive or synergistic effects

between these beneficial microorganisms in supporting plant growth in saline conditions. However, the interactions among PGPB, PGP fungi, plants and stressful conditions have not been studied in depth. Moreover, despite the fact that mycorrhizal helper bacteria (MHB) (Garbaye and Bowen 1989), as well as fungal endophytic bacteria (Bonfante-Fasolo and Scannerini 1977), were discovered some time ago for ECM fungi, to our knowledge no studies have been performed on the possible enhancement of plant tolerance to salt stress by the combination of ECM fungi and these bacteria. Studies dealing with the coinoculation of bacteria and fungi on plants exposed to salt stress have mainly been focused on the rhizobia–AM fungi–legume tripartite symbioses (Hatimi 1999; Diouf et al. 2005; Rabie and Almadini 2005).

In Northern Africa, the salt-tolerant tree *Acacia cyanophylla* is used to stabilize the coastal dunes, increase saline soil fertility and produce large amounts of wood and forage. Soil salinization and the low availability of water induce severe reductions of plant height and dry biomass production (Hatimi 1999). Nevertheless, the inhibitory physiological alterations of *A. cyanophylla* that were induced by salt stress were moderated in the presence of *Bradyrhizobium* sp. Moreover, the tolerance of the plant to salinity increased significantly when this symbiosis between *A. cyanophylla* and *Bradyrhizobium* sp. was associated with a mixture of endomycorrhizal fungi isolated from coastal dune soils. In plants grown in the presence of salt and treated with both microsymbionts, the observed reduction of dry matter was lower than when plants were inoculated with any one of these organisms (Hatimi 1999). Similar results were obtained by combining *Glomus intraradices* and *Bradyrhizobia* sp. with salt-stressed *Acacia auriculiformis* (A. Cunn. ex Benth.) or *Acacia mangium* (Willd.) seedlings (Diouf et al. 2005).

An increase of plant tolerance to salt stress was observed in fava bean (*Vicia faba*) plants inoculated with *Glomus clarum*. And the presence of nitrogen fixing rhizobia in mycorrhizal fava bean plants resulted in an additional increase of plant tolerance to salt. Moreover, the inhibitory effects of salinity on nitrogen fixation were significantly reduced by preinoculating the fava bean plants with the AM fungus (Rabie and Almadini 2005).

A clear example of the impact that salt stress can have on the interactions between mycorrhiza and bacteria has recently been described by Gamalero et al. (2008a, 2009). Under nonstressful conditions, the ACC deaminase-expressing strain *P. putida* UW4 stimulated the development of the AM fungus *G. rosea,* and this positive cooperation between microorganisms induced synergistic effects on plant growth. Unfortunately, with this system, this synergism disappeared when the plants were exposed to high levels of salt (Gamalero et al. 2009). The lack of these positive interactions in the presence of salt may be explained in several different ways. For example, plants exposed to salt stress can release different root exudates that may decrease or abolish the synergism between the microorganisms, even to the point where they are in competition with one another. Alternatively, since salt stress can induce an increase in the level of ethylene in the stressed plant, under these conditions, the amount of ACC deaminase that is synthesized by the bacterial strain may become insufficient to reduce the ethylene due to both the

salt stress and to the localized plant response that occurs as a direct consequence of AM colonization. Given that both PGPB and fungi can separately ameliorate some of the effects of salt stress, there is a paucity of studies where these two types of microorganisms are utilized together. Nevertheless, there is every reason to believe that, with the right combinations of bacteria and fungi, the salt stress of many important agricultural plants may be decreased significantly. In turn, this would increase crop productivity on saline soils and facilitate agricultural practice on some marginal lands.

## 1.7 Conclusion

1. Given the current reluctance on the part of many consumers worldwide to embrace the use as foods of genetically modified plants, it may be advantageous to consider the use of either PGPB or fungi (or a combination of the two) as a means of promoting plant growth in the presence of otherwise inhibitory levels of salt.
2. Using PGPB and/or fungi instead of genetically manipulated plants is also advantageous when one considers the large number of different plants, the many cultivars of those plants, and the multiplicity of genes that would need to be engineered into plants to ensure that they are salt tolerant.
3. PGPB or fungi may be readily selected and/or manipulated to maximally protect a wide range of different salt-stressed plants. Thus, it is easier to select and/or modify a few dozen soil bacteria than hundreds or even thousands of different plant cultivars.
4. The wide-scale use of PGPB and/or fungi may decrease the worldwide dependence on agricultural chemicals. Moreover, it is a technology that is readily accessible to farmers in both developed and developing countries.
5. The successful implementation of this approach has already been demonstrated in the field to a limited extent. However, the large-scale use of this technology would benefit from additional studies, particularly those directed toward understanding how the synergism between PGPB and fungi might be facilitated.

**Acknowledgments** The work from our laboratories that is cited in this manuscript has been financially supported by grants from the Italian MIUR and by the Natural Sciences and Engineering Research Council of Canada.

## References

Abeles FB, Morgan PW, Saltveit ME Jr (1992) Ethylene in plant biology. Academic, New York

Al-Karaki GN (2006) Nursery inoculation of tomato with arbuscular mycorrhizal fungi and subsequent performance under irrigation with saline water. Sci Hort 109:1–7

Allen EB, Cunningham GL (1983) Effects of vesicular arbuscular mycorrhizae on Distichlis spicata under three salinity levels. New Phytologist 93: 227–236

Aroca R, Porcel R, Ruiz-Lozano JM (2007) How does arbuscular mycorrhizal symbiosis regulate root hydraulic properties and plasma membrane aquaporins in *Phaseolus vulgaris* under drought, cold or salinity stresses? New Phytol 173:808–816

Awad AS, Edwards DG, Campbell LC (1990) Phosphorus enhancement of salt tolerance of tomato. Crop Sci 30:123–128

Azcón R, Atrash EEI (1997) Influence of arbuscular mycorrhizae and phosphorus fertilization on growth, nodulation and $N^2$ fixation in *Medicago sativa* at four salinity levels. Biol Fertil Soil 24:81–86

Aziz A, Martin-Tanguy J, Larher F (1998) Stress-induced changes in polyamine and tyramine levels can regulate proline accumulation in tomato leaf discs treated with sodium chloride. Physiol Plant 104:195–202

Bacilio M, Rodriguez H, Moreno M, Hernandez J-P, Bashan Y (2004) Mitigation of salt stress in wheat seedlings by a *gfp*-tagged *Azospirillum lipoferum*. Biol Fertil Soil 40:188–193

Bandou E, Lebailly F, Muller F, Dulormne M, Toribio A, Chabrol J, Courtecuisse R, Plenchette C, Prin Y, Duponnois R, Thiao M, Sylla S, Dreyfus B, Bâ AM (2006) The ectomycorrhizal fungus *Scleroderma bermudense* alleviates salt stress in seagrape (*Coccoloba uvifera* L.) seedlings. Mycorrhiza 16:559–565

Barassi CA, Ayrault G, Creus CM, Sueldo RJ, Sobrero MT (2006) Seed inoculation with *Azospirillum* mitigates NaCl effects on lettuce. Sci Hort 109:8–14

Barriuso J, Solano BR, Fray RG, Cámara M, Hartmann A, Mañero FJG (2008) Transgenic tomato plants alter quorum sensing in plant growth-promoting rhizobacteria. Plant Biotechnol J 6:442–452

Bartels D, Sunkar R (2005) Drought and salt tolerance in plants. Crit Rev Plant Sci 24:23–58

Bashan Y, Holguin G (1998) Proposal for the division of plant growth-promoting rhizobacteria into two classifications: biocontrol-PGPB (plant growth-promoting bacteria) and PGPB. Soil Biol Biochem 30:1225–1228

Berta G, Fusconi A, Trotta A (1993) VA mycorrhizal infection and the morphology and function of root systems. Environ Exp Bot 33:159–173

Berta G, Trotta A, Fusconi A, Hooker J, Munro M, Atkinson D, Giovannetti M, Marini S, Fortuna P, Tisserant B, Gianinazzi-Pearson V, Gianinazzi S (1995) Arbuscular mycorrhizal induced changes to plant growth and root system morphology in *Prunus cerasifera* L. Tree Physiol 15:281–293

Berta G, Fusconi A, Hooker JE (2002) Arbuscular mycorrhizal modifications to plant root systems: scale, mechanisms and consequences. In: Gianinazzi S, Schüepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkäuser, Basel, pp 71–85

Berta G, Sampo S, Gamalero E, Massa N, Lemanceau P (2005) Suppression of *Rhizoctonia* root-rot of tomato by *Glomus mossae* BEG12 and *Pseudomonas fluorescens* A6RI is associated with their effect on the pathogen growth and on the root morphogenesis. Eur J Plant Pathol 111:279–288

Bever JD, Pringle A, Schultz PA (2002) Dynamics within the plant-arbuscular mycorrhizal fungal mutualism: testing the nature of community feedback. In: van der Heijden MGA, Sanders IE (eds) Mycorrhizal ecology. Springer, Heidelberg, Germany, pp 267–292

Blaha G, Stelzl U, Spahn CM, Agrawal RK, Frank J, Nierhaus KH (2000) Preparation of functional ribosomal complexes and effect of buffer conditions on tRNA positions observed by cryoelectron microscopy. Method Enzymol 317:292–309

Blumwald E (2000) Sodium transport and salt tolerance in plants. Curr Opin Cell Biol 12:431–434

Bois G, Bertrand A, Piché Y, Fung M, Khasa DP (2006) Growth, compatible solute and salt accumulation of five mycorrhizal fungal species grown over a range of NaCl concentrations. Mycorrhiza 16:99–109

Bonfante-Fasolo P, Scannerini S (1977) Cytological observations on the mycorrhiza *Endogone flammicorona-Pinus strobus*. Allionia 22:23–34

Brown ME (1974) Seed and root bacterization. Annu Rev Phytopathol 12:181–197
Cantrell IC, Linderman RG (2001) Preinoculation of lettuce and onion with VA mycorrhizal fungi reduces deleterious effects of soil salinity. Plant Soil 233:269–281
Cao W-H, Liu J, Zhou Q-Y, Cao Y-R, Zheng SF, Du B-X, Zhang J-S, Chen SY (2006) Expression of tobacco ethylene receptor NTHK1 alters plant responses to salt stress. Plant Cell Environ 29:1210–1219
Carvajal M, Martinez V, Alcarez CF (1999) Physiological function of water channels as affected by salinity in roots of paprika pepper. Physiol Plant 105:95–101
Carvajal M, Cerda A, Martinez V (2000) Does calcium ameliorate the negative effect of NaCl on melon root water transport by regulating aquaporin activity? New Phytol 145:439–447
Champagnol F (1979) Relationships between phosphate nutrition of plants and salt toxicity. Phosphorus Agric 76:34–43
Chen DM, Ellul S, Herdman K, Cairnay JWG (2001) Influence of salinity on biomass production by Australian *Pisolithus* spp. isolates. Mycorrhiza 11:231–236
Cheng Z, Park E, Glick BR (2007) 1-Aminocyclopropane-1-carboxylate deaminase from *Pseudomonas putida* UW4 facilitates the growth of canola in the presence of salt. Can J Microbiol 53:912–918
Colla G, Rouphael Y, Cardarelli M, Tullio M, Rivera CM, Rea E (2008) Alleviation of salt stress by arbuscular mycorrhizal in zucchini plants grown at low and high phosphorus concentration. Biol Fertil Soil 44:501–509
Cooke JC, Lefor MW (1990) Comparison of vesicular–arbuscular mycorrhizae in plants from disturbed and adjacent undisturbed regions of a coastal salt marsh in Clinton, Connecticut, USA. Environ Manage 14:131–137
Copetta A, Lingua G, Berta G (2006) Effects of three AM fungi on growth, distribution of glandular hairs, and essential oil production in *Ocimum basilicum* L. var. Genovese. Mycorrhiza 16:485–494
Cuartero J, Fernandez-Munoz R (1999) Tomato and salinity. Sci Hortic 78:83–125
Daniels BA, Trappe JM (1980) Factors affecting spore germination of the vesicular–arbuscular mycorrhizal fungus *Glomus epigaeus*. Mycologia 72:457–471
Danneberg G, Latus C, Zimmer W, Hundeshagen B, Schneider-Poetsch HJ, Bothe H (1992) Influence of vesicular-arbuscular mycorrhiza on phytohormone balances in maize (*Zea mays* L.). J Plant Physiol 141:33–39
Davison J (1988) Plant beneficial bacteria. Biotechnology 6:282–286
Dharmasiri N, Estell M (2004) Auxin signaling and regulated protein degradation. Trends Plant Sci 9:302–308
Dionisio-Sese ML, Tobita S (1998) Antioxidant responses of rice seedling to salinity stress. Plant Sci 135:1–9
Diouf D, Duponnois R, Ba AT, Neyra M, Lesueur D (2005) Symbiosis of *Acacia auriculiformis* and *Acacia mangium* with mycorrhizal fungi and *Bradyrhizobium* spp. improves salt tolerance in greenhouse conditions. Funct Plant Biol 32:1143–1152
Dixon RK, Garg VK, Rao MV (1993) Inoculation of *Leucaena* and *Prosopis* seedlings with *Glomus* and *Rhizobium* species in saline soil: rhizosphere relations and seedling growth. Arid Soil Res Rehabil 7:133–144
Duke ER, Johnson CR, Koch KE (1986) Accumulation of phosphorus, dry matter and betaine during NaCl stress of split-root citrus seedlings colonised with vesicular–arbuscular mycorrhizal fungi on zero, one or two halves. New Phytol 104:583–590
Egamberdieva D, Kamilova F, Validov S, Gafurova L, Kucharova Z, Lugtenberg BJJ (2008) High incidence of plant growth-stimulating bacteria associated with the rhizosphere of wheat grown on salinated soil in Uzbekistan. Environ Microbiol 10:1–9
El Beltagy A, Khalifa M, Hall M (1979) Salinity in relation to ethylene. Egypt J Hortic 6:269–271
Elias KS, Safir GR (1987) Hyphal elongation of *Glomus fasciculatus* in response to root exudates. Appl Environ Microbiol 53:1928–1933

Fallik E, Sarig S, Okon Y (1994) Morphology and physiology of plant roots associated with *Azospirillum*. In: Okon Y (ed) *Azospirillum*/Plant associations. CRC, Boca Raton, FL, pp 77–85

Feng G, Zhang FS, Li XL, Tian CY, Tang C, Rengel Z (2002) Improved tolerance of maize plants to salt stress by arbuscular mycorrhiza is related to higher accumulation of soluble sugars in roots. Mycorrhiza 12:185–190

Flowers T (2004) Improving crop salt tolerance. J Exp Bot 55:307–319

Frommel MI, Nowak J, Lazarovits G (1991) Growth enhancement and development modifications of in vitro grown potato (*Solanum tuberosum* ssp. *tuberosum*) as affected by a nonfluorescent *Pseudomonas* sp. Plant Physiol 96:928–936

Gamalero E, Martinotti MG, Trotta A, Lemanceau P, Berta G (2002) Morphogenetic modifications induced by *Pseudomonas fluorescens* A6RI and *Glomus mosseae* BEG12 in the root system of tomato differ according to plant growth conditions. New Phytol 155:293–300

Gamalero E, Trotta A, Massa N, Copetta A, Martinotti MG, Berta G (2004) Impact of two fluorescent pseudomonads and an arbuscular mycorrhizal fungus on tomato plant growth, root architecture and P acquisition. Mycorrhiza 14:185–192

Gamalero E, Berta G, Massa N, Glick BR, Lingua G (2008) Synergistic interactions between the ACC deaminase-producing bacterium *Pseudomonas putida* UW4 and the AM fungus *Gigaspora rosea* positively affect cucumber plant growth. FEMS Microbiol Ecol 64:459–467

Gamalero E, Berta G, Massa N, Glick BR, Lingua G (2009) Interactions between *Pseudomonas putida* UW4 and *Gigaspora rosea* BEG9 and their consequences on the growth of cucumber under salt stress conditions. This paper has been accepted for publication in Journal of Applied Microbiology 10.1111/j.1356-2672.2009.04414.x

Garbaye J, Bowen GD (1989) Stimulation of ectomycorrhizal infection of *Pinus radiata* by some microorganisms associated with the mantle of ectomycorrhizas. New Phytol 112:383–388

Garg N, Manchanda G (2008) Effect of arbuscular mycorrhizal inoculation on salt-induced nodule senescence in *Cajanus cajan* (Pigeonpea). J Plant Growth Regul 27:115–124

Ghorbanli M, Ebrahimzadeh H, Sharifi M (2004) Effects of NaCl and mycorrhizal fungi on antioxidative enzymes in soybean. Biol Plant 48:575–581

Gianinazzi-Pearson V, Branzanti B, Gianinazzi S (1989) *In vitro* enhancement of spore germination and early hyphal growth of a vesicular–arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. Symbiosis 7:243–255

Giri B, Mukerji KG (2004) Mycorrhizal inoculant alleviates salt stress in *Sesbania aegyptiaca* and *Sesbania grandiflora* under field conditions: evidence for reduced sodium and improved magnesium uptake. Mycorrhiza 14:307–312

Giri B, Kapoor R, Agarwal L, Mukerji KG (2004) Pre-inoculation with arbuscular mycorrhizae helps Acacia auriculiformis grow in degraded Indian wasteland soil. Communications in soil science and plant analysis 35: 193–204

Giri B, Kapoor R, Mukerji KG (2003) Influence of arbuscular mycorrhizal fungi and salinity on growth, biomass, and mineral nutrition of *Acacia auriculiformis*. Biol Fertil Soil 38:170–175

Giri B, Kapoor R, Mukerji KG (2007) Improved tolerance of *Acacia nilotica* to salt stress by arbuscular mycorrhiza, *Glomus fasciculatum* may be partly related to elevated K/Na ratios in root and shoot tissues. Microb Ecol 54:753–760

Glick BR (1995) The enhancement of plant growth by free-living bacteria. Can J Microbiol 41:109–117

Glick BR (2004) Bacterial ACC deaminase and the alleviation of plant stress. Adv Appl Microbiol 56:291–312

Glick BR, Penrose DM, Li J (1998) A model for the lowering of plant ethylene concentrations by plant growth promoting bacteria. J Theor Biol 190:63–68

Glick BR, Cheng Z, Czarny J, Duan J (2007a) Promotion of plant growth by ACC deaminase-containing soil bacteria. Eur J Plant Pathol 119:329–339

Glick BR, Todorovic B, Czarny J, Cheng Z, Duan J, McConkey B (2007b) Promotion of plant growth by bacterial ACC deaminase. Crit Rev Plant Sci 26:227–242

Gollotte A, van Tuinen D, Atkinson D (2004) Diversity of arbuscular mycorrhizal fungi colonising roots of the grass species *Agrostis capillaris* and *Lolium perenne* in a field experiment. Mycorrhiza 14:111–117

Hall MA, Smith AR (1995) Ethylene and the responses of plants to stress. Bulg J Plant Physiol 21:71–79

Hamdia MA, Shaddad MAK, Doaa MM (2004) Mechanisms of salt tolerance and interactive effects of *Azospirillum brasilense* inoculation on maize cultivars grown under salt stress conditions. Plant Growth Regul 44:165–174

Hartmond U, Schaesberg NV, Graham JH, Syvertsen JP (1987) Salinity and flooding stress effects on mycorrhizal and nonmycorrhizal citrus rootstock seedlings. Plant Soil 104:37–43

Hatimi A (1999) Effect of salinity on the association between root symbionts and *Acacia cyanophylla* Lind.: growth and nutrition. Plant Soil 216:93–101

Hernandez JA, Olmos E, Corpas FJ, Sevilla F, del Rio LA (1995) Salt induced oxidative stress in chloroplasts of pea plants. Plant Sci 105:151–167

Hirrel MC, Gerdemann JW (1980) Improved growth of onion and bell pepper in saline soils by two vesicular–arbuscular mycorrhizal fungi. Soil Sci Soc Am J 44:654–655

Ho I (1987) Vesicular–arbuscular mycorrhizae of halophytic grasses in the Alvard desert of Oregon. Northwest Sci 61:148–151

Hoefnagels MH, Broome SW, Shafer SR (1993) Vesicular–arbuscular mycorrhizae in salt marshes in North Carolina. Estuaries 16:851–858

Holguin G, Glick BR (2001) Expression of the ACC deaminase gene from *Enterobacter cloacae* UW4 in *Azospirillum brasilense*. Microb Ecol 41:281–288

Hutchinson LJ (1990) Studies on the systematic of ectomycorrhizal fungi in axenic culture. IV. The effects of some selected fungi toxic compounds upon linear growth. Canadian Journal of Botany 68:2172–2178

Jahromi F, Aroca R, Porcel R, Ruiz-Lozano JM (2008) Influence of salinity on the in vitro development of *Glomus intraradices* and on the in vivo physiological and molecular responses of mycorrhizal lettuce plants. Microb Ecol 55:45–53

Jiang M, Zhang J (2002) Water stress-induced abscisic acid accumulation triggers the increased generation of reactive oxygen species and up-regulates the activities of antioxidant enzymes in maize leaves. J Exp Bot 53:2401–2410

Johnson-Green PC, Kenkel NC, Booth T (1995) The distribution and phenology of arbuscular mycorrhizae along an inland salinity gradient. Can J Bot 73:1318–1327

Jones RA, El-Abd SO (1989) Prevention of salt-induced epinasty by α-aminooxyacetic acid and cobalt. Plant Growth Regul 8:315–323

Juniper S (1996) The effect of sodium chloride on some vesicular–arbuscular mycorrhizal fungi. PhD Thesis, The University of Western Australia

Juniper S, Abbott LK (2006) Soil salinity delays germination and limits growth of hyphae from propagules of arbuscular mycorrhizal fungi. Mycorrhiza 16:371–379

Kende H (1993) Ethylene biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 44:283–307

Kernaghan G, Hambling B, Fung M, Khasa D (2002) *In vitro* selection of Boreal ectomycorrhizal fungi for use in reclamation of saline–alkaline habitats. Restor Ecol 10:1–9

Khalvati MA, Hu Y, Mozafar A, Schmidhalter U (2005) Quantification of water uptake by arbuscular mycorrhizal hyphae and its significance for leaf growth, water relations, and gas exchange of barley subjected to drought stress. Plant Biol 7:706–712

Khan MA, Rizvi Y (1994) Effect of salinity, temperature, and growth-regulators on the germination and early seedling growth of *Atriplex Griffithii* var stocksii. Can J Bot 72:475–479

Kloepper JW, Scher FM, Laliberte M, Tipping B (1986) Emergence-promoting rhizobacteria: description and implications for agriculture. In: Swinburne TR (ed) Iron, siderophores, and plant disease. Plenum, New York, pp 155–164

Kloepper JW, Lifshitz R, Zablotowicz RM (1989) Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol 7:39–43

Krishnamurthy R, Bhagwat KA (1989) Polyamines as modulators of salt tolerance in rice cultivars. Plant Physiol 91:500–504

Kukreja S, Nandwal AS, Kumar N, Sharma SK, Sharma SK, Unvi V, Sharma PK (2005) Plant water status, $H_2O_2$ scavaging enzymes, ethylene evolution and membrane integrity of *Cicer arietinum* as affected by salinity. Biol Plant 49:305–308

Lambert B, Joos H (1989) Fundamental aspects of rhizobacterial plant growth promotion research. Trends Biotechnol 7:215–219

Langenfeld-Heyser R, Gao J, Ducic T, Ph T, Lu CF, Fritz E, Gafur A, Polle A (2007) *Paxillus involutus* mycorrhiza attenuate NaCl-stress responses in the salt-sensitive hybrid poplar *Populus*×*canescens*. Mycorrhiza 17:121–131

Levy Y, Dodd J, Krikun J (1983) Effect of irrigation water salinity and rootstock on the vertical distribution of vesicular–arbuscular mycorrhiza in citrus roots. New Phytol 95:397–403

Lingua G, Sgorbati S, Citterio A, Fusconi A, Trotta A, Gnavi E, Berta G (1999) Arbuscular mycorrhizal colonization delays nucleus senescence in leek root cortical cells. New Phytol 141:161–169

Manschke M, Berta G, Gianinazzi S, Moncousin C (1995) Effect of endomycorrhizal infection on root system development in the apple rootstock *(Malus domestica* Borkh.) M26, morphogenesis. In: Azcon C, Barea JM, Ocampo J (eds) Mycorrhizas in integrated systems: from genes to plant development. . Office for Official Publications of the European Communities, Brussels, Luxemburg, pp 349–352

Marulanda A, Azcon R, Ruiz-Lozano JM (2003) Contribution of six arbuscular mycorrhizal fungal isolates to water uptake by *Lactuca sativa* plants under drought stress. Physiol Plant 119:526–533

Mayak S, Tirosh T, Glick BR (2004) Plant growth-promoting bacteria that confer resistance in tomato to salt stress. Plant Physiol Biochem 42:565–572

McMillen B, Juniper S, Abbott LK (1998) Inhibition of hyphal growth of a vesicular–arbuscular mycorrhizal fungus in soil containing sodium chloride limits the spread of infection from spores. Soil Biol Biochem 30:1639–1646

Muhsin TM, Zwiazek JJ (2002a) Colonization with *Hebeloma crustuliniforme* increases water conductance and limits shoot sodium uptake in white spruce (*Picea glauca*) seedlings. Plant Soil 238:217–225

Muhsin TM, Zwiazek JJ (2002b) Ectomycorrhizas increase apoplastic water transport and root hydraulic conductivity in *Ulmus americana* seedlings. New Phytol 153:153–158

Munns R (2002) Comparative physiology of salt and water stress. Plant Cell Environ 25:239–250

Nadeem SM, Zahair ZA, Naveed M, Arshad M (2007) Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC deaminase activity. Can J Microbiol 53:1141–1149

Nguyen H, Calvo Polanco M, Zwiazek JJ (2006) Gas exchange and growth responses of ectomycorrhizal *Picea mariana, Picea glauca,* and *Pinus banksiana* seedlings to NaCl and $Na_2SO_4$. Plant Biol 8:646–652

O'Donnell PJ, Calvert C, Atzorn R, Wasternack C, Leyser HMO, Bowles DJ (1996) Ethylene as a signal mediating the wound response of tomato plants. Science 274:1914–1917

Ojala JC, Jarrell WM, Menge JA, Johnson ELV (1983) Influence of mycorrhizal fungi on the mineral nutrition and yield of onion in saline soil. Agron J 75:255–259

Ouziad F, Wilde P, Schmelzer E, Hildebrandt U, Bothe H (2006) Analysis of expression of aquaporins and $Na^+/H^+$ transporters in tomato colonized by arbuscular mycorrhizal fungi and affected by salt stress. Environ Exp Bot 57:177–186

Parida AK, Das AB (2005) Salt tolerance and salinity effects on plants: a review. Ecotoxicol Environ Saf 60:324–349

Parker R, Flowers TJ, Moore AL, Harpham NVJ (2006) An accurate and reproducible method for proteome profiling of the effects of salt stress in the rice leaf lamina. J Exp Bot 57:1109–1118

Patten CL, Glick BR (1996) Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol 42:207–220

Patten CL, Glick BR (2002) The role of bacterial indoleacetic acid in the development of the host plant root system. Appl Environ Microbiol 68:3795–3801

Paul D, Dineshkumar N, Nair S (2006) Proteomics of a plant growth-promoting rhizobacterium, *Pseudomonas fluorescens* MSP-393, subjected to salt shock. World J Microbiol Biotechnol 22:369–374

Penrose DM, Glick BR (2001) Levels of 1-aminocyclopropane-1-carboxylic acid (ACC) in exudates and extracts of canola seeds treated with plant growth-promoting bacteria. Can J Microbiol 47:368–372

Pfeiffer CM, Bloss HE (1988) Growth and nutrition of guayule (*Parthenium argentatum*) in a saline soil as influenced by vesicular arbuscular mycorrhiza and phosphorus fertilization. New Phytol 108:315–321

Pivato B, Mazurier S, Lemanceau P, Siblot S, Berta G, Mougel C, van Tuinen D (2007) *Medicago* species affect the community composition of arbuscular mycorrhizal fungi associated with roots. New Phytol 176:197–210

Pond EC, Menge JA, Jarrell WM (1984) Improved growth of tomato in salinized soil by vesicular–arbuscular mycorrhizal fungi collected from saline soils. Mycologia 76:74–84

Poss JA, Pond EC, Menge JA, Jarrell WM (1985) Effect of salinity on mycorrhizal onion and tomato in soil with and without additional phosphate. Plant Soil 88:307–319

Príncipe A, Alvarez F, Castro MG, Zachi L, Fischer SE, Mori GB, Jofré E (2007) Biocontrol and PGPR features in native strains isolated from saline soils of Argentina. Curr Microbiol 55:314–322

Rabie GH, Almadini AM (2005) Role of bioinoculants in development of salt-tolerance of *Vicia faba* under salinity stress. Afr J Biotechnol 4:210–222

Richard AJ, El-Abd SO (1989) Prevention of salt-induced epinasty by a-aminooxyacetic acid and cobalt. Plant Growth Regul 8:315–323

Rozema J, Arp W, Van Diggelen J, Van Esbroek M, Broekman R, Punte H (1986) Occurrence and ecological significance of vesicular–arbuscular mycorrhiza in the salt marsh environment. Acta Bot Neerl 35:457–467

Ruiz-Lozano JM, Azcon R, Gomez M (1996) Alleviation of salt stress by arbuscular-mycorrhizal *Glomus* species in *Lactuca sativa* plants. Physiol Plant 98:767–772

Sairam RK, Tyagi A (2004) Physiology and molecular biology of salinity stress tolerance in plants. Curr Sci 86:407–421

Sannazzaro AI, Ruiz OA, Alberto EO, Menendez AB (2006) Alleviation of salt stress in *Lotus glaber* by *Glomus intraradices*. Plant Soil 285:279–287

Sannazzaro AI, Echeverria M, Alberto EO, Ruiz OA, Menendez AB (2007) Modulation of polyamine balance in *Lotus glaber* by salinity and arbuscular mycorrhiza. Plant Physiol Biochem 45:39–46

Saravanakumar D, Samiyappan R (2006) ACC deaminase from *Pseudomonas fluorescens* mediated saline resistance in groundnut (*Arachis hypogea*) plants. J Appl Microbiol 102:1283–1292

Schellenbaum L, Berta G, Ravolanirina F, Tisserant B, Giainazzi S, Fitter AH (1991) Influence of endomycorrhizal infection on root morphology in a micropropagated woody plant species (*Vitis vinifera* L.). Ann Bot 67:135–141

Sengupta A, Chaudhuri S (1990) Vesicular arbuscular mycorrhiza (VAM) in pioneer salt marsh plants of the Ganges River Delta in West Bengal (India). Plant Soil 122:111–113

Sergeeva E, Shah S, Glick BR (2006) Growth of transgenic canola (*Brassica napus* cv. Westar) expressing a bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene on high concentrations of salt. World J Microbiol Biotechnol 22:277–282

Sharifi M, Ghorbanli M, Ebrahimzadeh A (2007) Improved growth of salinity-stressed soybean after inoculation with salt pre-treated mycorrhizal fungi. J Plant Physiol 164:1144–1151

Shibli RA, Kushad M, Yousef GG, Lila MA (2007) Physiological and biochemical responses of tomato microshoots to induced salinity stress with associated ethylene accumulation. Plant Growth Regul 51:159–169

Simon-Sarkadi L, Kocsy G, Sebestyen Z (2002) Effect of salt stress on free amino acid and polyamine content in cereals. Acta Biol Szegediensis 46:73–75

Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, London, p 605

Smith GS, Middleton KR, Edmonds AS (1980) Sodium nutrition of pasture plants.1. Translocation of sodium and potassium in relation to transpiration rates. New Phytol 84:603–612

Spychalla JP, Desbough SL (1990) Superoxide dismutase, catalase, and alpha-tocopherol content of stored potato tubers. Plant Physiol 94:1214–1218

Tester M, Davenport R (2003) $Na^+$ tolerance and $Na^+$ transport in higher plants. Ann Bot 91: 503–527

Tian CY, Feng G, Li XL, Zhang FS (2004) Different effects of arbuscular mycorrhizal fungal isolates from saline or non-saline soil on salinity tolerance of plants. Appl Soil Ecol 26:143–148

Tresner HD, Hayes JA (1971) Sodium chloride tolerance of terrestrial fungi. Appl Environ Microbiol 22:210–213

van Duin WE, Rozema J, Ernst WHO (1989) Seasonal and spatial variation in the occurrence of vesicular–arbuscular mycorrhiza in salt marsh plants. Agric Ecosyst Environ 29:107–110

van Peer R, Schippers B (1989) Plant growth responses to bacterization with selected *Pseudomonas* spp. strains and rhizosphere microbial development in hydroponic cultures. Can J Microbiol 35:456–463

Verslues PE, Agarwal M, Katiyar-Agarwal S, Zhu J, Zhu JK (2006) Methods and concepts in quantifying resistance to drought, salt and freezing, abiotic stresses that affect plant water status. Plant J 45:523–539

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M, Heier T, Huckelhoven R, Neumann C, von Wettstein D, Franken P, Kogel KH (2005) The endophytic fungus *Piriformospora indica* reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci USA 102:13386–13391

Whipps JM (1990) Carbon utilization. In: Lynch JM (ed) The rhizosphere. Wiley, Chichester, UK, pp 59–97

White PJ, Broadley MR (2001) Chloride in soils and its uptake and movement within the plant: A review. Ann Bot 88:967–988

Yi H, Calvo Polanco M, MacKinnonc MD, Zwiazek JJ (2008) Responses of ectomycorrhizal *Populus tremuloides* and *Betula papyrifera* seedlings to salinity. Environ Exp Bot 62:357–363

Yildrim E, Taylor AG, Spittler TD (2006) Ameliorative effects of biological treatments on growth of squash plants under salt stress. Sci Hortic 111:1–6

Yue HT, Mo WP, Li C, Zheng YY, Li H (2007) The salt stress relief and growth promotion effect of Rs-5 on cotton. Plant Soil 297:139–145

Zhang J-S, Xi C, Shen Y-G, Shen S-Y (2001) A two component gene (*NTHK1*) encoding a putative ethylene-receptor homolog is both developmentally and stress regulated in tobacco. Theor Appl Genet 102:815–824

Zhang J, Jia W, Yang J, Ismail AM (2006) Role of ABA in integrating plant responses to drought and salt stresses. Field Crop Res 97:111–119

Zuccarini P (2007) Mycorrhizal infection ameliorates chlorophyll content and nutrient uptake of lettuce exposed to saline irrigation. Plant Soil Environ 53:283–289

# Chapter 2
# Recent Advances in Plant Growth Promotion by Phosphate-Solubilizing Microbes

**Almas Zaidi, Mohammad Saghir Khan, Munees Ahemad, Mohd Oves, and P.A. Wani**

**Abstract** Most soils contain large reserves of total phosphorus (P), but its fixation and precipitation with soil constituents cause a major P-deficiency and severely restrict the growth and yield of plants. The use of chemical P-fertilizers is obviously the best means to circumvent P-deficiency, but their use is always limited due to its spiraling cost. In order to increase the availability of P and to reduce the use of chemical fertilizers, solubilization of insoluble P by phosphate-solubilizing microorganisms has provided an alternative to chemical phosphatic fertilizer. Besides P, these organisms promote the growth of plants by $N_2$ fixation, enhancement of other plant nutrients, synthesizing phytohormones, suppressing plant diseases (bio-control) and reducing the toxicity of ethylene through 1-aminocyclopropane-1carboxylate (ACC) deaminase. In this chapter, attention is paid to understanding the fundamental and molecular basis as to how precisely these microbes, notably bacteria and fungi, help plants to grow better in P-deficient soils. Effective use of such microbes is likely to result in an ideal cropping system with a lesser impact on the environment through decreased application of chemical fertilizers.

## 2.1 Introduction

Phosphorus is one of the major nutrients limiting plant growth. In contrast, P is required for growth and development of plants and promotes $N_2$ fixation. It is also involved in photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis, and respiration (Saber et al. 2005; Fernández et al. 2007). However, most of the soils throughout the world are P-deficient (Batjes 1997) and, therefore, require P to replenish the P-demand by crop plants. Worldwide, 5.7 billion hectares

M.S. Khan(✉), A. Zaidi, M. Ahemad, M. Oves and P.A. Wani
Faculty of Agricultural Sciences, Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, U.P., India
e-mail: khanms17@rediffmail.com

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_2, 

contain too little available P for sustaining optimal crop production (Hinsinger 2001), and P-ion concentration in most soils varies from 0.1 to 10 μM while P required for optimal growth ranges from 1 to 5 μM for grasses and from 5 to 60 μM for high P-demanding crops, such as, tomato (*Lycopersicon esculentum*) and pea (*Pisum sativum*) (Raghothama 1999). Suboptimal levels of P can, however, lead to a 5–15% loss in the yield of plants (Hinsinger 2001). To circumvent the P-deficiency, synthetic P-fertilizers are applied. The repeated and injudicious applications of these fertilizers, however, lead to (1) the loss of soil fertility, (2) disturbance to microbial diversity and their associated metabolic activities, and (3) reduced yield of agronomic crops. Moreover, after application, a considerable amount of P is rapidly transformed into less available forms by forming a complex with Al or Fe in acid soils or with Ca in calcareous soils (Goldstein 1986; Toro 2007) before plant roots have had a chance to absorb it. This has led to the search for environment-friendly and economically feasible alternative strategies for improving crop production in low or P-deficient soils. Phosphorus is present in soils both in organic and inorganic forms. Of these, organic forms, as found in humus and other organic materials including decayed plant, animal and microbial tissues, is an important reservoir of immobilized P accounting for about 20–80% of total soil P (Richardson 1994). Organic P compounds can be slowly mineralized as available inorganic P or they can be immobilized as part of the soil organic matter (Mckenzie and Roberts 1990). The process of mineralization/solubilization or immobilization affected by rhizosphere microbes, especially phosphate-solubilizing microorganisms (PSM), serves as an alternative to chemical phosphatic fertilizers and provides the available forms of P to plants (Bojinova et al. 2008; Oliveira et al. 2008).

Besides providing P to the plants, the PSM(s) also facilitate the growth of plants by stimulating the efficiency of $N_2$ fixation, accelerating the accessibility of other trace elements and by synthesizing important growth promoting substances (Wani et al. 2007a; Mittal et al. 2008), including siderophores (Wani et al. 2007b) and antibiotics (Lipping et al. 2008), and providing protection to plants against soil-borne pathogens (Hamdali et al. 2008). Accordingly, these microbial communities when used singly (Poonguzhali et al. 2008; Chen et al. 2008) or in combination with other rhizosphere microbes (Zaidi and Khan 2006; Wani et al. 2007c; Vikram and Hamzehzarghani 2008) have shown substantial measurable effects on plants in conventional agronomic soils. Furthermore, bacterial strains capable of solubilizing P have also been isolated from various niches of saline–alkali soils. For example, strain RMLU-26, identified as *Xanthomonas campestris* with the ability to efficiently solubilize P, was subjected to *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) for development of mutants. The wild-type and mutant strains of *X. campestris* revealed a differential response to various stress factors (high pH, temperature, and salt concentration). Both the wild and mutant strains revealed substantial P-solubilization, but percent $P_2O_5$ solubilization by both strains revealed a steep decline in tricalcium phosphate (TCP) solubilization with an increase in NaCl concentration from 0.5 to 10% along with a concomitant drop in pH of the medium

from 8 to 4.5 in wild-type and 4 in mutant strain. However, the mutant strain displayed a 1.5- to 2-fold increase in P-solubilization compared to the wild-type strain when grown in the presence of NaCl. The overall improved tolerance of the strains to alkalinity and salinity could be due to accumulation and/or secretion of specific solute (xanthan), suggesting that these strains could also be used as a bio-inoculant under stressed soil environments (Sharan et al. 2008). In the following section, particular attention is paid to identifying such microbes and the mechanisms by which they solubilize insoluble P and promote the growth of plants. Special attention is further paid to the exploitation of microbial communities endowed with P-solubilizing activity for their use in agricultural practices in different agro-ecological niches.

## 2.2 Strategies for Isolation and Inoculant Development

Phosphate-solubilizing microbes form an integral bio-component of soils and affect the fertility of soils through biogeochemical cycles. Numerous rhizosphere microorganisms capable of dissolving insoluble P have been reported (Henri et al. 2008; Hameeda et al. 2008). Of these, the important genera of P-solubilizing bacteria include *Bacillus* and *Pseudomonas* (Illmer and Schinner 1992; Wani et al. 2007a), while *Aspergillus* and *Penicillium* form the important fungal genera (Souchie et al. 2006; Pandey et al. 2008). Other bacteria reported as P-solubilizers include *Rhodococcus*, *Arthrobacter*, *Serratia, Chryseobacterium*, *Gordonia*, *Phyllobacterium*, *Arthrobacter*, *Delftia* sp. (Wani et al. 2005; Chen et al. 2006), *Azotobacter* (Kumar et al. 2001), *Xanthomonas* (de Freitas et al. 1997), and *Enterobacter*, *Pantoea*, and *Klebsiella* (Chung et al. 2005). Furthermore, symbiotic nitrogenous rhizobia, which fix atmospheric nitrogen into ammonia and export the fixed nitrogen to the host plants, have also shown PS activity. For instance, *Rhizobium leguminosarum* bv. *trifolii* (Abril et al. 2007), *R. leguminosarum* bv. *viciae* (Alikhani et al. 2007) and *Rhizobium* species nodulating *Crotalaria* species (Sridevi et al. 2007) improved plant P-nutrition by mobilizing inorganic and organic P. These organisms are ubiquitous but vary in density and mineral PS (mps) ability from soil to soil or from one production system to another. They are generally isolated from rhizosphere and nonrhizosphere soils, rhizoplane, phyllosphere, and RP deposit area soil and even from stressed soils using serial plate dilution method or by enrichment culture technique. The viable microbial preparations possessing P-solubilizing activity are generally termed microphos. The microphos production involves three critical stages: (1) screening and selection and in vitro evaluation of P-solubilizing potentials of the microbial strains, (2) selection of carriers, mixing of inocula with selected carriers and proper development of microbial inoculants, and (3) testing of the quality of inoculants in terms of persistence of P-solubilizing activity, viable microbial load per gram of carrier, and proper distribution to the farmers. Since 1948, when Gerretsen suggested that microbes could dissolve not

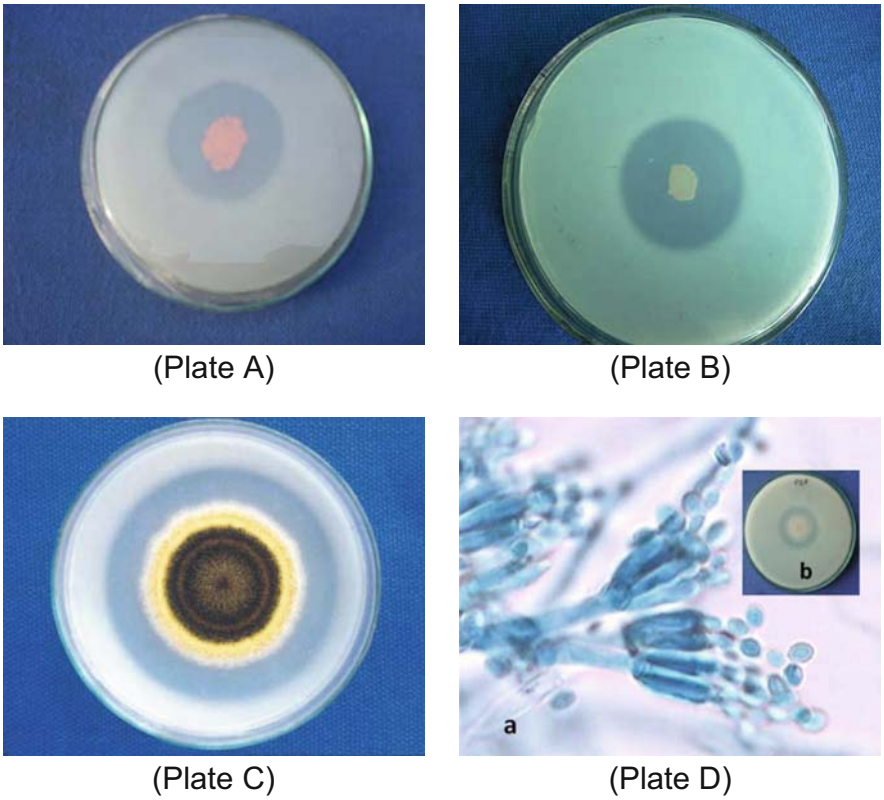



**Fig. 2.1** Solubilization of tricalcium phosphate on Pikovskaya by species of (Plate A) *Serratia* (Plate B) *Bacillus* (Plate C) *Aspergillus* and (Plate D) *Penicillium*

easily available forms of soil P and play an important role in providing P to plants, numerous methods and media, such as Pikovskaya (Pikovskaya 1948), bromophenol blue dye method (Gupta et al. 1994), and National Botanical Research Institute P (NBRIP) medium (Nautiyal 1999), have been proposed.

Both bacterial and fungal strains exhibiting PS activity are detected by the formation of clear halo (a sign of solubilization) around their colonies (Fig. 2.1). Due to inconsistency and variations in PS activity, these cultures are repeatedly subcultured. Once the efficient PS organisms have been selected, the release of P by PS organisms is quantitatively assayed. The phosphate-solubilizing microbes showing greater solubilization (both qualitatively and quantitatively) of insoluble P under in vitro conditions are selected for bulk production for ultimate transmission to the farmers. For instance, use of fungi as inoculants for increasing plant P-nutrition has been demonstrated by the successful commercial release of *Penicillium bilaiae* (JumpStart; Philom Bios, Saskatoon, Canada) and *Penicillium radicum* (PR-70 RELEASE; Bio-Care Technology, Somersby, Australia).

## 2.3 Mechanisms of P-Solubilization: A General Account

The solubilization of P-compounds by naturally abundant P-solubilizing microbes is common under in vitro conditions (Souchie et al. 2007; Song et al. 2008). Indeed, soil microorganisms are effective in releasing P from inorganic P through solubilization (Toro 2007; Wani et al. 2007b, c) and from organic pools of total soil P by mineralization (Bishop et al. 1994; Ponmurugan and Gopi 2006). The microbial biomass in soil also contains a significant quantity of immobilized P that is accessible for uptake by plants (Brookes et al. 1984; Oberson et al. 2001). The mechanisms used by PS microbes to solubilize mineral phosphate have been investigated by several workers (Cunningham and Kuiack 1992; Illmer and Schinner 1995; Song et al. 2008). Generally, the mps trait is correlated with the production of organic acids (OA) via the direct oxidation pathway that occurs on the outer face of the cytoplasmic membrane, with concomitant drop in pH value. The inverse correlation between pH of the culture and the release of P consolidates the hypothesis of OA involvement in the solubilization of insoluble P. The OA(s) released by PS microbes, notably bacteria (Table 2.1) and fungi (Table 2.2), chelate mineral ions or drop the pH to bring P into solution (Maliha et al. 2004; Pradhan and Sukla 2005). The OA produced both by bacteria and fungi in turn leads to acidification of

**Table 2.1** Organic acids involved in P-solubilization and produced by PS bacteria

| Bacterial communities | Organic acids produced | References |
|---|---|---|
| *Burkholderia cepacia* DA23 | Gluconic acid | Song et al. (2008) |
| *Pseudomonas corrugata* (NRRL B-30409) | Gluconic, 2-ketogluconic acid | Trivedi and Sa (2008) |
| *Citrobacter* sp. DHRSS | Acetic and gluconic acid | Patel et al. (2008) |
| *Burkholderia, Serratia, Ralstonia* and *Pantoea* | Gluconic acid | Elizabeth et al. (2007) |
| *Bacillus, Rhodococcus*, *Arthrobacter*, *Serratia* and one *Chryseobacterium*, *Delftia*, *Gordonia, Phyllobacterium*, *Arthrobacter ureafaciens*, *Phyllobacterium myrsinacearum*, *Rhodococcus erythropolis* and *Delftia* sp. | Citric acid, gluconic acid, lactic acid, succinic acid, propionic acid | Chen et al. (2006) |
| *Enterobacter intermedium* | 2-ketogluconic | Hwangbo et al. (2003) |
| *Bacillus amyloliquefaciens*, *B. licheniformis*, *B. atrophaeus*, *Penibacillus macerans*, *Vibrio proteolyticus*, *xanthobacter agilis*, *Enterobacter aerogenes*, *E. taylorae*, *E. asburiae*, *Kluyvera cryocrescens*, *Pseudomonas aerogenes*, *Chryseomonas luteola* | Lactic, itaconic, isovaleric, isobutyric, acetic | Vazquez et al. (2000) |
| *Pseudomonas cepacia* | Gluconic, 2-ketgluconic | Bar-Yosef et al. (1999) |
| *Bacillus polymyxa*, *B. licheniformis*, *Bacillus* spp. | Oxalic, citric | Gupta et al. (1994) |

**Table 2.2** Organic acids produced by phosphate solubilizing fungi

| Organism | Predominant acids | References |
|---|---|---|
| *Aspergillus niger* | Gluconic acid, oxalic acid | Chuang et al. (2007) |
| *Penicillium oxalicum* | Malic acid, gluconic acid, oxalic acid | Shin et al. (2006) |
| *Aspergillus flavus*, *A. niger*, *Penicillium canescens* | Oxalic, citric, gluconic succinic | Maliha et al. (2004) |
| *Penicillium rugulosum* | Citric, gluconic acid | Reyes et al. (2001) |
| *A. niger* | Succinic acid | Vazquez et al. (2000) |
| *Penicillium variabile* | Gluconic acid | Fenice et al. (2000) |
| *Penicillium rugulosum* | Gluconic | Reyes et al. (1999) |
| *Penicillium radicum* | Gluconic | Whitelaw et al. (1999) |
| *P. variabile* | Gluconic | Vassilev et al. (1996) |
| *A. niger* | Citric, oxalic,gluconic | Illmer et al. (1995) |
| *A. awamori*, *A. foetidus*, *A. terricola*, *A. amstelodemi*, *A. tamari* | Oxalic, citric | Gupta et al. (1994) |
| *A. japonicus*, *A. foetidus* | Oxalic, citric gluconic succinic, tartaric acid | Singal et al. (1994) |

microbial cells and their surroundings and, consequently, the release of P-ions from the P-mineral by $H^+$ substitution for $Ca^{2+}$ (Goldstein 1994). However, a lot of fixed P in acidic soil (such as red soil) accumulates Fe or Al ions, and no correlation was found between pH and the amount of P-solubilized (Asea et al. 1988). It is still unexplained why no substantial amounts of OA production could be detected from some PS microorganisms (Illmer and Schinner 1992; Chen et al. 2006). For this reason, alternative possibilities other than OA for insoluble inorganic P-solubilization have been proposed. Such mechanisms include the release of $H^+$, production of chelating substances and inorganic acids (Khan et al. 2007b). In this context, Illmer and Schinner (1995) suggested that the OA released by the bacterial cells are not the only means of P-solubilization and, hence, acidification does not seem to be the only mechanism of solubilization, as the ability to reduce the pH in some cases did not correlate with the ability to solubilize mineral P. The chelating ability of the OA is also important, as it has been shown that the addition of 0.05 M EDTA to the medium has the same solubilizing effect as inoculation with *Penicillium bilaii* (Kucey 1988). In another study, Altomare et al. (1999) investigated the capability of the plant growth-promoting and bio-control fungus *Trichoderma harzianum* T-22 to solubilize in vitro insoluble minerals including rock phosphate. OA were not detected in culture filtrates and, hence, the authors concluded that the insoluble P could be solubilized by mechanisms other than acidification process. In this study, the fungal-solubilizing activity was attributed both to chelation and to reduction processes, which could also play a role in the bio-control of plant pathogens. Among nodule bacteria, e.g., *Rhizobium/Bradyrhizobium*, the PS activity of *Rhizobium* was associated with the production of 2-ketogluconic acid which was abolished by the addition of NaOH, indicating that PS activity of this organism was entirely due to its ability to reduce the pH of the medium (Halder and

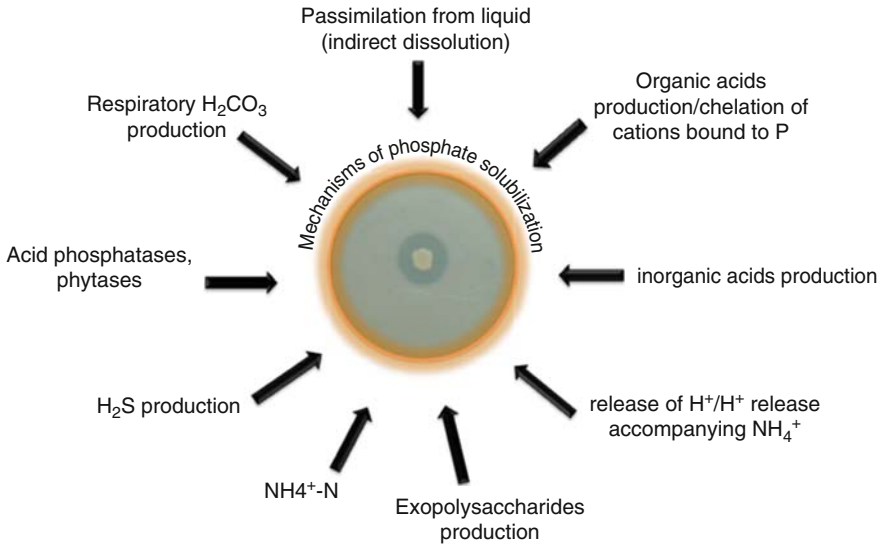



**Fig. 2.2** Mechanisms of P-solubilization by phosphate solubilizing bacteria

Chakrabarty 1993). However, the detailed biochemical and molecular mechanism of P-solubilization by symbiotic nodule bacteria are not conclusive. In addition to OA, the solubilization of insoluble P by inorganic acid, e.g., HCl (Kim et al. 1997), and involvement of the $H^+$ pump, as reported in *Penicillium rugulosum*, is suggested (Reyes et al. 1999).

Recently, four bacterial strains of *Enterobacter* sp. (EnHy-401), *Arthrobacter* sp. (ArHy-505), *Azotobacter* sp. (AzHy-510) and *Enterobacter* sp. (EnHy-402), possessing the ability to solubilize TCP, were used to assess the role of exopolysaccharide (EPS) in the solubilization of P (Yi et al. 2008). These PS bacteria produced a significant amount of EPS and demonstrated a strong ability for P-solubilization. Of these, the strain EnHy-401 with the highest EPS and OA production had a stronger capacity for P-solubilization than the others. Further studies demonstrated that addition of EPS into the medium could increase the amount of P-solubilized by OA, but it failed to release P from TCP alone. The synergistic effects of EPS and OA on TCP-solubilization varied with the origin and the concentration of EPS in medium. EPS produced by EnHy-401 was most effective in promoting P release at an optimal concentration in the medium. The increase in P-solubilization by EPS was attributed mainly to the participation of EPS which led to a change in the homeostasis of P-solubilization, pushing it towards P-dissolving by holding free P in the medium, consequently resulting in greater P released from insoluble P. It was, therefore, suggested that EPS with the ability of P-holding may be a novel important factor in the microbial dissolution of TCP except for OA. A general mechanism how PS organisms bring out solubilization of insoluble P is presented in Fig. 2.2.

### 2.3.1 Enzymatic Dissolution of Phosphates by Phosphate-Solubilizing Microbes

Organic compounds containing P is mineralized in soil by phosphatases, phytases and phosphonatases and C–P lyases. The efficiency of microbial phosphatases, a widely distributed exoenzyme, in dissolution and mineralization of organic P-compounds in the rhizosphere and P-uptake by plants has been reported (Rodriguez and Fraga 1999; Rodriguez et al. 2006). Among phosphatases, acid phosphatase (To-O et al. 2000) is commonly found in fungi (To-O et al. 1997; Omar and Abd-Alla 2000) and activates the release of inorganic P from P-esters (Nozawa et al. 1998). Acid phosphatase has been detected in vacuoles and vesicles (Ruch and Motta 1987; Saito 1995) and other intracellular and extracellular (Ruch and Motta 1987) organs of fungi like phenotypic mutants of *Aspergillus tubingensis* (Varenyam et al. 2007). For instance, the enzyme was found in the vacuoles of ungerminated conidia of *Colletotrichum graminicola,* and also during germination (Schadeck et al. 1998a, b). Under in vitro conditions, phosphatase activity is identified by a distinct zone of clearing (transparent yellow halos) around fungal colonies on Pikovskaya medium or agar plates supplemented with 1-naphthyl P as a chromogenic substrate (Goud et al. 2008). *Aspergillus*, *Emmericella* and *Penicillium,* isolated from arid and semiarid regions of India, have shown phytin and glycerophosphate hydrolyzing activity (Yadav and Tarafdar 2003). In this report, the extracellular (E) phosphatases released by fungi were less than their intracellular (I) counterpart, and the E:I ratio of fungi ranged from 0.39 to 0.86 for acid phosphatase and 0.29 to 0.41 for alkaline phosphatases. The efficiency of hydrolysis of organic P-compounds of fungi varied from 2.12 to 4.85 μg $min^{-1}g^{-1}$ for glycerophosphate to 0.92–2.10 μg $min^{-1}g^{-1}$ for phytin. The trend of efficiency was: *Aspergillus* sp. > *Emmericella* sp. > *Penicillium* sp. Similar phosphatase activity is also reported for *A. caespitosus* and *Mucor rouxii* (Guimarães et al. 2006). Another attractive use of P-dissolving enzymes is the solubilization of soil organic P through phytate degradation mediated by enzyme phytase. In its basic form, phytate is the primary source of inositol and the major stored form of P in plant seeds and pollen, and is a major component of organic P in soil (Richardson 1994). Although the ability of plants to obtain P directly from phytate is very limited, yet the growth and P-nutrition of *Arabidopsis* plants supplied with phytate was significantly improved when they were genetically transformed with the phytase gene (phyA) derived from *Aspergillus niger* (Richardson et al. 2001a). This led to an increase in P-nutrition to such an extent that the growth and P-content of the plant was equivalent to control plants supplied with inorganic P. A similar increase in utilization of inositol P by plants in the presence of PS fungus (*A. niger)* capable of producing phytase is reported (Richardson et al. 2001b; Vassilev et al. 2007). Therefore, developing inoculants with high phosphatase and phytase activity would be of great practical interest for augmenting plant nutrition and reducing P-pollution in soil.

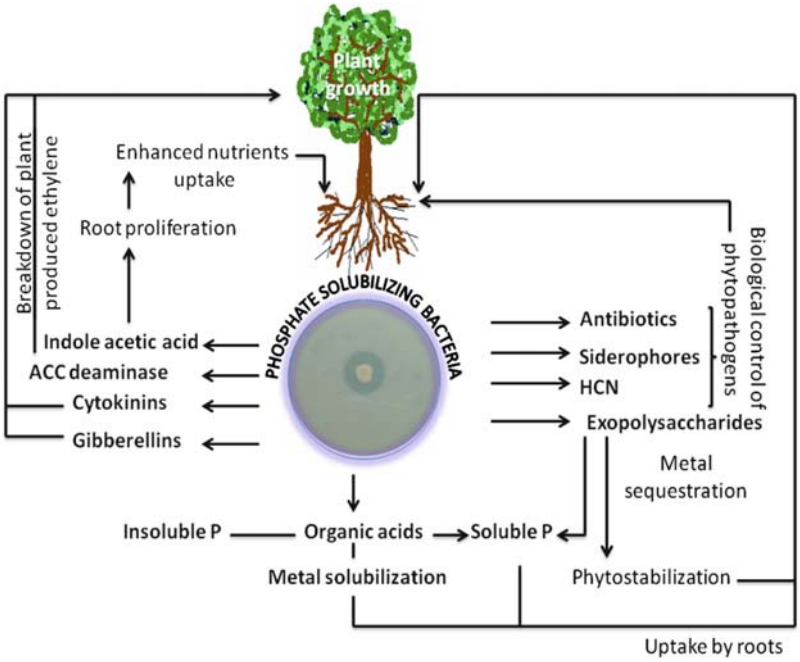



**Fig. 2.3** Mechanism of growth promotion by phosphate solubilizing bacteria

## 2.4 Mechanism of Plant Growth Promotion by P-Solubilizing Microbes

PS microbes are well known for making soluble P accessible for uptake by plants. They can also facilitate growth and development of plants by producing essential nutrients (Thomas et al. 2005) or by changing the concentration of plant growth-promoting substances including phytohormones such as indoleacetic acid (Wani et al. 2007a, b), through asymbiotic or symbiotic $N_2$ fixation (Zaidi 1999; Zaidi and Khan 2007), soil conditioning, exhibiting bio-control activity (Pandey et al. 2006), by synthesizing siderophores (Vassilev et al. 2006), antibiotics, and cyanide (Lipping et al. 2008), by synthesizing an ACC deaminase that can modulate plant ethylene levels ( Anandham et al. 2008; Poonguzhali et al. 2008), and by solubilizing or reducing the toxicity of metals (bioremediation) (Khan et al. 2009). These mechanisms can probably be active simultaneously or sequentially at different stages of plant growth. However, the intrinsic ability of PS microbes for synthesizing the growth-promoting substances varies considerably under different ecological niches. The mechanisms involved in plant growth promotion (Fig. 2.3) and growth regulators produced by PS organisms are presented in Table 2.3.

**Table 2.3** Growth-promoting substances released by phosphate solubilizing bacteria

| Phosphate solubilizing bacteria | Plant growth-promoting traits | References |
|---|---|---|
| *Dyella ginsengisoli, Burkholderia kururiensis, Pandoraea* sp. strain ATSB30 | Siderophore, IAA, salicylic acid, ACC deaminase | Anandham et al. (2008) |
| *Pseudomonas* sp. | ACC deaminase, IAA, siderophore | Poonguzhali et al. (2008) |
| *Bacillus subtilis* | IAA, siderophore, antifungal activity | Singh et al. (2008) |
| *Serratia marcescens* | IAA, siderophore, HCN | Selvakumar et al. (2008) |
| *Pseudomonas fluorescens* | ACC deaminase | Shaharoona et al. (2008) |
| *Acinetobacter* sp., *Pseudomonas* sp. | ACC deaminase, IAA, antifungal activity, $N_2$-fixation | Indiragandhi et al. (2008) |
| *Enterobacter* sp. | ACC deaminase, IAA, siderophore | Kumar et al. (2008) |
| *Burkholderia* | ACC deaminase, IAA, siderophore, heavy metal solubilization | Jiang et al. (2008) |
| *Pseudomonas jessenii* | ACC deaminase, IAA, siderophore, heavy metal solubilization | Rajkumar and Freitas (2008) |
| *Pseudomonas aeruginosa* | ACC deaminase, IAA, siderophore | Ganesan (2008) |
| *Azotobacter* sp., *Mesorhizobium* sp., *Pseudomonas* sp., *Bacillus* sp. | IAA, siderophore, antifungal activity, ammonia production, HCN | Ahmad et al. (2008) |
| *P. aeruginosa*, *P. plecoglossicida* and *P. mosselii* | Siderophore, IAA, protease, cellulase and HCN | Jha et al. (2008) |
| *Bacillus* spp. | IAA, siderophores, ammonia production, HCN, chromium reduction, metal solubilization | Wani et al. (2007a, 2007b, 2007c) |
| *Mesorhizobium loti* MP6 | HCN, IAA | Chandra et al. (2007) |
| *Pseudomonas sp., Bacillus sp.* | IAA, siderophore | Rajkumar et al. (2006) |

### 2.4.1 Phosphate-Solubilizing Microbes as Bio-Control Agent

Phosphate-solubilizing microorganisms as a group form an integral component of soils. In addition to providing P to plants, PS microorganisms also act as a bio-control agent and promote the growth of plants by suppressing the soilborne phytopathogens. Several in vitro studies show the potential of PS microorganisms for the simultaneous synthesis and release of pathogen-suppressing metabolites, mainly siderophores, and lytic enzymes (Pandey et al. 2006; Rane et al. 2008). Potential application of PS microbes as bio-control agents is reviewed and discussed.

Bio-control ability of PS bacterium *Pseudomonas aeruginosa* ID 4365, a bio-control agent of groundnut (*Arachis hypogaea*) phytopathogens of marine origin, was previously attributed to the production of pyoverdin type of siderophores. However, pyoverdin-rich supernatants of this organism have shown better antifungal activity compared to equivalent amounts of purified pyoverdin indicating the presence of undetected metabolite(s) in pyoverdin-rich supernatants. In addition

to pyoverdin, the strain produced additional siderophores, i.e., pyochelin and salicylic acid, and two broad spectrum antifungal compounds, i.e., pyocyanin and phenazine-1-carboxylic acid, and exhibited antifungal activity (Rane et al. 2008). Similarly, P-solubilizer *Pseudomonas putida* exhibited antifungal activity against phytopathogenic fungi in Petri dish assays, and produced chitinase, β-1,3-glucanase, salicylic acid, siderophore, and hydrogen cyanide. The plant growth-promotion and antifungal properties were demonstrated through a maize (*Zea mays*)-based bioassay under greenhouse conditions. Although the bacterial inoculation demonstrated a substantial increase in plant biomass, it stimulated bacterial and suppressed fungal counts in the rhizosphere. This study leads to a better understanding of the microbial diversity of the colder regions as well as to understanding the potential biotechnological applications of native microbes (Pandey et al. 2006). Among the fungal P-solubilizers, *Trichoderma* species are the most commonly studied bio-control microorganisms which also exhibit plant growth-promoting activity (Harman and Bjorkman 1998). While the mechanisms of bio-control have been well investigated, those responsible for the growth promotion by *T. harzianum* have not been extensively studied. In one study, Altomare et al. (1999) investigated the capability of the plant growth-promotion and bio-control activity of *T. harzianum* T-22 to solubilize in vitro insoluble minerals including RP. OA were not detected in the culture filtrates and, hence, the authors concluded that acidification was probably not the major mechanism of solubilization as the pH never fell below 5. The fungal-solubilizing activity was attributed both to chelation and to reduction processes, which also play a role in the bio-control of plant pathogens. A similar bio-control effect of PS filamentous fungi against *Fusarium* wilt in tomato (*Fusarium oxysporum* f. sp. *lycopersici*; *Fol*) was reported by Khan and Khan (2001). Root-dip applications of *B. subtilis*, *P. fluorescens*, *Aspergillus awamori, A. niger*, and *Penicillium digitatum* declined the rhizosphere population of Fol. Tomato yield was enhanced, being greatest with *A. awamori* and *P. digitatum*. Direct soil–plant inoculation with *A. niger*, *A. awamori*, and *P. digitatum* decreased the rhizosphere Fol population by 23–49% while the tomato yield increased by 28–53% in field experiments. The authors propose that OA produced by these microorganisms may inhibit fungal infection but that other metabolites such as bulbiformin and phenazin could also be involved, particularly in the treatments with *B. subtilis* and *P. fluorescens*. Research using *P. variabile* P16 demonstrated increased glucose oxidase (GOD) production in the presence of polysaccharides, which were found to serve as activators of defensive systems in this filamentous fungus (Petruccioli et al. 1999). In fact, GOD activity can play a significant role in antibiosis in the soil environment, and $H_2O_2$ enzymatically produced is cytotoxic for microorganisms. In another report, PS fungus *P. oxalicum* showed strong antibiotic activity against pathogenic fungi, including *Sclerotinia sclerotiorum*, a widespread pathogenic fungus that severely attacks rapeseed (*Brassica napus*) (Lipping et al. 2008). The combination of P-solubilization and bio-control activity of PSM(s) could prove very effective in promoting the growth of plants both in conventional and stressed soils of different agro-ecosystems.

**Table 2.4** Phosphate solubilizing microbes with bio-control activity

| Strain species | Bio-control activity | Metabolite with bio-control activity | References |
|---|---|---|---|
| *Pseudomonas aeruginosa* ID 4365 | + | Siderophores and phenazines | Rane et al. (2008) |
| *P. aeruginosa*, *P. plecoglossicida* and *P. mosselii* | + | Protease, cellulase | Jha et al. (2008) |
| *P. putida* | + | Chitinase, β-l,3-glucanase, salicylic acid, siderophore, and hydrogen cyanide | Pandey et al. (2006) |
| *Aspergillus niger* | + | Siderophores | Vassilev et al. (2006) |
| *A. niger, A. awamori Penicillium digitatum* | + | Organic acids | Khan and Khan (2001, 2002) |
| *P. variabile* P16 | − | Glucose oxidase | Petruccioli et al. (1999) |
| *Trichoderma harzianum* T22 | + | Siderophores | Altomare et al. (1999) |

+ Proved, − not proved: modified from Nikolay et al. (2006)

Some of the metabolites released both by PS bacteria and fungi are presented in Table 2.4.

## 2.5 Molecular Engineering of P-Solubilizing Bacteria

Studies at the molecular level in order to understand how precisely the PS microbes brings out the solubilization of insoluble P were inconclusive (Rodriguez et al. 2006). However, several phosphatase-encoding genes have been cloned and characterized, and a few genes involved in mps have been isolated (Table 2.5) and characterized (Fraga-Vidal et al. 2003; Krishnaraj and Goldstein 2001). Genetic manipulation of PS bacteria to improve their ability to improve plant growth may include cloning genes involved in both mps and organic P-solubilization (ops), followed by their expression in selected rhizobacterial strains. For example, Goldstein and Liu (1987) have shown that mps activity is genetically coded in a gene cluster on plasmids of microbes endowed with PS activity. They further transferred this gene cluster to an *E. coli* strain that did not previously possess PS activity but could demonstrate that the transferred gene was expressed in the transgenic *E. coli* strain. They have also found that the gene expression and mps activity of bacteria is affected by the presence of soluble P in the medium (feed-back regulation). Chromosomal insertion of these genes under appropriate promoters is another interesting approach (Rodriguez and Fraga 1999). Rodríguez et al. (2000) carried out a genetic construction using the broad host range vector pKT230 and plasmid pMCG898, which encode the *Erwinia herbicola* pyrroloquinoline quinone (PQQ) synthase, a gene involved in mps. The final construct was transformed and

**Table 2.5** Cloning of genes involved in mineral phosphate solubilization

| Microorganisms | Gene or plasmid | Features | Reference |
|---|---|---|---|
| *Serratia marcescens* | pKG3791 | Produces gluconic acid and solubilizes P | Krishnaraj and Goldstein (2001) |
| *Rahnella aquatilis* | pKIM10 | Solubilizes P and produces gluconic acid in E. coli DH5α | Kim et al. (1998) |
| *Enterobacter agglomerans* | pKKY | Solubilizes P in *E. coli* 109; Does not lower pH | Kim et al. (1997) |
| *Pseudomonas cepacia* | Gab Y | Produces gluconic acid and solubilizes mineral P in *E. coli* JM109; No homology with PQQ genes | Babu-Khan et al. (1995) |
| *Erwinia herbicola* | Mps | Produces gluconic acid and solubilizes mineral P in *E. coli* HB101; Probably involved in PQQ synthesis | Goldstein and Liu (1987) |

expressed in *E. coli* MC1061, and the recombinant plasmids were transferred to *Burkholderia cepacia* IS-16 and *Pseudomonas* sp. PSS recipient cells by conjugation. Clones containing recombinant plasmids produced higher clearing halos in plates with insoluble P as the unique (P) source, in comparison to those strains without plasmids, demonstrating the heterologous expression of the *E. herbicola* gene in the recipient strains. This genetic manipulation allowed the increase in mps ability of both strains, enhancing their potential as growth promoters of agricultural crops. In addition, the subcloning of the gene encoding the PhoC acid phosphatase from *Morganella morganii* (phoC gene), in a vector that permits stable chromosomal integration of this gene in plant growth-promoting bacteria, has been reported (Fraga-Vidal et al. 2003). Another important rhizosphere-competent bacteria (*Pseudomonas* spp.) can form gluconic acid through the oxidative metabolism and overexpression of PQQ synthase and glucose dehydrogenase (GDH) genes make them functionally a better PS organism. Interestingly, in addition to its role in P-solubilization, PQQ also plays an important role in beneficial traits, such as antifungal activity and induced systemic resistance (ISR) of *Enterobacter intermedium*, possibly by acting as a cofactor for several enzymes including GDH (Han et al. 2008). In yet another approach, the mps genes can directly be transferred into the target bacteria by over-/underexpression of genes followed by the selection of transformants with mps ability. Such an approach has been used to obtain mps genes from *Synechosystis* PCC 6803 in *E. coli* (Gyaneshwar et al. 1998). However, it remains to be seen if this will also be effective in other bacteria. Genetic engineering could also help in increasing the survival of the inoculant strains by incorporating the abilities to utilize certain nutrients better than the rest of the microbial populations (Glick and Bashan 1997). In addition, genes for utilization of salicylate were transferred to growth-promoting bacteria and the recombinant bacterium was able to survive and enhance plant growth better than the wild-type (Colbert et al. 1993).

## 2.6 Crop Improvement by P-Solubilizing Microbes

### *2.6.1 Inoculation Effects of Phosphate-Solubilizing Bacteria*

Phosphate-solubilizing bacteria in general enhance the growth of plant by providing soluble P to plants. However, they also affect the growth and development of plants by other mechanisms. For example, PS strains of *Pseudomonas* capable of synthesizing IAA, ACC deaminase, and siderophores in vitro, increased the root elongation and biomass of Chinese cabbage (*Brassica rapa)* but had no effect on P-uptake of plants, suggesting that the growth promotion by PS strain could be due to the production of phytohormones or mechanisms other than P-solubilization (Poonguzhali et al. 2008). Similarly, a significant increase in biomass and total P of winter wheat (*Triticum aestivum*) following a single inoculation of *Phosphobacterium* (strain 9320-SD) under both pot and field conditions has been reported; although no obvious difference was found in plant height (Chen et al. 2006). Cold-tolerant PS *Serratia marcescens* with inherent plant growth-promoting traits (IAA, HCN and siderophore production) has also been found to significantly enhanced plant biomass and nutrient uptake of wheat seedlings grown in cold temperatures (Selvakumar et al. 2008). Two plant growth-promoting PSB, *Pseudomonas fluorescens* and *P. fluorescens* biotype F, having ACC deaminase showed a profound effect on growth, yield, and nutrient use efficiency of wheat under simultaneously varying levels of all the three major nutrients, N, P, and K (at 0, 25, 50, 75, and 100% of recommended doses). Results of pot and field trials revealed that the growth-promoting efficacy of these strains decreased with increasing rates of NPK added to the soil. In most of the cases, significant negative linear correlations were recorded between percentage increases in growth and yield parameters of wheat caused by inoculation and increasing levels of applied NPK fertilizers. They speculated that, under low fertilizer application, the ACC deaminase activity of PS strains might have caused reduction in the synthesis of stress (nutrient)-induced inhibitory levels of ethylene in the roots through ACC hydrolysis into $NH_3$ and α-ketobutyrate. This study suggested that *Pseudomonads* could be used in combination with appropriate doses of fertilizers for better plant growth and savings of fertilizers (Shaharoona et al. 2008). A similar increase in biomass of maize plants following inoculation of two efficient strains of *Serratia marcescens* (EB 67) and *Pseudomonas* sp. (CDB 35) has been reported (Hameeda et al. 2008). Of these PS bacteria, strain EB 67 increased the biomass by 99% while strain CDB 35 demonstrated an increase of 94%. Increase in plant biomass at 48 and 96 days after sowing was 66 and 50%, respectively, with EB 67 and 51 and 18%, respectively, with CDB 35 under field conditions. Seed bacterization with strain EB 67 and CDB 35 increased the grain yield of field-grown maize by 85 and 64%, respectively. Similarly, *B. subtilis* having antifungal activity against *Macrophomina phaseolina* and other phytopathogens, including *Fusarium oxysporum* and *Rhizoctonia solani* and plant growth-promoting attributes (IAA and siderophore production) isolated from chirpine (*Pinus roxburghii*) rhizosphere, resulted in 44 and 94% increases in

root and shoot dry weights of chirpine, respectively (Singh et al. 2008). Phosphate-solubilizing *Acinetobacter* sp. (PSGB04) and *Pseudomonas* sp. (PRGB06) with plant growth-promoting traits ($N_2$ fixation, IAA, salicylic acid production) isolated from the larval guts of diamondback moths (*Plutella xylostella*) have also been tested for their effects on growth of canola (*Brassica napus*) and tomato (Indiragandhi et al. 2008). *Acinetobacter* sp. (PSGB04) significantly increased root length (41%), seedling vigor, and dry biomass (30%) of the canola test plants, whereas a substantial increase, greater than that of the control, was also recorded for the tomato plants when seeds were treated with *Pseudomonas* sp. (PRGB06) possessing antifungal activity.

Phosphate-solubilizing microbes cohabit in the rhizosphere with other agronomically beneficial microbes and could play an additive or synergistic role in the growth promotion of plants. Subsequently, dramatic increases in yield of various crops following seed or soil inoculation with PS organisms and other plant growth-promoting rhizobacteria (Afzal and Bano 2008) or AM fungus (Khan and Zaidi 2006; Ehteshami et al. 2007) under different agro-ecosystems have been reported. The inoculation of PS bacteria *Pseudomonas striata* and $N_2$-fixing bacterium *Rhizobium* sp. (*Vigna*) substantially increased the yield of greengram (*Vigna radiata* L.) (Khan et al. 1997, 1998) and nodulation, available P of soil as well as dry matter of the plants, grain yield, and P- and N-uptake by chickpea (*Cicer aeritinum*) (Wani et al. 2007b, c) compared to single inoculation of either PS bacteria or $N_2$ fixers. Similarly, the seed inoculation of chickpea with single, dual and triple inoculations of *Rhizobium*, *Bacillus subtilis* (OSU-142) and PS *Bacillus megaterium* (M-3) increased biological and chemical properties of chickpea (Elkoca et al. 2008). Substantial increases in the seed yield under different inoculation treatments ranged between 18% (*Rhizobium*) and 31% (*Rhizobium* + OSU-142 + M-3) over control. Generally, the increases in seed and total biomass yields were more pronounced in dual and triple inoculations. In another study, Wani et al. (2007c) demonstrated synergistic effects of $N_2$-fixing and PS rhizobacteria on chickpea plants. Legume grain yield and concentration and uptake of N and P were significantly increased following coinoculation with *Mesorhizobium* and PS *Pseudomonas* and *Bacillus* spp. The inoculation of *M. ciceri* with *Azotobacter chroococcum* and *Bacillus* tripled the seed yield and resulted in the highest grain protein (295 mg $g^{-1}$). An 8% increase in P concentration above the uninoculated control was observed in the case of a single inoculation with *Pseudomonas*, while the P-uptake was highest (2.14-fold above the uninoculated control) when *M. ciceri* was applied with *A. chroococcum* and *Bacillus*. They showed that the multiple inoculations with rhizospheric microorganisms can synergistically facilitate growth and yields and increase concentrations and uptake of N and P by field-grown chickpea, as also reported by Valverde et al. (2006). In another study, Canbolat et al. (2006) observed a significant increase in seedling growth, total dry matter accumulation and available P in barley (*Hordeum vulgare*) bioprimed with $N_2$-fixing and P-dissolving *Bacilli* suggesting that the $N_2$-fixing and PS bacterial strains could be used to save fertilizer application. It is also reported that PS *P. putida*, possessed with antifungal activity, antibiotic resistance and ability to

produce chitinase, β-1,3-glucanase, salicylic acid, siderophore, and HCN significantly increased the plant biomass in a maize-based bioassay under greenhouse conditions (Pandey et al. 2006). The effect of a combined inoculation of *Rhizobium,* a P-solubilizing *B. megaterium* subsp. *phosphaticum* strain-PB and a bio-control fungus *Trichoderma* spp. on the performance of chickpea under glasshouse and field conditions is also reported (Rudresh et al. 2005b). Tripartite inoculations exhibited increased germination, nutrient uptake, plant height, number of branches, nodulation, yield, and total biomass of chickpea compared to either individual inoculations or an uninoculated control. Increased growth and yield parameters were more pronounced when *T. harzianum* was inoculated along with the PS bacterium and *Rhizobium*.

Furthermore, the PS bacteria forms a strong association with AM fungi in P-deficient soils or RP-enriched soils and can release some P-ions from an otherwise sparingly soluble P source, which is tapped and translocated by the AM fungal hyphae to the plants. In this context, the composite inoculation of PS bacteria and AM fungi has been found to improve plant growth in a more sustainable manner in soil deficient in P (Zaidi et al. 2004; Zaidi and Khan 2007). During this intergeneric interaction, the AM fungi increases plant growth via improved uptake of nutrients, especially P, due to the exploration by the external hyphae of the soil beyond the root-hair zone where P is depleted. The AM fungi also produce plant hormones and increase the activity of $N_2$-fixing organisms in the root zone. In contrast, the PS bacteria could alter the composition of root exudates and plasticity which in turn may affect the colonization and development of AM fungi. These factors together facilitate the colonization and establishment of AM fungus onto the root system of plants and also alter microbial composition of rhizospheres, which in turn affect the competition between inoculated and native soil organisms. However, a thorough understanding of interactions between organisms of agronomic importance is required so that a bio-inoculant with multifaceted activity could be developed.

Phosphate-solubilizing microbes are reported to reduce the toxicity of metals and protect the plants against the toxic effects of these metals and consequently enhance the growth and yield of plants in contaminated soils (Wani et al. 2007a). For instance, Rajkumar et al. (2006) assessed the plant growth-promoting activity of Cr (vi) resistant plant growth-promoting bacteria having PS potential, *Pseudomonas* sp. and *Bacillus* sp., recovered from heavy metal contaminated soils, on the Indian mustard (*Brassica juncea*) with different concentrations of Cr (vi) added to soil. Inoculation of both strains promoted the growth of plants at 95.3 and 198.3 g of Cr (vi)/g soil by protecting the plants against the inhibitory effects of chromium, and probably due to the solubilization of P and production of IAA and siderophores. In another report, a heavy metal- and antibiotic-resistant bacterial strain, *Burkholderia* sp., isolated from metal-contaminated soils capable of solubilizing inorganic P, producing IAA, siderophore and ACC deaminase, was found to appreciably increase the biomass of maize and tomato plants (Jiang et al. 2008). Rajkumar and Freitas (2008) reported that the inoculation of metal-resistant plant growth-promoting *Pseudomonas sp. (*PsM6) and *P. jessenii* (PjM15) strains possessing intrinsic ability of P-solubilization ability for the utilization of ACC as the sole N source and

production of IAA. increased the growth and the uptake of Ni, Cu and Zn by *Ricinus communis,* grown in noncontaminated and contaminated soil. Application of cadmium-resistant plant growth-promoting *P. aeruginosa* exhibiting P-solubilization, ACC deaminase activity, siderophore production, and auxin synthesis, when used as inoculant for blackgram (*Vigna mungo* L.*)* plants grown in soil treated with a gradient of $CdCl_2$ concentration, reduced the toxicity of metal to plants (Ganesan 2008) . These and other associated data suggest that such PS microbes could be used as biofertilizer not only to provide P to plants but could also play an important role in reducing/detoxifying the effects of heavy metals in derelict soils.

### 2.6.2 Inoculation Effects of Phosphate-Solubilizing Fungi

The inoculation of P-solubilizing fungi is a promising technique because it can increase P-availability in soils fertilized with RP. Accordingly, several authors have reported a profound increase in dry weight yield, and in N- and P-contents of *Brassica chinensis* Linn. (Chuang et al. 2007) and soybean and faba bean (*Vicia faba*) (Abd-Alla et al. 2001) through inoculation of PS fungi. Recently, Mittal et al. (2008) observed the effect of six PS fungi, including two strains of *A. awamori* and four strains of *P. citrinum*, on growth and seed production of chickpea cv. GPF2 plants. The PS fungi was biocompatible and produced growth-promoting hormone, indole acetic acid (IAA), varying in concentration from 2.5 to 9.8 g $ml^{-1}$. Inoculation of four strains of *P. citrinum* exhibited a smaller stimulatory effect and increased the shoot height by 7%, seed number by twofold, and seed weight by 87% above uninoculated plants. However, a consortium of all the six fungal isolates showed no stimulatory effect on chickpea plants. Similarly, the inoculation of *P. oxalicum* (CBPS-3F-Tsa) used either alone or along with fused phosphates (FP) and RP, increased the growth and N and P accumulation in maize plants compared to control (Shin et al. 2006), while the mutant strain of *P. regulosum* ($Mps^{+}$) also stimulated the growth of maize plants as indicated by a 3.6–28.6% increase in dry matter yield. In the presence of RP, P-uptake by maize plants inoculated with the two mutants, $Mps^{++}$ and $Mps^{-}$, was not always in agreement with their P-solubilizing phenotypes (Reyes et al. 2002). Also, a substantial increase in plant height (1.4 times), plant weight (5.2–8.1 times) and root length (1.1–1.2 times) of maize following inoculation of PS fungus *Penicillium* sp. PS-113 is reported (Kang and Choi 1999). Babana and Antoun (2006) isolated two TPR (Tilemsi phosphate rock)-solubilizing fungi (TSM) *A. awamori* Nakazawa C1 and *P. chrysogenum* Thom C13 with high P-solubilizing trait from the rhizosphere of three wheat cultivars (Alkama Beri, Hindi Tossom and Tetra) and tested against wheat cv. Tetra fertilized with 30 kg $ha^{-1}$ P (added as TPR or DAP). *A. awamori* Nakazawa C1 increased the root dry matter yields by 60% while *P. chrysogenum* Thom C13 enhanced the root dry matter yields by 40%. A 3-year field trial on a Gangetic alluvial soil of India was conducted to examine how P demands of rice (*Oryza sativa*)–wheat cropping systems might be met with heavy initial dressings of RP.

Preplant inoculation of rice seedling roots or wheat seeds with P-solubilizing fungus (*A. Awamori)* led to a yield increase over noninoculated treatments of 0.09–0.22 $tha^{-1}$ in rice and 0.15–0.45 $tha^{-1}$ in wheat. The agronomic efficiency and recovery efficiency of fertilizer P in the rice–wheat system were highest (57.2 kg grain/kg P and 40.4%, respectively) under DAP-fertilized treatments. The agronomic efficiency ranged from 14.3 to 44.4 kg grain/kg P and the recovery efficiency ranged from 7.7 to 26.4% for phosphate rock treatments. The agronomic efficiency and recovery efficiency increased with increasing initial phosphate rock application rate and with P-solubilizing fungus inoculation (Dwivedi et al. 2004). In another study, PS fungi, *A. niger*, *A. fumigatus* and *P. pinophilum*, significantly increased the yield components of wheat and faba bean plants, *P. pinophilum* being the most efficient, which increased the yield of wheat grains by 28.9 and 32.8% in the soil treated with RP and superphosphate, respectively. Similarly, it increased the production of faba bean seeds by 14.7 and 29.4% with the same treatments, and the uptake of P by both plants significantly increased due to inoculation of the soil with the tested fungi (Wahid and Mehana 2000). Inoculation of *Trichoderma* spp. has also shown a substantial increase in growth and yield parameters of chickpea under both glasshouse and field trials (Rudresh et al. 2005).

Volcanic soils in the south of Chile have an elevated quantity of total P, which is hardly available due to its high P-fixation capacity. One strategy for increasing the availability of P for the vegetables that grow there would be to use PS fungi. In one assay conducted in a greenhouse on a volcanic soil, the effect of inoculation with *Penicillium albidum* on the growth of red clover (*Trifolium pratense*) including active inoculum [In (+)], inactive inoculum [In (−)] and without inoculum [In (0)] was reported. The In (+) significantly increased the root growth of the plants and the P mobilized by the shoot with In (+) was twofold higher than in the In (0) or In (−) treatments. In soil, available P was not different among the treatments but phosphatase activity in In (+) was higher in comparison to In (0). The author suggested that *P. albidum* could be developed as fungal inoculant to increase the productivity of crops in volcanic soils of Chile (Morales et al. 2007). Wakelin et al. (2007) tested the PS fungi *P. radicum*, *P. bilaiae* (strain RS7B-SD1), and an unidentified *Penicillium* sp. designated strain KC6-W2 for their ability to increase the growth and P nutrition of wheat and lentil (*Lens culinaris)* in three soils of neutral to alkaline pH reaction. The strongest plant growth-promoting strain was found *Penicillium* sp. (KC6-W2), which increased shoot growth and dry mass. Levels of increase by *Penicillium* sp. KC6-W2 ranged from 6.6 to 19% and were associated with increased uptake of P to the shoot. Inoculation of seed with *P. radicum* increased lentil growth by 5.5% in soil from Tarlee but did not affect plant growth in the eight other experiments. However, when significant, stimulation of plant growth promotion by *P. bilaiae* RS7B-SD1 was strong and showed a 15% increase in lentil shoot dry matter.

Nitrogen and P are the two major plant nutrients whose composite inoculation has shown a greater impact on the performance of various crops than those observed for single inoculation treatments. For example, when PS bacterium (*Bacillus* ) and asymbiotic $N_2$ fixer (*A. chroococcum*) were used in combination with PS fungus,

*P. variabile,* significantly enhanced the seed yield, grain protein and N- and P-accumulation in wheat (Khan and Zaidi 2007). The increase in overall performance of wheat was attributed to the ability of PS fungi to solubilize inorganic P. During interactions, the phytostimulator, *A. chroococcum*, besides providing N, produced considerable amounts of growth-promoting substances in the rhizosphere (Kucey et al. 1989). Combining an improved nutrient supply with N (*A. chroococcum*) and P (PS fungus) with plant growth promotion appears to have additive and possibly even multiplicative effects. Furthermore, a plant growth-promoting rhizobacterium, *Azospirillum brasilense* SP7, when applied with a bio-control fungus possessing PS activity (*T. harzianum* Rifai 1295-22) and RP at 1 mg $ha^{-1}$, significantly increased seed yield, total N and total P of field-grown beans (Mehmet et al. 2005). However, variations in the effectiveness of microbial combinations under field conditions are reported which may possibly be due to (1) variations in the survivability and colonization efficiency of the inoculated microbial cultures in the soils, (2) strong competition from the natural microbiota of the field soils, leading possibly to the exclusion of inoculated cultures from the rhizosphere, (3) differential rhizosphere effect of plants in harboring a target microbial strain (Pal 1998), (4) the modulation of the PS activity by specific root exudates (Goldstein et al. 1999; Dakora and Phillips 2002), (5) availability of inadequate nutrients in the rhizosphere to produce enough OA, (6) variation in the persistence of PS activity, and (7) genetic instability among inoculated strains. Furthermore, there are reports that also suggest the inhibitory effect of fungi on crop improvement (Zaidi et al. 2003). This inhibition has been due to high OA-secreting ability of fungi which in turn limits the growth of even associative partners that require neutral or alkaline growth conditions for their active metabolism. Moreover, the inhibitory effect of PS fungi on the associative partners could be due to the release of toxins in the growing environment which might affect the functional symbioses between rhizobia and their specific host plants. These data suggests that, before carrying out in situ experiments, the compatibility between the two associate members must be checked in vitro.

The use of AM fungi has been shown to possess the ability to increase nutrient uptake of plants by developing associations with roots of more than 80% of higher plant species (Marschner and Dell 1994; Schreiner et al. 1997). The combined inoculation of $N_2$ fixers, P-solubilizers and AM fungi has been found to stimulate plant growth more than inoculation of either organism used alone in certain situations when the soil is P-deficient (Khan et al. 2007). During this intergeneric pairing, the P-solubilizers interact well with the AM fungi in P-deficient soils or soils having RP (Poi et al. 1989). The PS fungi release some P-ions from otherwise sparingly soluble P sources which is tapped and translocated by the AM fungal hyphae to the plant (Azcon-Aguilar et al. 1986). Moreover, the P-solubilizers survives longer around mycorrhizal roots compared to nonmycorrhizal roots, and acts synergistically with AM fungi leading to increased plant growth. In this context, the coinoculation of AM fungus (*G. fasciculatum*) and PS fungus (*P. variabile* or *Penicillium* sp.) has been shown to facilitate chickpea (Zaidi and Khan 2007) and greengram (Khan and Zaidi 2006) growth more than single inoculation. However, when *Mesorhizobium* or *Bradyrhizobium* sp. (*Vigna*) was

simultaneously applied with dual inoculation of AM fungus and P-solubilizing fungi, the seed yield, grain protein and nutrient (N and P) accumulation was further enhanced in these legumes. The simplest interpretation of this fact is that mycorrhizal endophyte could be stimulated in quantity, efficiency, and longevity. The main effect of this mycorrhiza in improving plant growth is through improved uptake of nutrients, especially P, due to the exploration by the external hyphae of the soil beyond the root-hair zone where P is depleted. In addition, root exudation and plasticity might change by the activity of PS fungi, which could also affect mycorrhizal development. Moreover, the triple inoculation of nitrogen fixer (*Mesorhizobium*/*Bradyrhizobium)*, PS fungus (*P. variabile)* and AM fungus (*G. fasciculatum*) synergistically enhanced the dry matter accumulation, seed yield, grain protein and N and P of chickpea (Zaidi et al. 2003; Zaidi and Khan 2007) and greengram (Khan and Zaidi 2006). These results strongly suggested that a relationship existed between root colonization, P-uptake and growth promotion, which in turn profoundly enhanced the yield of crops.

## 2.7 Conclusion

In agricultural practices, to circumvent the P-deficiency, chemical P fertilizers are applied. However, the excessive and injudicious applications of these fertilizers leads to a severe threat to microbial diversity, soil microbial community structure, soil fertility and consequently the productivity of crops in different agro-ecosystems. Microbiologists and soil scientists are thus searching for an alternative to these P problems. Since the majority of soils the world over are deficient in plant-available P and since phosphatic fertilizers are expensive, focus is placed on the use of soil microorganisms endowed with PS ability, which could be used as inoculants to mobilize P from poorly available sources in soil. The use of such PSM(s) opens up a new horizon for better plant productivity besides reducing the reliance on chemical P and protecting the agro-ecosystems from the hazards of agrochemicals. The protection of the soil environment by applying PSM(s) could become a major breakthrough for plants grown in derelict soils. Moreover, the molecular engineering of these microbes has also provided a new insight into the promotion of crops in P-deficient soils. In this context, novel, genetically engineered and soil- and region-specific PSM(s) and technologies have to be developed, pilot tested and transferred to farmers in a relatively short time in order to improve plant P-nutrition and agro-ecosystem sustainability.

## References

Abril A, Zurdo-Piñeiro JL, Peix A, Rivas R, Velázquez E (2007) Solubilization of phosphate by a strain of *Rhizobium leguminosarum* bv. *trifolii* isolated from *Phaseolus vulgaris* in El Chaco Arido soil (Argentina). In: Velazquez E, Rodriguez-Berrueco C (eds) Book Series: Developments in Plant and Soil Sciences. Springer, The Netherlands, pp 135–138

Abd-Alla MH, Omar SA, Omar SA (2001) Survival of rhizobia/bradyrhizobia and a rock phosphate solubilising fungus on various carriers from some agro-industrial wastes and their effects on nodulation and growth of fababean and soybean. J Plant Nutr 24: 261–272

Afzal A, Bano A (2008) *Rhizobium* and phosphate solubilizing bacteria improve the yield and phosphorus uptake in wheat (*Triticum aestivum*). Int J Agric Biol 10:85–88

Ahmad F, Ahmad I, Khan MS (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol Res 163:173–181

Alikhani HA, Saleh-Rastin N, Antoun H (2007) Phosphate solubilization activity of rhizobia native to Iranian soils. In: Velazquez E, Rodriguez-Berrueco C (eds) Book Series: Developments in plant and soil sciences. Springer, The Netherlands, pp 135–138

Altomare C, Norvell WA, Bjorkman T, Harman GE (1999) Solubilization of phosphates and micronutrients by the plant growth promoting and biocontrol fungus *Trichoderma harzianum rifai* 1295–22. Appl Environ Microbiol 65:2926–2933

Anandham R, Gandhi PI, Madhaiyan M, Sa T (2008) Potential plant growth promoting traits and bioacidulation of rock phosphate by thiosulfate oxidizing bacteria isolated from crop plants. J Basic Microbiol . doi:10.1002/jobm.200700380

Asea PEA, Kucey RMN, Stewart JWB (1988) Inorganic phosphate solubilization by two *Penicillium* species in solution culture and soil. Soil Biol Biochem 20:459–464

Azcon-Aguilar C, Diaz-Rodriguez R, Barea JM (1986) Effect of soil microorganisms on spore germination and growth on the vesicular arbuscular mycorrhizal fungus *Glomus moseae*. Trans Br Mycol Soc 86:337–340

Babana AH, Antoun H (2006) Biological system for improving the availability of Tilemsi phosphate rock for wheat (*Triticum aestivum* L.) cultivated in Mali. Nutr Cycl Agroecosys 76:285–295

Babu-Khan S, Yeo C, Martin WL, Duron MR, Rogers R, Goldstein A (1995) Cloning of a mineral phosphate-solubilizing gene from *Pseudomonas cepacia*. Appl Environ Microbiol 61:972–978

Bar-Yosef B, Rogers RD, Wolfram JH, Richman E (1999) *Pseudomonas cepacia* mediated rock phosphate solubilization in kaolinite and montmorillonite suspensions. Soil Sci Soc Am J 63:1703–1708

Batjes NH (1997) A world data set of derived soil properties by FAO–UNESCO soil unit for global modelling. Soil Use Manage 13:9–16

Bishop ML, Chang AC, Lee RWK (1994) Enzymatic mineralization of organic phosphorus in a volcanic soil in Chile. Soil Sci 157:238–243

Bojinova D, Velkova R, Ivanova R (2008) Solubilization of Morocco phosphorite by *Aspergillus niger*. Biores Technol 99:7348–7353

Brookes PC, Powlson DS, Jenkinson DS (1984) Phosphorus in the soil microbial biomass. Soil Biol Biochem 16:169–175

Canbolat MY, Bilen S, Cakmakci R, Sahin F, Aydin A (2006) Effect of plant growth-promoting bacteria and soil compaction on barley seedling growth, nutrient uptake, soil properties and rhizosphere microflora. Biol Fertil Soils 42:350–357

Chandra S, Choure K, Dubey RC, Maheshwari DK (2007) Rhizosphere competent *Mesorhizobium loti* mp6 induces root hair curling, inhibits *Sclerotinia sclerotiorum* and enhances growth of Indian mustard (*Brassica campestris*). Brazil J Microbiol 38:124–130

Chen YP, Rekha PD, Arun AB, Shen FT, Lai WA, Young CC (2006) Phosphate solubilizing bacteria from subtropical soil and their tricalcium phosphate solubilizing abilities. Appl Soil Ecol 34:33–41

Chen Z, Ma S, Liu LL (2008) Studies on phosphorus solubilizing activity of a strain of phosphobacteria isolated from chestnut type soil in China. Biores Technol 99:6702–6707

Chuang CC, Kuo YL, Chao CC, Chao WL (2007) Solubilization of inorganic phosphates and plant growth promotion by *Aspergillus niger*. Biol Fertil Soils 43:575–584

Chung H, Park M, Madhaiyan M, Seshadri S, Song J, Cho H, Sa T (2005) Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. Soil Biol Biochem 37:1970–1974

Colbert SF, Hendson M, Ferri M, Schroth MN (1993) Enhanced growth and activity of a biocontrol bacterium genetically engineered to utilize salicylate. Appl Microbiol 59:2071–2076

Cunningham J, Kuiack C (1992) Production of citric and oxalic acids and solubilization of calcium phosphate by *Penicillium bilaii*. Appl Environ Microbiol 58:1451–1458

Dakora FD, Phillips DA (2002) Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant Soil 245:35–47

De Freitas JR, Banerjee MR, Germida JJ (1997) Phosphate-solubilizing rhizobacteria enhance the growth and yield but not phosphorus uptake of canola (*Brassica napus* L.). Biol Fertil Soils 24:358–364

Dwivedi BS, Singh VK, Dwivedi V (2004) Application of phosphate rock, with or without *Aspergillus awamori* inoculation, to meet phosphorus demands of rice–wheat systems in the Indo–Gangetic plains of India. Aust J Exp Agric 44:1041–1050

Ehteshami SMR, Aghaalikhani M, Khavazi K, Chaichi MR (2007) Effect of phosphate solubilizing microorganisms on quantitative and qualitative characteristics of maize (*Zea mays* L.) under water deficit stress. Pak J Biol Sci 10:3585–3591

Elizabeth P, Miguel S, Ball Maria M, Andrés YL (2007) Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region. Soil Biol Biochem 39:2905–2914

Elkoca E, Kantar F, Sahin F (2008) Influence of nitrogen fixing and phosphorus solubilizing bacteria on the nodulation, plant growth, and yield of chickpea. J Plant Nutr 31:157–171

Fenice M, Selbman L, Federici F, Vassilev N (2000) Application of encapsulated *Penicillium variabile* P16 in solubilization of rock phosphate. Biores Technol 73:157–162

Fernández LA, Zalba P, Gómez MA, Sagardoy MA (2007) Phosphate-solubilization activity of bacterial strains in soil and their effect on soybean growth under greenhouse conditions. Biol Fertil Soils 43:805–809

Fraga-Vidal R, Rodriguez HM, De Villegas TG (2003) Vector for chromosomal integration of the phoC gene in plant growth-promoting bacteria. In: Velazquez E, Rodrguez-Barrueco C (eds) First international meeting on microbial phosphate solubilization. Springer, Berlin, pp 239–244

Ganesan V (2008) Rhizoremediation of cadmium soil using a cadmium-resistant plant growth-promoting rhizopseudomonad. Curr Microbiol 56:403–407

Glick BR, Bashan Y (1997) Genetic manipulation of plant growth promoting bacteria to enhance biocontrol of phytopathogens. Biotechnol Adv 15:353–378

Goldstein AH (1986) Bacterial solubilization of mineral phosphates: historical perspectives and future prospects. Am J Altern Agric 1:57–65

Goldstein AH (1994) Involvement of the quinoprotein glucose dehydrohenase in the solubilization of exogenous phosphates by gran-negative bacteria. In: Torriani-Gorini A, Yagil E, Silver S (eds) Phosphate in microorganisms: cellular and molecular biology. ASM, Washington DC, pp 197–203

Goldstein AH, Liu ST (1987) Molecular cloning and regulation of a mineral phosphate solubilizing gene from *Erwinia herbicola*. Biotechnology 5:72–74

Goldstein AH, Braverman K, Osorino N (1999) Evidence for mutualism between a plant growing in a phosphate limited desert environment and a mineral phosphates solubilizing (MPS) rhizobacterium. FEMS Microbiol Ecol 30:295–300

Goud MJP, Goud JVS, Charya MAS (2008) Replica plate screening method for detecting phosphatase activity in basidiomycetes using 1-napthyl phosphate as a chromogenic substrate. Sci World J 3:13–15

Guimarães LHS, Peixoto-Nogueira SC, Michelin M, Rizzatti ACS, Sandrim VC, Zanoelo F, Aquino ACMM, Junior AB, De Lourdes M, Polizeli TM (2006) Screening of filamentous fungi for production of enzymes of biotechnological interest. Brazil J Microbiol 37:474–480

Gupta RR, Singal R, Shanker A, Kuhad RC, Saxena RK (1994) A modified plate assay for secreening phosphate solubilizing microorganisms. Gen Appl Microbiol 40:255–260

Gyaneshwar P, Naresh K, Parekh LJ (1998) Cloning of mineral phosphate solubilizing genes from *Synechocystis* PCC 6803. Curr Sci 74:1097–1099

Halder AK, Chakrabarty PK (1993) Solubilization of inorganic phosphate by *Rhizobium*. Folia Microbiol 38:325–330

Hamdali H, Hafidi M, Virolle MJ, Ouhdouch Y (2008) Rock phosphate-solubilizing Actinomycetes: screening for plant growth-promoting activities. World J Microbiol Biotechnol 24:2565–2575

Hameeda B, Harini G, Rupela OP, Wani SP, Reddy G (2008) Growth promotion of maize by phosphate-solubilizing bacteria isolated from composts and macrofauna. Microbiol Res 163:234–242

Han SH, Kim CH, Lee JH, Park JY, Cho SM, Park SK, Kim KY, Krishnan HB, Kim YC (2008) Inactivation of pqq genes of *Enterobacter intermedium* 60–2G reduces antifungal activity and induction of systemic resistance. FEMS Microbiol Lett 282:140–146

Harman GE, Bjorkman T (1998) Potential and existing uses of *Trichoderma* and *Gliocladium* for plant disease control and plant growth enhancement. In: Harman GE, Kubicek CP (eds) *Trichoderma* and *Gliocladium*, vol 2. Taylor and Francis, London, UK, pp 229–265

Henri F, Laurette NN, Annette D, John Q, Wolfgang M, François-Xavier E, Dieudonne N (2008) Solubilization of inorganic phosphates and plant growth promotion by strains of *Pseudomonas fluorescens* isolated from acidic soils of Cameroon. Afr J Microbiol Res 2:171–178

Hinsinger P (2001) Bio-availability of soil inorganic P in the rhizosphere as affected by root induced chemical changes: a review. Plant Soil 237:173–195

Hwangbo H, Park RD, Kim YW, Rim YS, Park KH, Kim TH, Such JS, Kim KY (2003) 2-ketogluconic acid production and phosphate solubilization by *Enterobacter intermedium*. Curr Microbiol 47:87–92

Illmer P, Schinner F (1992) Solubilization of inorganic phosphates by microorganisms isolated from forest soil. Soil Biol Biochem 24:389–395

Illmer P, Schinner F (1995) Solubilization of inorganic calcium phosphates-solubilization mechanisms soil. Soil Biol Biochem 27:257–263

Illmer P, Barbato A, Schinner F (1995) Solubilization of hardly soluble AlPO4 with P-solubilizing microorganisms. Soil Biol Biochem 27:265–270

Indiragandhi P, Anandham R, Madhaiyan M, Sa TM (2008) Characterization of plant growth-promoting traits of bacteria isolated from larval guts of diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae). Curr Microbiol 56:327–333

Jha BK, Pragash MG, Cletus J, Raman G, Sakthivel N (2008) Simultaneous phosphate solubilization potential and antifungal activity of new fluorescent pseudomonad strains, *Pseudomonas aeruginosa, P. plecoglossicida* and *P. mosselii*. World J Microbiol Biotechnol. doi:10.1007/s11274-008-9925-x

Jiang C, Sheng X, Qian M, Wang Q (2008) Isolation and characterization of a heavy metal-resistant *Burkholderia* sp. from heavy metal-contaminated paddy field soil and its potential in promoting plant growth and heavy metal accumulation in metal-polluted soil. Chemosphere 72:157–164

Kang SC, Choi MC (1999) Solid culture of phosphate solubilising fungus. Sanoeb Misaegmul Haghoeji 27:1–7

Khan MR, Khan SM (2001) Biomanagement of *Fusarium* wilt of tomato by the soil application of certain phosphate-solubilizing microorganisms. Int J Pest Manag 47:227–231

Khan MR, Khan SM (2002) Effect of root-dip treatment with certain phosphate-solubilizing microorganisms on the *Fusarium* wilt of tomato. Biores Technol 85:213–215

Khan MS, Zaidi A (2006) Influence of composite inoculations of phosphate solubilizing organisms and an arbuscular mycorrhizal fungus on yield, grain protein and phosphorus and nitrogen uptake by greengram. Arch Agron Soil Sci 52:579–590

Khan MS, Zaidi A (2007) Synergistic effects of the inoculation with plant growth promoting rhizobacteria and arbuscular mycorrhizal fungus on the performance of wheat. Turk J Agric For 31:355–362

Khan MS, Aamil M, Zaidi A (1997) Associative effect of Bradyrhizobium sp. (vigna) and phosphate solubilizing bacteria on moongbean [*Vigna radiata*(L.) wilczek]. Biojournal 10:101–106

Khan MS, Aamil M, Zaidi A (1998) Moongbean response to inoculation with nitrogen fixing and phosphate solubilizing bacteria. In: Deshmukh AM (ed) Biofertilizers and biopesticides. Technoscience, Jaipur, pp 40–48

Khan MR, Khan SM, Mohiddin FA (2007a) Effect of certain fungal and bacterial phosphate solubilizing microorganisms on the fusarial wilt of tomato. In: Velázquez E, Rodríguez-Barrueco C (eds) First International Meeting on Microbial Phosphate Solubilization. Springer, The Netherlands, pp 357–361

Khan MS, Zaidi A, Wani PA (2007b) Role of phosphate solubilizing microorganisms in sustainable agriculture: A review. Agron Sustain Dev 27:29–43

Khan MS, Zaidi A, Wani PA, Oves M (2009) Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. Environ Chem Lett 7: 1–19

Kim KY, Mcdonald GA, Jordan D (1997) Solubilization of hydroxypatite by *Enterobacter agglomerans* and cloned *Escherichia coli* in culture medium. Biol Fertil Soils 24:347–352

Kim KY, Jordan D, Krishnan HB (1998) Expression of genes from *Rahnella aquatilis* that are necessary for mineral phosphate solubilization in Escherichia coli. FEMS Microbiol Lett 159:121–127

Krishnaraj PU, Goldstein AH (2001) Cloning of a *Serratia marcescens* DNA fragment that induces quinoprotein glucose dehydrogenase-mediated gluconic acid production in *Escherichia coli* in the presence of stationary phase *Serratia marcescens*. FEMS Microbiol Lett 205:215–220

Kucey RMN (1988) Effect of *Penicillium bilaji* on the solubility and uptake of P and micronutrients from soil by wheat. Can J Soil Sci 68:261–270

Kucey RMN, Janzen HH, Legget ME (1989) Microbial mediated increases in plant available phosphorus. Adv Agron 42:199–228

Kumar V, Behl RK, Narula N (2001) Establishment of phosphate-solubilizing strains of *Azotobacter chroococcum* in the rhizosphere and their effect on wheat cultivars under greenhouse conditions. Microbiol Res 156:87–93

Kumar KV, Singh N, Behl HM, Srivastava S (2008) Influence of plant growth promoting bacteria and its mutant on heavy metal toxicity in *Brassica juncea* grown in fly ash amended soil. Chemosphere 72:678–683

Lipping Y, Jiatao X, Daohong J, Yanping F, Guoqing L, Fangcan L (2008) Antifungal substances produced by *Penicillium oxalicum* strain PY-1–potential antibiotics against plant pathogenic fungi. World J Microbiol Biotechnol 24:909–915

Maliha R, Samina K, Najma A, Sadia A, Farooq L (2004) Organic acids production and phosphate solubilization by phosphate solubilizing microorganisms under *in vitro* conditions. Pak J Biol Sci 7:187–196

Marschner H, Dell B (1994) Nutrient uptake in mycorrhizal symbiosis. Plant Soil 159:89–102

Mckenzie RH, Roberts TL (1990) Soil and fertilizers phosphorus update. Alberta Soil Science Workshop Proceedings, Edmonton, Alberta, pp 84–104

Mehmet O, Cevdet A, Oral D, Ali SM (2005) Single and double inoculation with *Azospirillum*/*Trichoderma* : the effects on dry bean and wheat. Biol Fertil Soils 41:262–272

Mittal V, Singh O, Nayyar H, Kaur J, Tewari R (2008) Stimulatory effect of phosphate-solubilizing fungal strains (*Aspergillus awamori* and *Penicillium citrinum*) on the yield of chickpea (*Cicer arietinum* L. cv. GPF2). Soil Biol Biochem 40:718–727

Morales A, Alvear M, Valenzuela E, Rubio R, Borie F (2007) Effect of inoculation with *Penicillium albidum*, a phosphate-solubilizing fungus, on the growth of *Trifolium pratense* cropped in a volcanic soil. J Basic Microbiol 47:275–280

Nautiyal CS (1999) An efficient microbiological growth medium for screening of phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265–270

Nikolay V, Maria V, Iana N (2006) Simultaneous P-solubilizing and biocontrol activity of microorganisms: potentials and future trends. Appl Microbiol Biotechnol 71:137–144

Nozawa M, Hu HY, Fujie K, Tanaka H, Urano K (1998) Quantitative detection of *Enterobacter cloacae* strain HO-I In bioreactor for chromate wastewater treatment using polymerase chain reaction (PCR). Water Res 32:3472–3476

Oberson A, Friesen DK, Rao IM, Bühler S, Frossard E (2001) Phosphorus transformations in an oxisol under contrasting land-use systems: The role of the microbial biomass. Plant Soil 237:197–210

Oliveira CA, Alves VMC, Marriel IE, Gomes EA, Scotti MR, Carneiro NP, Guimarães CT, Schaffert RE, Sá NMH (2008) Phosphate solubilizing microorganisms isolated from rhizosphere of maize cultivated in an oxisol of the Brazilian Cerrado Biome. Soil Biol Biochem . doi:10.1016/j.soilbio.2008.01.012

Omar SA, Abd-Alla MH (2000) Physiological aspects of fungi isolated from root nodules of faba bean (*Vicia faba* L.). Microbiol Res 154:339–347

Pal SS (1998) Interaction of an acid tolerant strain of phosphate solubilizing bacteria with a few acid tolerant crops. Plant Soil 198:169–177

Pandey A, Trivedi P, Kumar B, Palni LMS (2006) Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-alpine location in the Indian Central Himalaya. Curr Microbiol 53:102–107

Pandey A, Das N, Kumar B, Rinu K, Trivedi P (2008) Phosphate solubilization by *Penicillium* spp. isolated from soil samples of Indian Himalayan region. World J Microbiol Biotechnol 24: 97–102

Patel DK, Archana G, Kumar GN (2008) Variation in the nature of organic acid secretion and mineral phosphate solubilization by *Citrobacter* sp. DHRSS in the presence of different sugars. Curr Microbiol 56:168–174

Petruccioli M, Federici F, Bucke C, Keshavarz T (1999) Enhancement of glucose oxidase production by *Penicillium variabile* P16. Enzyme Microb Technol 24:397–401

Pikovskaya RI (1948) Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Microbiology 17:362–370

Poi SC, Ghosh G, Kabi MC (1989) Response of chickpea (*Cicer aeritinum* L.) to combined inoculation with *Rhizobium*, phosphobacteria and mycorrhizal organisms. Zentral fur Microbiol 114:249–253

Ponmurugan P, Gopi C (2006) *In vitro* production of growth regulators and phosphatase activity by phosphate solubilizing bacteria. Afr J Biotechnol 5:348–350

Poonguzhali S, Madhaiyan M, Sa T (2008) Isolation and identification of phosphate solubilizing bacteria from chinese cabbage and their effect on growth and phosphorus utilization of plants. J Microbiol Biotechnol 18:773–777

Pradhan N, Sukla LB (2005) Solubilization of inorganic phosphates by fungi isolated from agriculture soil. Afr J Biotechnol 5:850–854

Raghothama KG (1999) Phosphate acquisition. Annu Rev Plant Physiol Mol Biol 50:665–693

Rajkumar M, Freitas H (2008) Influence of metal resistant-plant growth-promoting bacteria on the growth of *Ricinus communis* in soil contaminated with heavy metals. Chemosphere 71: 834–842

Rajkumar M, Nagendran R, Lee KJ, Lee WH, Kim SZ (2006) Influence of plant growth promoting bacteria and $Cr^{6+}$ on the growth of Indian mustard. Chemosphere 62:741–748

Rane MR, Sarode PD, Chaudhari BL, Chincholkar SB (2008) Exploring antagonistic metabolites of established biocontrol agent of marine origin. Appl Biochem Biotechnol 151:665–675

Reyes I, Bernier L, Simard RR, Antoun H (1999) Effect of nitrogen source on the solubilization of different inorganic phosphates by an isolate of *Penicillium rugulosum* and two UV induced mutants. FEMS Micobiol Ecol 28:281–290

Reyes I, Bernier L, Antoun H (2002) Rock phosphate solubilization and colonization of maize rhizosphere by wild and genetically modified strains of *Penicillium regulosum*. Microb Ecol 44:39–45

Reyes I, Baziramakenga R, Bernier L, Antoun H (2001) Solubilization of phosphate rocks and minerals by a wild type strain and two UV induced mutants of *Penicillium regulosum*. Soil Biol Biochem 33: 1741–1747

Richardson AE (1994) Soil microorganisms and phosphorous availability. In: Pankhurst CE, Doube BM, Gupta VVSR (eds) Soil biota: management in sustainable farming systems. CSIRO, Victoria, Australia, pp 50–62

Richardson AE, Hadobas PA, Hayes JE (2001a) Extracellular secretion of *Aspergillus* phytase from *Arabidopsis* roots enables plants to obtain phosphorous from phytate. Plant J 25:641–649

Richardson AE, Hadobas PA, Hayes JE, O'Hara CP, Simpson RJ (2001b) Utilization of phosphorus by pasture plants supplied with myo-inositol hexaphosphate is enhanced by the presence of soil micro-organisms. Plant Soil 229:47–56

Rodriguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv 17:319–339

Rodriguez H, Fraga R, Gonzalez T, Bashan Y (2006) Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Plant Soil 287:15–21

Rodríguez H, Gonzalez T, Selman G (2000) Expression of a mineral phosphate solubilizing gene from *Erwinia herbicola* in two rhizobacterial strains. J Biotechnol 84:155–161

Ruch DG, Motta JJ (1987) Ultrastructure and cytochemistry of dormant basidiospores of *Psilocybe cubensis*. Mycologia 79:387–398

Rudresh DL, Shivaprakash MK, Prasad RD (2005a) Tricalcium phosphate solubilizing abilities of *Trichoderma* spp. in relation to P uptake and growth and yield parameters of chickpea (*Cicer arietinum* L.). Can J Microbiol 51:217–222

Rudresh DL, Shivaprakash MK, Prasad RD (2005b) Effect of combined application of *Rhizobium,* phosphate solubilizing bacterium and *Trichoderma* spp. on growth, nutrient uptake and yield of chickpea (*Cicer aritenium* L.). Appl Soil Ecol 28:139–146

Saber K, Nahla L, Ahmed D, Chedly A (2005) Effect of P on nodule formation and N fixation in bean. Agron Sustain Dev 25:389–393

Saito M (1995) Enzyme activities of the internal hypha and germinated spores of an arbuscular mycorrhizal fungus, *Gigaspora margarita*. New Phytol 129:425–431

Schadeck RJG, Buchi DF, Leite B (1998a) Ultrastructural aspects of *Colletotrichum graminicola* conidium germination, appressorium formation and penetration on cellophane membranes: focus on lipid reserves. J Submicrosc Cytol Pathol 30:555–561

Schadeck RJG, Leite B, Buchi DF (1998b) Lipid mobilization and acid phosphatase activity in lytic compartments during conidium dormancy and appressorium formation of *Colletotrichum graminicola*. Cell Struct Funct 23:333–340

Schreiner RP, Mihara KL, Mcdaniel H, Bethlenfalvay GJ (1997) Mycorrhizal fungi influence plant and soil functions and interactions. Plant Soil 188:199–209

Selvakumar G, Mohan M, Kundu S, Gupta AD, Joshi P, Nazim S, Gupta HS (2008) Cold tolerance and plant growth promotion potential of *Serratia marcescens* strain SRM (MTCC 8708) isolated from flowers of summer squash (*Cucurbita pepo*). Lett Appl Microbiol 46:171–175

Shaharoona B, Naveed M, Arshad M, Zahir ZA (2008) Fertilizer-dependent efficiency of *Pseudomonads* for improving growth, yield, and nutrient use efficiency of wheat (*Triticum aestivum* L.). Appl Microbiol Biotechnol 79:147–155

Sharan A, Shikha DNS, Gaur R (2008) *Xanthomonas campestris*, a novel stress tolerant, phosphate-solubilizing bacterial strain from saline–alkali soils. World J Microbiol Biotechnol 24:753–759

Shin W, Ryu J, kim Y, Yang J, Madhaiyan M, Sa T (2006) Phosphate solubilization and growth promotion of maize (*Zea mays* L.) by the rhizosphere soil fungus *Penicillium oxalicum.*18th World Congress of Soil Science. July 9–15, Philadelphia, Pennsylvania, USA

Singal R, Gupta R, Saxena RK (1994) Rock phosphate solubilization under alkaline conditions by *Aspergillus japonicus* and *A. foetidus*. Folia Microbiol 39:33–36

Singh N, Pandey P, Dubey R, Maheshwari DK (2008) Biological control of root rot fungus *Macrophomina phaseolina* and growth enhancement of *Pinus roxburghii* (Sarg.) by rhizosphere competent *Bacillus subtilis* BN1. World J Microbiol Biotechnol 24:1669–1679

Song OR, Lee SJ, Lee YS, Lee SC, Kim KK, Choi YL (2008) Solubilization of insoluble inorganic phosphate by *Burkholderia cepacia* DA23 isolated from cultivated soil. Brazil J Microbiol 39:151–156

Souchie EL, Azcón R, Barea JM, Saggin-Júnior OJ, Silva EMR (2006) Phosphate solubilization and synergism between P-solubilizing and arbuscular mycorrhizal fungi. Pesquisa Agropecuária Brasileira 41:1405–1411

Souchie EL, Abboud ACS, Caproni AL (2007) In vitro phosphate solubilization by rhizospheric microorganisms from pigeonpea. Biosci J 23:53–60

Sridevi M, Mallaiah KV, Yadav NCS (2007) Phosphate solubilization by *Rhizobium* isolates from *Crotalaria* species. J Plant Sci 2:635–639

Thomas GV, Shantaram MV, Saraswathy N (2005) Occurrence and activity of phosphate solubilizing fungi from coconut plantation soils. J Plant Sci 87:357–364

To-o K, Kamasaka H, Kusaka K, Kuriki T, Kometani K, Okada S (1997) A novel acid phosphatase from *Aspergillus niger* KU-8 that specifically hydrolyzes C-6 phosphate groups of phosphoryl oligosaccharides. Biosci Biotechnol Biochem 61:1512–1517

To-o K, Kamasaka H, Kuriki T, Okada S (2000) Substrate selectivity in *Aspergillus niger* KU-8 acid phosphatase II using phosphoryl oligosaccharides. Biosci Biotechnol Biochem 64:1534–1537

Toro M (2007) Phosphate solubilizing microorganisms in the rhizosphere of native plants from tropical savannas: An adaptive strategy to acid soils? In: Velazquez C, Rodriguez-Barrueco E (eds) Developments in Plant and Soil Sciences. Springer, The Netherlands, pp 249-–252

Trivedi P, Sa TM (2008) *Pseudomonas corrugata* (NRRL B-30409) mutants increased phosphate solubilization, organic acid production, and plant growth at lower temperatures. Curr Microbiol 56:140–144

Valverde A, Burgos A, Fiscella T, Rivas R, Velazquez E, Rodriguez-Barrueco C, Cervantes E, Chamber M, Igual JM (2006) Differential effects of coinoculations with *Pseudomonas jessenii* PS06 (a phosphate-solubilizing bacterium) and *Mesorhizobium ciceri* C-2/2 strains on the growth and seed yield of chickpea under greenhouse and field conditions. Plant Soil 287:43–50

Varenyam A, Savant VV, Reddy MS (2007) Phosphate solubilization by a wild type strain and UV-induced mutants of *Aspergillus tubingensis*. Soil Biol Biochem 39:695–699

Vassilev N, Fenice M, Federici F (1996) Rock phosphate solubilisation with gluconic acid produced by immobilized *Penicillium variable* P16. Biotecnol Tech 20: 585–588

Vassilev N, Vassileva M, Nikolaeva I (2006) Simultaneous P-solubilizing and biocontrol activity of microorganisms: potentials and future trends. Appl Microbiol Biotechnol 71:137–144

Vassilev N, Vassileva M, Bravo V, Fernández-Serrano M, Nikolaeva I (2007) Simultaneous phytase production and rock phosphate solubilization by *Aspergillus niger* grown on dry olive wastes. Ind Crop Prod 26:332–336

Vazquez P, Holguin G, Puente M, Elopez CA, Bashan Y (2000) Phosphate solubilizing microorganisms associated with the rhizosphere of mangroves in a semi arid coastal lagoon. Biol Fertil Soil 30:460–468

Vikram A, Hamzehzarghani H (2008) Effect of phosphate solubilizing bacteria on nodulation and growth parameters of greengram (*Vigna radiate* L. Wilczec). Res J Microbiol 3:62–72

Wahid OA, Mehana TA (2000) Impact of phosphate solubilizing fungi on the yield and phosphorus uptake by wheat and faba bean plants. Microbiol Res 155:221–227

Wakelin SA, Gupta VVSR, Harvey PR, Ryder MH (2007) The effect of *Penicillium* fungi on plant growth and phosphorus mobilization in neutral to alkaline soils from southern Australia. Can J Microbiol 53:106–115

Wani PA, Zaidi A, Khan AA, Khan MS (2005) Effect of phorate on phosphate solubilization and indole acetic acid (IAA) releasing potentials of rhizospheric microorganisms. Ann Plant Prot. Sci 13:139–144

Wani PA, Khan MS, Zaidi A (2007a) Chromium reduction, plant growth promoting potentials and metal solubilization by *Bacillus* sp. isolated from alluvial soil. Curr Microbiol 54:237–243

Wani PA, Khan MS, Zaidi A (2007b) Co-inoculation of nitrogen fixing and phosphate solubilizing bacteria to promote growth, yield and nutrient uptake in chickpea. Acta Agron Hung 55: 315–323

Wani PA, Khan MS, Zaidi A (2007c) Synergistic effects of the inoculation with nitrogen fixing and phosphate solubilizing rhizobacteria on the performance of field grown chickpea. J Plant Nutr Soil Sci 170:283–287

Whitelaw MA, Harden TJ, Helyar KR (1999) Phosphate solubilization in solution culture by the soil fungus *Penicillium radicum*. Soil Biol Biochem 32:655–665

Yadav RS, Tarafdar JC (2003) Phytase and phosphatase producing fungi in arid and semi-arid soils and their efficiency in hydrolyzing different organic P compounds. Soil Biol Biochem 35: 745–751

Yi Y, Huang W, Ge Y (2008) Exopolysaccharide: a novel important factor in the microbial dissolution of tricalcium phosphate. World J Microbiol Biotechnol 24:1059–1065

Zaidi A (1999) Synergistic interactions of nitrogen fixing microorganisms with phosphate mobilizing microorganisms. PhD Thesis, Aligarh Muslim University, Aligarh

Zaidi A, Khan MS (2006) Co-inoculation effects of phosphate solubilizing microorganisms and G*lomus fasciculatum* on green gram *Bradyrhizobium* symbiosis. Turk J Agric For 30:223–230

Zaidi A, Khan MS (2007) Stimulatory effects of dual inoculation with phosphate solubilizing microorganisms and arbuscular mycorrhizal fungus on chickpea. Aust J Exp Agric 47:1016–1022

Zaidi A, Khan MS, Amil M (2003) Interactive effect of rhizotrophic microorganisms on yield and nutrient uptake of chickpea (*Cicer arietinum* L.). Eur J Agron 19:15–21

Zaidi A, Khan MS, Aamil M (2004) Bio-associative effect of rhizospheric microorganisms on growth, yield and nutrient uptake of greengram. J Plant Nutr 27:599–610

# Chapter 3
# Developing Beneficial Microbial Biofilms on Roots of Non legumes: A Novel Biofertilizing Technique

**Gamini Seneviratne, RMMS Thilakaratne, APDA Jayasekara, KACN Seneviratne, KRE Padmathilake, and MSDL De Silva**

**Abstract** Biofilms are often complex communities of multiple microbial species and remain attached to surfaces or with interfaces. Such beneficial biofilms can be developed in vitro and be used as biofertilizers (biofilmed biofertilizers, BBs) and biocontroling agents for nonlegumes, when applied at high cell densities. This chapter describes research studies conducted so far in this field with special attention into development of biofilms of $N_2$-fixing bacteria and P-solubilizing fungi. When these two distinct microbes were cocultured in vitro, the bacteria colonized fungal mycelia to form the biofilms. The biofilms showed higher rates of biological nitrogen fixation and organic acid production, which was directly proportional to the synthesis of indoleacetic acid-like substances, than microbes when used alone. The plant growth-promoting effects of such BBs were evaluated using rice (*Oryza sativa*), tea (*Camellia sinensis*), wheat (*Triticum aestivum*), and anthurium (*Anthurium andraeanum*). The biofilms formed nodule-like structures or "pseudonodules" on roots of such plants. For rice and tea, the results showed that recommended chemical fertilizers may be reduced by about 50% while applying BBs. Since this field of research is in its infancy, both laboratory and field experiments are required to fully explore the potential of this emerging biotechnological approach in the future.

G. Seneviratne (✉), RMMS Thilakaratne and KRE Padmathilake
Biological Nitrogen Fixation Project, Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka
e-mail: gaminis@ifs.ac.lk

APDA Jayasekara and MSDL De Silva
Tea Researh Institute of Sri Lanka, Talawakele, Sri Lanka

KACN Seneviratne
Royal Botanic Gardens, Peradeniya, Sri Lanka

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement,*
DOI: 10.1007/978-3-642-01979-1_3, 

## 3.1 Introduction

Although microorganisms have historically been studied as planktonic (freely swimming) cells in many ecosystems, it is common to find assembling of microorganisms adherent to each other and/or surfaces and embedded in a matrix of polymers, which are then known as biofilms (Harrison et al. 2005; Rudrappa et al. 2008). The assemblage leads to metabolic cooperation among the microbes (Davey and O'toole 2000). Newly developed microbiological and molecular biological methods clearly show that most bacteria live as biofilms formed on various biotic and abiotic surfaces (Romanova et al. 2006).

A biofilm consists of microbial cells (algal, fungal, bacterial and/or other microbial) and sticky extracellular polymeric substances (EPS), which provide structure and protection to the community (Vandevivere and Kirchman 1993; Seneviratne 2003). The EPS is composed of polysaccharides, proteins, nucleic acids and other substances which help protect the biofilm organisms from various environmental stress factors, such as UV radiation, extreme pH conditions, osmotic shock, dehydration, antimicrobial substances, predators, etc. (Costerton et al. 1987; Stewart and Costerton 2001; Romanova et al. 2006). As the biofilm microbes behave like a group within the EPS coating, external environmental stresses and attacks of competitors are tolerable (Seneviratne and Jayasinghearachchi 2005). This is why microorganisms prefer to exist in the biofilm mode rather than the planktonic stage. As an example, when *Pseudomonas putida* cells were incubated in the presence of *Saccharomyces cerevisiae* in grape (*Vitis vinifera*) juice or in a synthetic medium containing various concentrations of glucose, their stationary phase survival improved dramatically (Romano and Kolter 2005), possibly through biofilm formation. Similarly, when introduced into soils after coupling with a common soil fungus to form biofilms, rhizobial cells were observed to perform better than when they were used alone (Seneviratne and Jayasinghearachchi 2005).

Biofilms occur naturally in animals, plants and the environment. These biofilmed communities could be harmful/pathogenic or beneficial (Morikawa 2006). Beneficial biofilms attached to the plant roots of some crops may help cycle nutrients as well as biocontrol of pests and diseases and, consequently, improve the productivity of crops. However, the density in the soil of such naturally occurring beneficial biofilms is too low to have a significant effect. This was reflected by the success of the microbial inoculation into a soil planted with rice (G. Seneviratne, unpublished). Critical cell density-dependant quorum sensing is a prerequisite for biofilm formation (Kong et al. 2006), which is frequently not attainable in the soil solution under natural conditions. Further, the naturally occurring biofilms are N-deficient for optimal action, which may be overcome by incorporating $N_2$ fixers to them (Seneviratne 2008). Therefore, the development of such biofilms in vitro and their application as biofertilizers are essential for augmenting agricultural productivity (Bandara et al. 2006). This chapter highlights the recent advances in research, focusing on the potential effect of the BBs on non leguminous crops.

## 3.2 Effects of Beneficial Microbial Biofilms

With the first in vitro development and observation of interactions between common nonmycorrhizal soil fungi (e.g., *Penicillium* spp.) and rhizobia forming the biofilms (Seneviratne and Jayasinghearachchi 2003), a series of studies were conducted to assess their potentials as microbial agents in plant growth. Microbes used in this study were mainly $N_2$-fixing bacteria and P-solubilizing fungi. When they were cocultured in vitro, the bacteria attached and colonized on fungal mycelia to form the biofilms, known as fungal–bacterial biofilm (FBB). When the bacterium is a *Rhizobium* species, they are called fungal–rhizobial biofilm (FRB). It was observed that the interaction in the FRB fixed $N_2$ biologically, as revealed by nitrogenase activity and N-accumulation, which was not observed when a rhizobial strain was used alone as a monoculture (Jayasinghearachchi and Seneviratne 2004a). The rhizobial strain used here was *Bradyrhizobium elkanii* SEMIA 5019, a soybean (*Glycine max*)-nodulating strain with a high $N_2$-fixing capacity. A recent study showed the enhanced release of organic acids and plant growth-promoting substances by developed FBB/FRB, leading to an increase of 25% in dry matter accumulation in early grown rice over the monocultured conventional inocula (Seneviratne et al. 2008a). It was also observed that there was a significant negative relationship between pH and indoleacetic acid-like substances (IAAS) production in liquid culture media of the biofilms, but not in mixed cultures without biofilm formation of a large collection of microbes (Seneviratne et al. 2008b). Thus, when biofilms were formed, high acidity reflected high production of IAAS. The high acidity is generally important for pathogen suppression. The biofilmed inocula can also be used effectively to enhance biosolubilization of rock phosphate, due to high acid production (Jayasinghearachchi and Seneviratne 2006a; Seneviratne and Indrasena 2006). Moreover, the biofilmed inocula can be used for successful establishment of introduced beneficial microorganisms in plants for biocontrol of diseases. For instance, a *Pleurotus ostreatus–Pseudomonas fluorescens* biofilm (FBB) increased endophytic colonization of tomato (*Lycopersicon lycopersicum*) by *P. fluorescens*, a biocontroling agent, by over 1,000%, compared to inoculation with *P. fluorescens* alone under in vitro conditions (Jayasinghearachchi and Seneviratne 2006b).

## 3.3 Developing Beneficial Biofilms on Roots of Non legumes

It has now been clearly shown under axenic conditions that the monocultures of *Rhizobium* sp. inoculated into nonleguminous plant roots develop biofilms on the root surface (Santaella et al. 2008). Recent studies conducted under axenic conditions demonstrated that the inoculation of the FRB helped maintain a higher cell density of rhizobia on the root system of wheat than the inoculation of rhizobial monocultures (Fig. 3.1). The monocultures developed clusters of rhizobial cells on

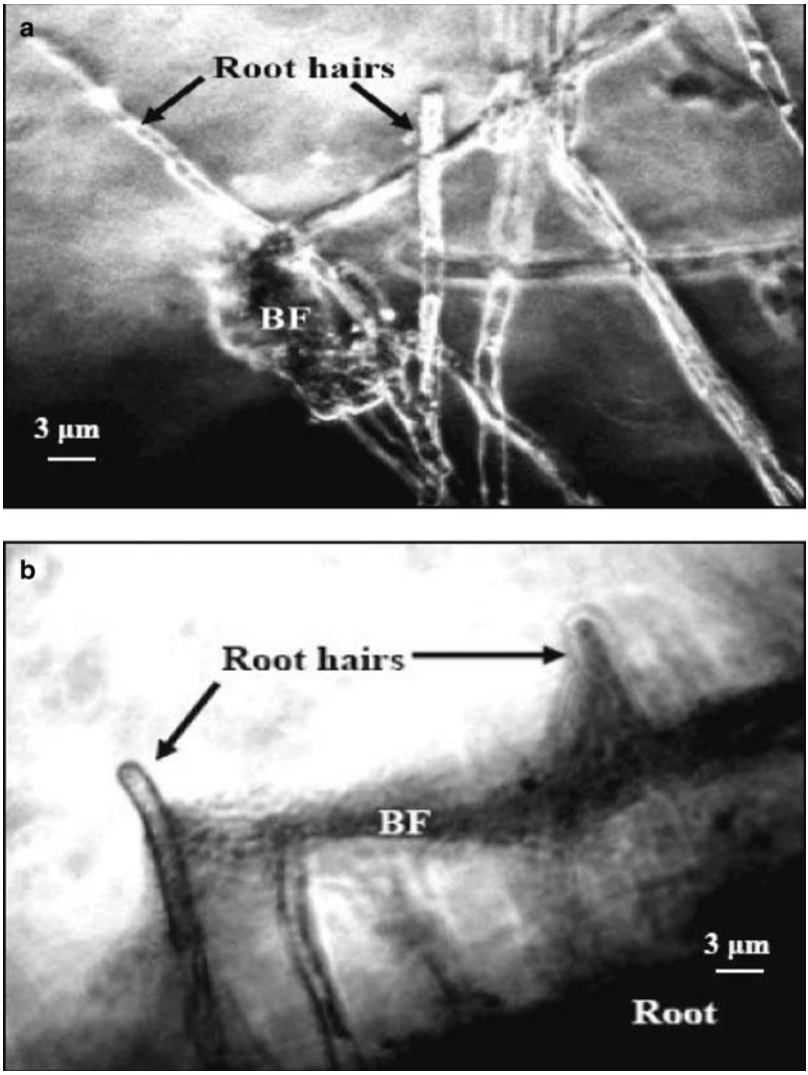



**Fig. 3.1** Rhizobial biofilms (*BF*) developed on root hairs of wheat, when rhizobial monocultures (**a**) or fungal–rhizobial biofilms (*FRB*) (**b**) was inoculated under axenic conditions. The inoculation of the FRB helped maintain a higher cell density of rhizobia on the root hairs than the inoculation of the monoculture

the root hairs whereas fungal mycelium of the FRB linked root hairs, which provided support to maintain the higher cell density (Seneviratne et al. 2008b). Thus, the FRB may act as nodule-like structures or "pseudonodules" capable of fixing $N_2$ biologically on roots of such non legumes, as reported earlier by Jayasinghearachchi and Seneviratne (2004b). The FBB/FRB can also develop biofilms

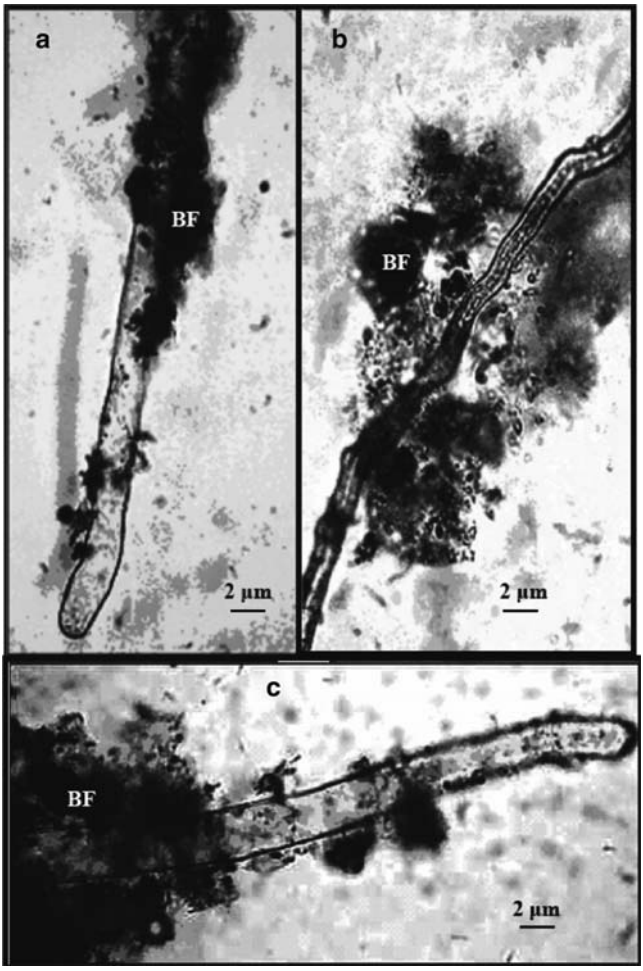



**Fig. 3.2** Root hairs of rice (**a**), tea (**b**) and *Anthurium* (**c**) colonized by microbial biofilms (*BF*), when fungal–bacterial biofilms (*FBB*) or fungal–rhizobial biofilms (*FRB*) were inoculated under axenic conditions. Darkness is due to cotton blue stain absorbed by the EPS produced by the BF

on root hairs of other non legumes, when inoculated under axenic conditions. Root hairs of rice, tea and *Anthurium* have been found heavily colonized with biofilm formation, as evidenced by the EPS produced by the biofilms (Fig. 3.2). It was reported recently that the adsorption of rhizobia to biotic surfaces like plant roots is governed by the rhizobial adhesion protein RapA1 (Mongiardini et al. 2008). It can therefore be speculated that this adhesion protein, which may also have contributed to attachment and formation of the FBB/FRB, is common in many bacteria.

## 3.4 Effects of Biofilmed Biofertilizers on Plant Growth and Yield

When the FBB/FRB formulated as the BBs were applied to the growing medium of plants, they formed biofilms on the root system, as discussed earlier. These biofilms have shown their positive effects on growth and yield of crops. For instance, in a soil pot experiment, when rice was grown in the absence of chemical fertilizers, it was found that the shoot growth of this crop improved when the number of beneficial microbial species of the BBs was increased (Fig. 3.3). Conventional biofertilizers with single microbial species or monocultures increased the shoot biomass by only 7% compared to control. In contrast, the inoculation of BBs containing three microbes FBB/FRB (two bacteria + one fungus) increased the dry matter accumulation in shoots substantially by 25%. However, when a tripartite culture of FBB/FRB was used with 50% of the recommended rates of chemical fertilizers (urea 100 kg N $ha^{-1}$, triple super phosphate 13 kg P $ha^{-1}$ and muriate of potash 28 kg K $ha^{-1}$) for rice, plant biomass increased by ca. 55% compared to those observed for 100% application of the recommended fertilizers alone (Table 3.1). This was mainly attributed to increased root dry weight. Increasing the recommended fertilizer level up to 100% with the BBs, however, did not increase the plant growth. Both panicle formation and seed yield per hill were comparable between the BBs + 50% of the recommended fertilizers and the 100% of the recommended fertilizers alone (G. Seneviratne, unpublished).

In a nursery trial of tea with BBs of diazotrophic bacteria and/or recommended chemical fertilizers (T65; sulfate of ammonia, mono-ammonium phosphate, sulfate of potash and epsom salt; 15:20:15:15 (weight ratio) 80 kg per 10,000 plants during nursery period), there was a positive correlation between net photosynthetic rate and relative growth rate of leaf area (Fig. 3.4a). When two-bacterial BBs were applied, there was a propensity of increasing photosynthesis compared to one-bacterial BBs. Moderate application of 50% of the recommended fertilizers with the two-bacterial BBs helped increase leaf growth compared to other microbial fertilizer treatments and even 100% of the recommended fertilizer application. Soil C after harvest of the tea plants of the nursery was positively related to shoot/root ratio of the plants (Fig. 3.4b). The two-bacterial BBs with the moderate fertilizer level showed the highest shoot/root ratio and the soil C, which could possibly be due to increased growth of fine roots (Zavahir et al. 2008) and subsequent rapid turnover. The application of recommended fertilizers alone resulted in a low shoot/root ratio and soil C. The high shoot/root ratio with the two-bacterial BBs and the moderate N application maintained a low transpiration rate (Fig. 3.4c). However, the plants treated with the recommended fertilizers alone showed a high transpiration rate, as reflected by early wilting when exposed to sunlight (Jayasekara et al. 2008). The application of BBs with the N fertilizer helped increase leaf N of tea due to increased root growth (Fig. 3.4d). In a field experiment, young tea applied with

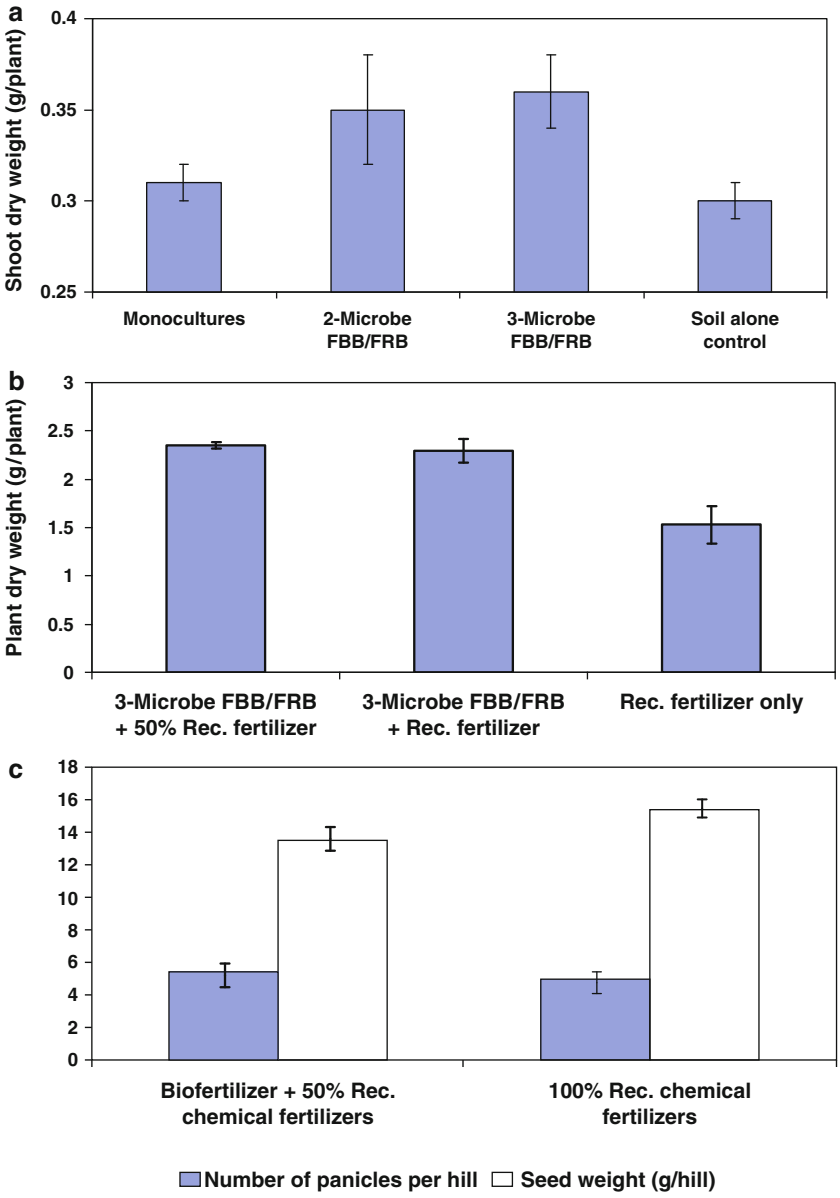



**Fig. 3.3** Shoot growth of rice (**a**) in a soil pot experiment, when the number of beneficial microbial species of the fungal–bacterial biofilms (*FBB*) and fungal–rhizobial biofilms (*FRB*), formulated and applied as biofilmed biofertilizers (*BBs*) was increased, in comparison to microbial monocultures. Total plant growth (**b**), when a three-microbe FBB/FRB was coupled with 50% or 100% of the recommended chemical fertilizers, or 100% of the recommended fertilizers alone. Panicle formation and seed yield of rice (**c**), when the BBs were coupled with 50% of the recommended chemical fertilizers or 100% of the recommended fertilizers alone. Vertical bars show standard error

**Table 3.1** Dry matter accumulation in shoot, root and whole plant of rice grown in pot soil treated with a tripartite FBB/FRB with 50% or 100% of recommended chemical fertilizer, or 100% of the recommended fertilizer alone

| Treatment | Shoot dry weight (g per plant) | Root dry weight (g per plant) | Total dry weight (g per plant) |
|---|---|---|---|
| Tripartite FBB/FRB + 50% recommended fertilizer | 1.95a ± 0.03 | 0.40a ± 0.001 | 2.35a ± 0.03 |
| Tripartite FBB/FRB + 100% recommended fertilizer | 1.99a ± 0.10 | 0.29b ± 0.02 | 2.29ab ± 0.12 |
| 100% recommended fertilizer alone | 1.42a ± 0.19 | 0.10c ± 0.01 | 1.53b ± 0.19 |
| CV (%) | 14.5 | 6.20 | 13.4 |

Mean ±SE. Values in each column followed by a same letter are not significantly different at 5% probability level

four-microbe FRB (three bacteria + one fungus) alone produced a leaf weight of 506 kg $ha^{-1}$ compared to 431 kg $ha^{-1}$ with 100% of the recommended chemical fertilizers alone, at the first tipping.

Anthurium plantlets treated with four-microbe FRB and 50% of recommended chemical fertilizers in an inert particle medium showed a higher relative growth rate of plant dry weight than that of the 100% of the recommended fertilizers alone, in early growth (Fig. 3.5). The four-microbe FRB alone marginally supported the plant growth, possibly due to low microbial biomass of the biofilm, in the absence of the fertilizer nutrients. These findings suggested that the input of chemical fertilizers can be reduced by 50% during the vegetative growth phase and possibly for the entire crop of such non legumes, which could be a huge economic gain in terms of fertilizer saving.

The BBs once applied to a root establish an association between the root and the biofilm, as shown in the conceptual model presented in Fig. 3.6. The moderate application of the chemical fertilizer nutrients helps increase the microbial biomass of the biofilm, which in turn tends to increase the microbial efficiency or the functionality, as the concentrations of the fertilizer nutrients, particularly N depletes. The biofilm acts as a nodule-like structure or a pseudonodule-fixing $N_2$. This fixed N may be transferred to the root, and in return the root may supply carbon sources to the biofilm, the processes of which need future investigations. The release of organic acids by the biofilm helps suppress microbial pathogens (Browning et al. 2006) as well as increase mineralization of soil nutrients in the rhizosphere (Seneviratne and Jayasinghearachchi 2005). Moreover, plant growth hormones, such as IAA produced by the biofilms (Bandara et al. 2006), should increase the growth of roots and mycorrhizal fungi. In this manner, this association constitutes an excellent metabolic cooperation that helps the healthy growth of the plant. In addition, the BBs are also important in replenishing beneficial microbial communities in deteriorated soils due to heavy use of chemical inputs and intensive cropping (Seneviratne 2009).

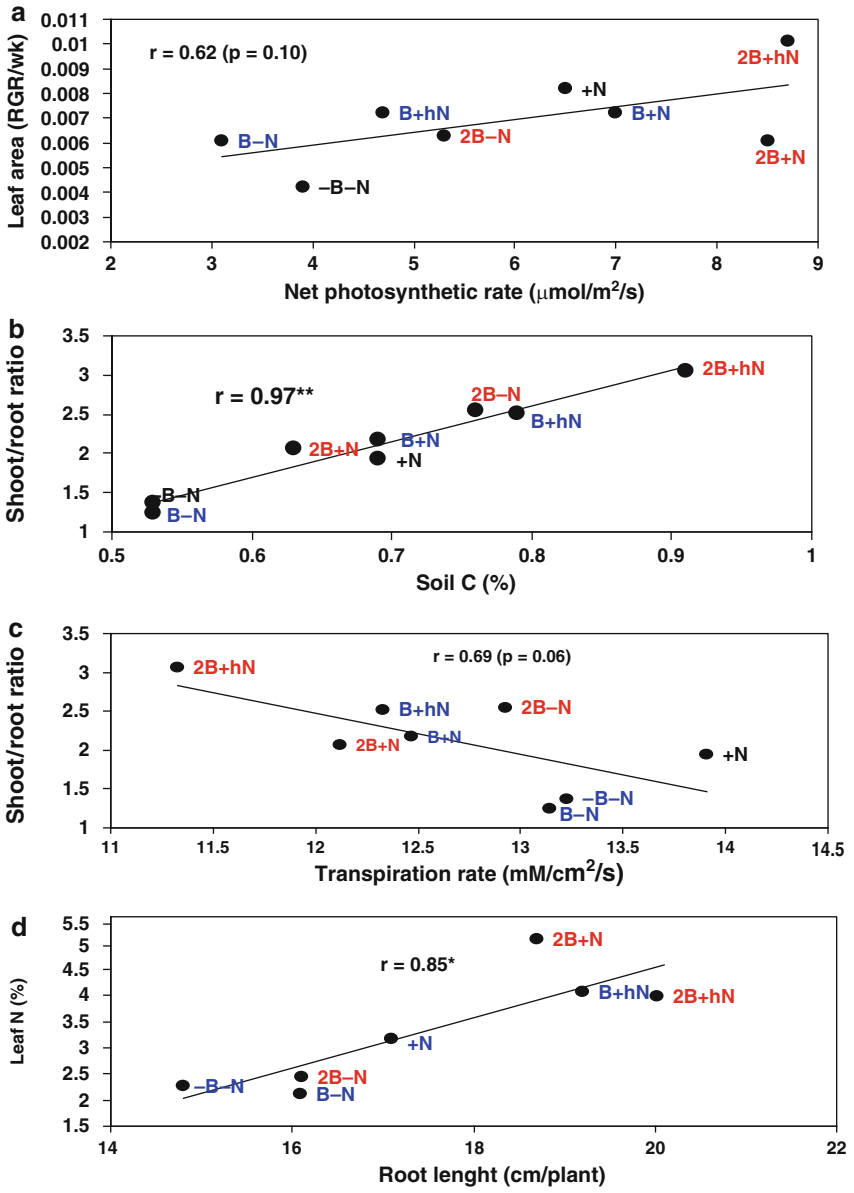



**Fig. 3.4** Relationships among plant and soil parameters in a nursery trial of tea, when biofilmed biofertilizers (*BBs*) of diazotrophic bacteria and/or recommended chemical fertilizers were applied. Correlations between (**a**) net photosynthetic rate and relative growth rate of leaf area (**b**) soil C after harvest of the plants and shoot/root ratio (**c**) shoot/root ratio and transpiration rate and (**d**) leaf N and root growth. Treatments included no bacteria (*−B*), one (*B*) or two (*2B*) bacteria, and half (*hN*) or full (*N*) recommendation of chemical fertilizers, or no fertilizers (*−N*)

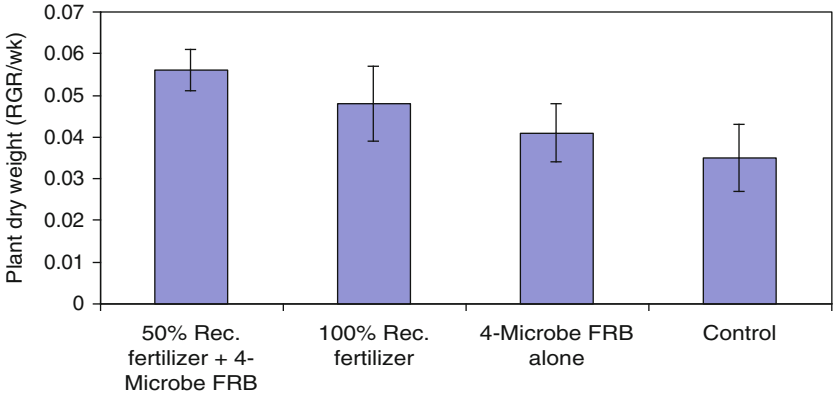



**Fig. 3.5** Relative growth rate (*RGR*) of plant dry weight of Anthurium plantlets treated with a four-microbe fungal–rhizobial biofilm (*FRB*) alone, four-microbe *FRB* and 50% of recommended chemical fertilizers or 100% of the recommended fertilizers alone, in an inert particle medium during early growth. Vertical bars show standard error

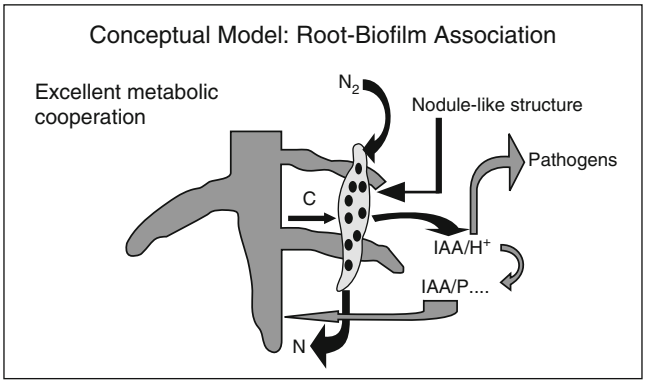



**Fig. 3.6** Conceptual model showing the association established between the root and the biofilm, when the biofilmed biofertilizers (*BBs*) were applied to a root of a non legume

## 3.5 Conclusion

It is clear from various studies that the FBB/FRB when formulated as BBs and applied to non legumes enhance the plant growth in the presence of even moderate levels of chemical fertilizers. The biofilms with a higher number of beneficial microbial species (higher order biofilms) increased the plant growth possibly due to improved microbial activities. However, selection of combinations of microbes possessing the highest efficiency, simultaneous biofertilizing and biocontroling activities is a key factor in the preparation and formulation of BBs. Diverse forms of the BBs can serve as a source to increase $N_2$ fixation, promote nutrient uptake

and to manage plant diseases in different agro-ecosystems. Therefore, both laboratory and field trials are required to realize the full impact of this biotechnological approach for sustainable crop production.

## References

Bandara WMMS, Seneviratne G, Kulasooriya SA (2006) Interactions among endophytic bacteria and fungi: effects and potentials. J Biosci 31:645–650

Browning M, Wallace DB, Dawson C, Alm SR, Amador JA (2006) Potential of butyric acid for control of soil-borne fungal pathogens and nematodes affecting strawberries. Soil Biol Biochem 38:401–404

Costerton JW, Geesy GG, Cheng KJ (1987) Bacterial biofilms in nature and disease. Annu Rev Microbiol 41:435–464

Davey ME, O'toole GA (2000) Microbial biofilms: from ecology to molecular genetics. Microbiol Mol Biol Rev 64:847–867

Harrison JJ, Turner RJ, Marques LLR, Ceri H (2005) Biofilms. Am Sci 93:508–515

Jayasekara APDA, Seneviratne G, De Silva MSDL, Jayasinghe LASP, Prematunga P (2008) Preliminary investigations on the potential applications of biofilmed biofertilizers for tea nurseries. In: Nainanayake NPAD, Everard JMDT (eds) Proceedings of the second symposium on plantation crop research-export competitiveness through quality improvements. Coconut Research Institute, Lunuwila, Sri Lanka, pp 170–175

Jayasinghearachchi HS, Seneviratne G (2004a) Can mushrooms fix atmosphericnitrogen? J Biosci 23:293–296

Jayasinghearachchi HS, Seneviratne G (2004b) A bradyrhizobial-*Penicillium* spp biofilm with nitrogenase activity improves $N_2$ fixing symbiosis of soybean. Biol Fertil Soil 40:432–434

Jayasinghearachchi HS, Seneviratne G (2006a) Fungal solubilization of rockphosphate is enhanced by forming fungal-rhizobia biofilms. Soil Biol Biochem 38:405–408

Jayasinghearachchi HS, Seneviratne G (2006b) A mushroom-fungus helps improve endophytic colonization of tomato by *Pseudomonas fluorescence* through biofilm formation. Res J Microbiol 1:83–89

Kong KF, Vuong C, Otto M (2006) *Staphylococcus* quorum sensing in biofilm formation and infection. Int J Med Microbiol 296:133–139

Mongiardini EJ, Ausmees N, Pérez-Giménez J, Althabegoiti MJ, Quelas JI, López-García SL, Lodeiro AR (2008) The rhizobial adhesion protein RapA1 is involved in adsorption of rhizobia to plant roots but not in nodulation. FEMS Microbiol Ecol 65:279–288

Morikawa M (2006) Beneficial biofilm formation by industrial bacteria *Bacillus subtilis* and related species. J Biosci Bioeng 101:1–8

Romano JD, Kolter R (2005) *Pseudomonas-Saccharomyces* interactions: influence of fungal metabolism on bacterial physiology and survival. J Bacteriol 187:940–948

Romanova YM, Smirnova TA, Andreev AL, Il'ina TS, Didenko LV, Gintsburg AL (2006) Formation of biofilms as an example of the social behaviour of bacteria. Microbiology 75:481–485

Rudrappa T, Biedrzycki ML, Bais HP (2008) Causes and consequences of plant-associated biofilms. FEMS Microbiol Ecol 64:153–166

Santaella C, Schue M, Berge O, Heulin T, Achouak W (2008) The exopolysaccharide of *Rhizobium* sp. YAS34 is not necessary for biofilm formation on *Arabidopsis thaliana* and *Brassica napus* roots but contributes to root colonization. Environ Microbiol 10:2150–2163

Seneviratne G (2003) Development of eco-friendly, beneficial microbial biofilms. Curr Sci 85:1395–1396

Seneviratne G (2008) Biological nitrogen fixation: potential biotechnological applications beyond biofertilizers. Curr Sci 95:7

Seneviratne G (2009) Collapse of beneficial microbial communities and deterioration of soil health: a cause for reduced crop productivity. Curr Sci 96:633

Seneviratne G, Indrasena IK (2006) Nitrogen fixation in lichens is important for improved rock weathering. J Biosci 31:639–643

Seneviratne G, Jayasinghearachchi HS (2003) Mycelial colonization by *Bradyrhizobia* and *Azorhizobia*. J Biosci 28:243–247

Seneviratne G, Jayasinghearachchi HS (2005) A rhizobial biofilm with nitrogenase activity alters nutrient availability in a soil. Soil Biol Biochem 37:1975–1978

Seneviratne G, Zavahir JS, Bandara WMMS, Weerasekara MLMAW (2008a) Fungal-bacterial biofilms: their development for novel biotechnological applications. World J Microbiol Biotechnol 24:739–743

Seneviratne G, Kecskés ML, Kennedy IR (2008b) Biofilmed biofertilisers: novel inoculants for efficient nutrient use in plants. In: Kennedy IR, Choudhury ATMA, Kecskés ML, Rose MT (eds) Efficient nutrient use in rice production in Vietnam achieved using inoculant biofertilisers. Proceedings of a project (SMCN/2002/073) workshop held in Hanoi, Vietnam, 12–13 October 2007. ACIAR Proceedings No. 130, ACIAR, Canberra, pp 126–130

Stewart P, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. Lancet 358:135–138

Vandevivere P, Kirchman DL (1993) Attachment stimulates exopolysaccharide synthesis by a bacterium. Appl Environ Microbiol 59:3280–3286

Zavahir JS, Jayasekara APDA, Seneviratne G, De Silva MSDL (2008) Potential application of biofilms: a new approach for tea gardens. Tea Bull 20:1–6

# Chapter 4
# Role of 1-Aminocyclopropane-1-carboxylate deaminase in *Rhizobium*–Legume Symbiosis

**Javed Musarrat, Abdulaziz A Al Khedhairy, Saud Al-Arifi, and Mohammad Saghir Khan**

**Abstract** Rhizobia form symbiotic relationships with leguminous plants and convert atmospheric nitrogen into ammonia which can be utilized by host legume plants. Symbiotic nitrogen fixation is important not only for the production of protein-rich legumes but also to improve the fertility of soils. Nodulation in the roots of legumes is regarded as an initiating event in the onset of $N_2$ fixation. Inhibition of nodulation restricts the $N_2$-fixing ability of legumes, which may occur due to variety of reasons. For instance, ethylene, as a stress hormone, inhibits nodulation in legumes. However, its action is naturally offset by an enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, produced by rhizobia. This enzyme cleaves plant-produced ACC into ammonia and α-ketobutyrate thereby lowering the ethylene level. As a consequence of decreased ethylene levels, nodulation is increased. Moreover, the ACC deaminase-containing plant growth-promoting rhizobacteria including symbiotic $N_2$ fixers reduce the physiological damage to plants caused by other environmental factors. In this chapter, the focus is to understand how the rhizobial ACC deaminase lowers the ethylene level in legumes and overcomes the inhibitory effects of ethylene on nodulation. The strategy adopted by rhizobial species to promote nodulation by adjusting ethylene levels could be exploited as an effective tool for legume improvement in different agro-ecological niches.

J. Musarrat (✉), A.A.Al Khedhairy and S. Al-Arifi
College of Science, King Saud University, Riyadh, SA
e-mail: musarratj1@yahoo.com

M.S. Khan
Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh-202002, U.P., India

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_4, 

## 4.1 Introduction

Microbial communities, in general, predominate in or around the plant root surfaces compared to bulk soil, and play a crucial role in maintaining soil health and plant development (Kloepper et al. 1980). This "rhizosphere effect" occurs primarily owing to the release of as much as 40% of photosynthates (e.g., sugars, organic acids and amino acids, etc.) from the plant roots (Lynch and Whipps 1991; Nelson 2004). The microorganisms use these exudates as nutrients and rapidly colonize the roots of plants (VanLoon and Glick 2004). Root-colonizing bacteria commonly referred to as "rhizobacteria" may remain confined to the root surface (rhizoplane), or enter the root interior and behave as endophytes (Sturz et al. 2000). Such rhizobacterial strains, including both free-living and symbiotic bacteria, capable of facilitating plant growth after inoculation on to seeds or when already present in soils, are called plant growth-promoting rhizobacteria (PGPR) (Kloepper et al. 1980; Zahir et al. 2004). Of these PGPR, soil bacteria belonging to various genera of the order rhizobiales (collectively called rhizobia) invade legume roots in nitrogen-limiting environments, leading to the formation of a highly specialized organ, the root nodule, on compatible legume roots or stems, and converts atmospheric $N_2$ into $NH_3$ and exports the fixed N to the host plants (Long 1989). Besides promoting the growth of plants by providing N to plants, rhizobia also facilitate the growth of plants by preventing the deleterious effects of one or more phytopathogenic organisms (e.g., *Botrytis cinerea* and *Fusarium oxysporum* ) by synthesizing an array of antibiotics, such as phenazines (Krishnan et al. 2007) and phytohormones like IAA (Mandal et al. 2007; Sridevi and Mallaiah 2007; Wani et al. 2007) and siderophores (Storey et al. 2006; Wani et al. 2008) which can solubilize and sequester iron and make it available to plants, and solubilize not easily available forms of phosphorus (Abd-Alla 1994; Alikhani et al. 2006).

The gaseous hormone ethylene produced endogenously by plants has many strong effects on plant development and has been shown to act as a secondary signal in the induction of plant defenses (Ecker 1995). Ethylene has been involved in many physiological processes, such as seed germination, tissue differentiation, formation of root and shoot primordial, root elongation, lateral bud development, flowering initiation, anthocyanin synthesis, flower opening and organ senescence, fruit aroma and ripening, storage product hydrolysis, and leaf and fruit abscission (Frankenberger and Arshad 1995; Spaink 1997). In addition, ethylene acts as a messenger of biotic and abiotic stresses (Herder et al. 2006). It also plays a major role in the beneficial rhizobial and arbuscular mycorrhizal symbioses (Guinel and Geil 2002). Ethylene is produced and sensed in response to a wide variety of environmental and developmental factors, including salinity, drought, water logging, heavy metals, pathogenicity and nodulation (Abeles et al. 1992; Spaink 1997). Despite acting as a plant growth regulator, ethylene at higher concentrations inversely affects many physiological stages of plants including nodulation by rhizobia in various legumes (Hirsch and Fang 1994; Oldroyd et al. 2001; VanLoon et al. 2006). For instance, ethylene has been shown to inhibit nodule development in

alfalfa (*Medicago sativa*) (Ligero et al. 1991; Glick et al. 2007a) and pea (*Pisum sativum*) (Guinel and Geil 2002; Cheng et al. 2008). However, *Medicago truncatula,* a hypernodulating mutant sickle, is ethylene insensitive (Penmetsa and Cook 1997) and has shown altered auxin transport regulation during nodulation (Prayitno et al. 2006).

Similar to the model proposed for free-living bacteria, the strains of $N_2$-fixing bacteria also synthesize a multimeric (homodimeric or homotrimeric) enzyme, 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Glick 1995; Glick et al. 1999), which facilitates symbiosis by metabolizing the immediate biosynthetic precursor (ACC) of ethylene (Glick et al. 1994; Mayak et al. 1999) into ammonia and α-ketobutyrate (Glick et al. 1998). Hence, ACC deaminase decreases ethylene levels in host plants (Ma et al. 2003a, 2004; Glick 2005) and consequently improves the overall growth of plants (Glick et al. 2007b; Contesto et al. 2008). This enzyme was first detected in *Pseudomonas* sp. strain ACP and the yeast, *Hansenula saturnus* (Honma and Shimomura 1978; Honma 1993; Minami et al. 1998) and since then, it has been reported in numerous other PGPR strains (Shaharoona et al. 2007; Naveed et al. 2008; Duan et al. 2008) including large number of symbiotic $N_2$-fixing bacteria, such as *Rhizobium leguminosarum* bv. *viciae*, *R. hedysari*, *Rhizobium* spp., *Mesorhizobium* and *Bradyrhizobium* (Belimov et al. 2005; Hontzeas et al. 2005; Blaha et al. 2006; Bajgiran et al. 2008). When the bacteria possessing ACC deaminase are bound to the seed coat of a developing seedling, they act as a sink for ACC ensuring that the ethylene level does not increase to the point where root growth is impaired. By facilitating the formation of longer roots, these bacteria may enhance the survival of some seedlings, especially during the first few days after the seeds are planted. Plants treated with ACC deaminase-containing bacteria have longer roots (Hall et al. 1996; Shah et al. 1998) and help plants to better resist the inhibitory effects of stress ethylene on growth imposed by heavy metals (Reed and Glick 2005; Safronova et al. 2006; Zhang et al. 2008), pathogens (Wang et al. 2000; Belimov et al. 2007), drought (Zahir et al. 2008; Arshad et al. 2008), flooding (Grichko and Glick 2001; Farwell et al. 2007), and high salt (Cheng et al. 2007; Yang et al. 2008; Jalili et al. 2008). Thus, understanding the role of rhizobial ACC deaminase in reducing the effect of ethylene would help to develop the rhizobial inoculant containing ACC deaminase activity that could become useful in sustaining growth and development of legumes under stress conditions by reducing stress-induced ethylene production.

## 4.2 Rhizobia–Legume Symbiosis: An Overview

The interaction between rhizobia and their corresponding specific legume host plants leading to nodule formation is a complex process that requires a continuous and adequate signal exchange between the plant and the bacteria (Perret et al. 2000; Bartsev et al. 2004). The symbiotic associates show a high degree of mutual specificity

which is often mediated by the exchange of signal compounds (Long and Staskawicz 1993; Spaink 1997). During this interaction, rhizobia are attracted by root exudates and colonize plant root surfaces. Flavonoids, the plant signal compounds present in the exudates, trigger the transcription of bacterial nodulation (*nod*) genes leading thereby to the synthesis of lipochito-oligosaccharide signals called Nod factors (Perret et al. 2000). These signal compounds in turn cause the legume root hairs to curl. Nod factors together with additional microbial signals, such as polysaccharides and secreted proteins, allow bacteria attached to root hairs to penetrate the root through a tubular structure called the infection thread, through which the rhizobia enter, move into root hair, and subsequently reach to the dividing cortical cells. When the thread reaches the primordium, the bacteria are released into the plant cytoplasm, where they differentiate into endosymbiotic form, the $N_2$-fixing bacteroids. Inside the central nodule cells, rhizobia are housed as symbiosome that are horizontally acquired organelles and are involved in the enzymatic reduction of atmospheric nitrogen to ammonia and make this N accessible to their hosts. In return, the bacteria are supplied with carbohydrates in a protected environment. The host plant, however, regulates the number of nodules formed, the maturation of nodules, and the $N_2$ fixation of the nodules dependent upon available nitrogen.

Despite substantial progress in biological sciences, the mechanisms regulating legume root nodule development are still poorly understood, and very few regulatory genes have been cloned and characterized. For instance, *EFD* (ethylene response factor, ERF, required for nodule differentiation), a gene that is upregulated during nodulation in *M. truncatula*, has recently been characterized (Vernié et al. 2008). The EFD transcription factor belongs to the ERF group V, which contains ERN1, 2, and 3, three ERFs involved in Nod factor signaling. The role of EFD in the regulation of nodulation has been examined through the characterization of a null deletion mutant (*efd-1*), RNA interference, and overexpression studies (Vernié et al. 2008). EFD is a negative regulator of root nodulation and infection by *Rhizobium*. It is required for the formation of functional nitrogen-fixing nodules and affects the plant and bacteroid differentiation processes occurring beneath the nodule meristem. Furthermore, EFD also activate a cytokinin primary response gene Mt *RR4* that encodes a type-A response regulator. Induction of Mt *RR4* leads to the inhibition of cytokinin signaling with the suppression of new nodule initiation and activation of differentiation as cells leave the nodule meristem. Thus, a key regulator linking early and late stages of nodulation and the regulation of the cytokinin pathway are important both for nodule initiation and development.

Rhizobia, in general, produce both indeterminate and determinate types of nodules. Indeterminate nodules are characterized by different zones: (1) the distal meristem, where bacteria are internalized, (2) an interzone with amyloplast accumulation and differentiation of bacteroids, and (3) a fixation zone that includes plant cells and a senescent zone (Pawlowski and Bisseling 1996; Timmers et al. 1999; Jeroen et al. 2006). In comparison, determinate nodules are typically round shaped and are derived from the cessation of meristem activity after nodule initiation and growth of the nodule mainly by cell expansion (Jeroen et al. 2006).

## 4.3 Effect of Ethylene on Symbiosis

Legumes form a mutualistic symbiosis with rhizobia. Although the interaction is beneficial to the plant, the symbiosis is tightly regulated. The gaseous plant hormone ethylene has been found to be involved in many physiological events including regulation of symbiotic process in legumes. For instance, many plants require a burst of ethylene to break seed dormancy (Esashi and Esashi 1991) but, following germination, a sustained high level of ethylene may inhibit root elongation (Jackson 1991). It is reported that root growth is stimulated at low concentrations of ethylene but is markedly decreased at higher concentrations. Another interesting response of plant species to ethylene is the massive production of root hairs. Ethylene may also modify shoot growth (Bajgiran et al. 2008) and nodule formation in legumes by rhizobia (Table 4.1). However, the mechanism by which ethylene inhibits nodulation is unclear, and the position at which it acts during the

**Table 4.1** Effect of ethylene on *Rhizobium*–legume symbiosis

| Legume | Host specific rhizobia | Effect on nodulation | References |
|---|---|---|---|
| *Medicago truncatula* | *Sinorhizobium meliloti* | Initiation of infection threads, Inhibits the Nod factor signal transduction pathway, Inhibits the maintenance of calcium spiking | Oldroyd et al. (2001) |
| *Glycine max* cv. Gong jiao 6301-1 | *Bradyrhizobium japonicum* | Decrease in nodule number | Xie et al. (1996) |
| *Pisum sativum* cv. Rondo | *Rhizobium leguminosarum* bv. *viciae* | Decrease in nodule number | Lee and LaRue (1992) |
| *Melilotus alba* U389 | *S. meliloti* | Decrease in nodule number | Lee and LaRue (1992) |
| *Pisum sativum* cv. Sparkle | *R. leguminosarum* bv. *viciae* | Decrease in nodule number; blockage of infection thread elongation in inner cortex | Lee and LaRue (1992) |
| *Pisum sativum* cv. Feltham First | *R. leguminosarum* bv. *viciae* | Inhibition of root extension, decrease in nodule numbers and nitrogen fixation | Goodlass and Smith (1979) |
| *Trifolium repense* cv.Huia | *R. trifoli* | Decrease in nodule number and nitrogen fixation | Goodlass and Smith (1979) |
| *Phaseolus vulgaris* var. Pencil Podded Black Wax | *Rhizobium* sp. | Decrease in nodule number and nitrogen fixation | Grobbelaar et al. (1971) |

Modified from Okazaki et al. (2004)

complex symbiotic process is inconclusive. For example, inoculation with sinorhizobia and bradyrhizobia enhanced ethylene production in the roots of alfalfa and soybean (*Glycine max*) plants (Ligero et al. 1986, 1991; Suganuma et al. 1995). In contrast, there are several reports that suggest that exogenously applied ethylene inhibits nodulation in legumes by blocking cortical cell division and reducing the number of cortical infections (Lee and LaRue 1992; Spaink 1997). The inhibition of nodule formation could be a result of developmental effects that ethylene exerts on the plant. For example, ethylene is required for the elongation of root hairs (Tanimoto et al. 1995; Pitts et al. 1998); a function clearly involved in the invasion of rhizobia, but simultaneously inhibits cell division (Goodlass and Smith 1979), a function that would be detrimental to nodule formation. However, a report suggests that the induction of root hair growth by nod factors occurs independently of ethylene (Heidstra et al. 1997). Moreover, the synthesis of endogenous ethylene in legume (e.g., pea) controls the position of nodule primodia formation (Heidstra et al. 1997). Additionally, the exogenously applied ethylene inhibits infection thread elongation in inner cortex and root growth (Xie et al. 1996), and also reduces nodule number (Peters and Crist-Estes 1989; Lee and LaRue 1992) and the morphology of nodules (Fernandez-Lopez et al. 1998) formed on the root systems and, consequently, nitrogen fixation. The production of ethylene in legume roots has been found to increase after application of rhizobial cells (Ligero et al. 1986), nitrate (Ligero et al. 1986) and nod factor (van Spronsen et al. 1995), suggesting that these factors control nodulation through their effects on the levels of ethylene. For example, the fate of rhizobial infection in the root hairs of legumes has been proposed to be regulated by the levels of ethylene in the underlying plant cortex (Guinel and Geil 2002); a low level of ethylene, allowing proper disposition of the cytoskeleton, is probably required for successful entry of the infection thread in the outermost layer of cortical cells, whereas higher levels of the hormone induce abortion of the infection thread by inducing crosslinking of its matrix glycoproteins. This hypothesis is substantiated by much evidence, for instance, *sickle*, an ethylene-insensitive mutant of barrel medic, has a very high persistence of infection threads (Penmetsa and Cook 1997), while *brz*, a potential ethylene-oversensitive mutant of pea, had much higher numbers of aborted infection threads than those observed for wild-type plants. Ethylene effect has commonly been reported in legumes that form indeterminate nodules. It has been shown to inhibit nodule development in pea (Guinel and Sloetjes 2000; Guinel and Geil 2002), *Trifolium repens* (Goodlass and Smith 1979), and *M. sativa* (Peters and Crist-Estes 1989). On the other hand, the nodulation response of determinate nodules to ethylene is variable and species-dependent. For example, ethylene inhibited nodulation in *Lotus japonicus* and *Macroptilium atropurpureum* (Nukui et al. 2000) but failed to alter nodulation in soybean (Schmidt et al. 1999; Suganuma et al. 1995), although the number of nodules on soybean cv. Bragg was reduced by ethylene. Also, *Sesbania rostrata* is less sensitive to ethylene because exogenous ACC or CEPA (2-chloroethylphosphonic acid) did not negatively influence root nodulation (Xie et al. 1996). In *S. rostrata* roots, differential ethylene concentrations may

determine the fate of nodule development by influencing the persistence of the nodule meristem (Fernandez-Lopez et al. 1998). Differential ethylene concentrations could be caused by mechanisms interfering with ethylene production, diffusion, or perception. Ethylene, thus, (1) regulates the formation of the infection thread, (2) regulates the maintenance of the nodule meristems, as reported in *S. rostrata*, and (3) inhibits the maintenance of calcium spiking after induction by nod factor (Oldroyd et al. 2001).

However, when ethylene pressure is removed from plants, a substantial increase in nodule formation occurred in *M. trunculata* (Oldroyd et al. 2001), *M. sativa* (Nukui et al. 2000), *L. japonicus* (Bras et al. 2000), pea (Lorteau et al. 2001), and *M. atropurpureum* (Nukui et al. 2000). Since legume root nodulation is dependent on the density of *Rhizobium* in the root environment (Kucey and Hynes 1989), it is, therefore, possible that ethylene controls nodulation by also limiting rhizobial multiplication. To validate this hypothesis, Tamimi and Timko (2003) suggested that ethylene could modulate an early step in the Nod factor signal transduction pathway indicating that the effect of ethylene on nodulation is not limited to the host plant but might also affect the bacterial symbiont.

## 4.4 Rhizobial Strategies for Decreasing Ethylene Levels

Legume production depends heavily on biologically fixed nitrogen (BNF) derived from the symbiotic interactions between rhizobia and its specific host plants. Despite a phytohormone, ethylene acts as a negative factor in the nodulation process**.** Conversely, nodulation can be promoted when plants are treated with ethylene inhibitors or antagonists (Peters and Crist-Estes 1989; Yuhashi et al. 2000). Interestingly, recent discoveries suggest that rhizobia employ several strategies to reduce the amount of ethylene and consequently its inhibitory effect on nodulation. In this context, two potential strategies have been reported for lowering the ethylene effect. One strategy involves an ethylene biosynthesis inhibitor, called rhizobiotoxine, an enol–ether amino acid [2-amino-4-(2-amino-3-hydroxypropoxy)- trans-3-butenoic acid] produced by rhizobia (Okazaki et al. 2007) that inhibits ACC synthase while the other involves an enzyme, ACC deaminase, produced by the symbiotic nitrogen-fixing bacteria (Duan et al. 2008; Bajgiran et al. 2008). Of these, rhizobiotoxine are secreted outside rhizobial cells probably by a rhizobiotoxine transporter and delivered to plants. Although rhizobiotoxin is best known for its chlorosis-inducing activity on leguminous plants (Owens and Wright 1965; Owens 1973), the recent studies have shown that rhizobiotoxine plays a positive role in nodule development through its inhibition of ethylene biosynthesis. The positive and necessary role of rhizobiotoxine in mutualistic symbiosis was demonstrated by Duodu et al. (1999) who suggested that rhizobiotoxine is required for *Bradyrhizobium elkanii* for efficient nodulation in greengram (*Vigna radiata* L. Wilczek). Since then, the role of rhizobiotoxine in symbiosis improvement has been reported for other legumes (Yuhashi et al 2000; Sugawara et al. 2006).

The cumulative evidence suggests that rhizobitoxine-producing bacteria modulate plant–microbe interactions via ethylene in the rhizosphere and phyllosphere environments. In addition, rhizobitoxine-producing capability might be utilized as tools in agriculture and biotechnology (Sugawara et al. 2006). The reduction in ethylene levels by synthesizing rhizobiotoxin is, however, limited to slow growing rhizobial species. Therefore, how fast growing rhizobia alleviate the toxicity of ethylene needs to be addressed since they could enhance nodulation as well. Hence, an alternative strategy for reduction in the effects of ethylene on plants involves ACC deaminase. The extent of ACC deaminase in rhizobial strains varies greatly. For example, out of the total 33 strains of rhizobia collected from 30 different sites across Saskatchewan, Canada, only 11% had ACC deaminase activity (Duan et al. 2008), while in another study, around 38% *Rhizobium* spp. displayed ACC activity (Ma et al. 2003a).

Rhizobia capable of producing ACC deaminase are able to reduce ethylene concentration in the plant cells in the immediate vicinity of the infection threads. Therefore, the infection threads containing ACC deaminase-producing rhizobial cells can better suppress the defense signals in the plant cell and increase the persistence of the infection threads. This leads to higher nodule numbers as reported in plants inoculated with *S. meliloti* Rm11466 as well as a higher portion of nodules formed by Rm11466 when the plants were coinoculated with both *S. meliloti* Rm5356 and Rm11466. In addition, since a high percentage of infection threads abort before a nodule is formed, infection threads containing Rm11466 could suppress the development of those containing Rm5356 by reaching nodule primordia and forming nodules faster. This also accounts for the higher competitiveness of Rm11466 (Ma et al. 2004). Gage et al. (1996) observed that *S. meliloti* proliferates inside the infection threads of alfalfa. The population of bacteria inside the threads originates from the clonal expansion of a small number of founder cells which enter the threads early, and only the bacterial cells of the threads will eventually be released into the cytoplasm of the plant cell as reported by Ma et al. (2004). It is also suggested that *S. meliloti* cells travel down the infection threads by proliferation instead of swimming, since bacteria do not have flagella when inside the threads. However, what nutrient source(s) *S. meliloti* cells utilize to proliferate in the infection threads is not known. Although the ACC concentrations in the infection threads are expected to be low, the possible ability of the ACC deaminase-producing cells to utilize ACC as an extra nutrient source makes the bacterium proliferate better in the infection threads than those that do not have this enzyme. Therefore, infecting cells that produce ACC deaminase are more likely to reach nodule primordia and form mature nodules, which results in the higher competitiveness of *S. meliloti* Rm11466 than of Rm5356 .

In order to explain how ACC deaminase-containing PGPR can lower plant ethylene levels and in turn stimulate plant growth, a model (Fig. 4.1) similar to those originally proposed by Glick et al. (1998) has also been reported for reducing the levels of ethylene in legumes by rhizobia (Ma et al. 2003a, 2004). According to this model, the reduction in the concentration of ethylene by ACC deaminase

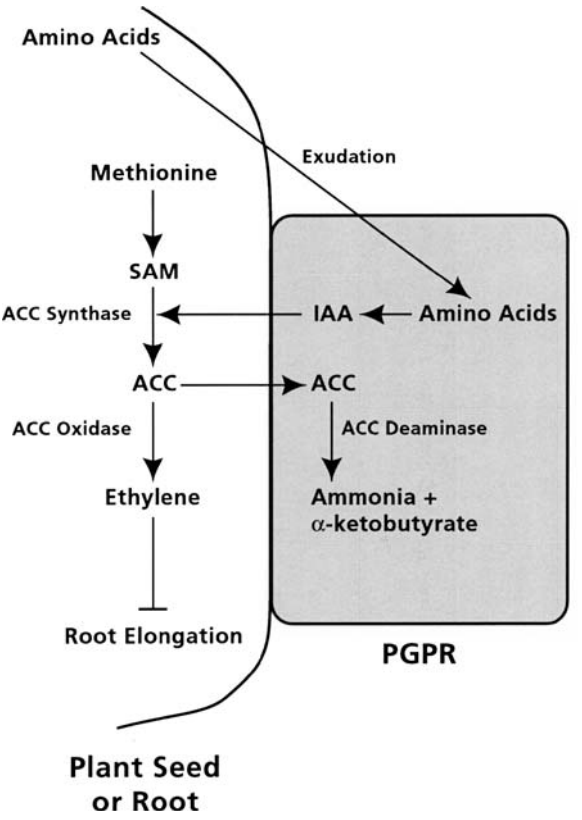



**Fig. 4.1** Schematic representation of mechanisms through which the PGPR attached to either a seed or plant root lowers ethylene concentration and prevents ethylene inhibition of root elongation. The (⊥) indicates inhibition. IAA indicates indoleacetic acid, ACC 1-aminocyclopropane-1- carboxylic acid and SAM, *S*-adenosyl-methionine (adapted from Glick et al. 1998)

involves: (1) binding or colonization of organisms onto the surface of either the seed or root of a developing plant; (2) synthesis and release of IAA by bacterial strains in response to tryptophan and other small molecules present in the seed or root exudates; some of IAA secreted could be used by the plants; the IAA so released, together with endogenous plant IAA, stimulate plant cell proliferation and elongation, or it can also induce the activity of ACC synthase to produce ACC; (3) exudation of the plant's ACC along with other small molecules such as sugars, organic acids and amino acids (Penrose and Glick 2001); (4) uptake of exudates by the bacteria which is used as a source of nutrients; and (5) cleavage of ACC by ACC deaminase forming ammonia and α-ketobutyrate, which are further metabolized by the bacteria. Thus, the uptake and cleavage of ACC by ACC deaminase containing $N_2$ fixers decreases the amount of ACC and thereby acts as a sink for it. In turn, the

decreased level of ACC lowers the levels of ethylene and alleviates its potential inhibitory effect and consequently help plants to grow better under ethylene stressed environment.

## 4.5 Genes Involved in the Synthesis of ACC Deaminase and Nodulation Enhancement

1-amino cyclopropane-1-carboxylic acid deaminase (AcdS) is an enzyme that degrades the precursor of plant hormone ethylene. The AcdS activity has been identified in many soil bacteria (Hameeda et al. 2006; Cheng et al. 2007) including rhizobia, which is reported to play an important role in plant growth promotion. Genes encoding ACC deaminase have been reported in PGPR (Venkadasamy et al. 2008) including *B. japonicum* (Kaneko et al. 2002), *Mesorhizobium loti* (Kaneko et al. 2000; Sullivan et al. 2002; Uchiumi et al. 2004), *R. leguminosarum* and *R. gallicum* (Ma et al. 2003a; Duan et al. 2008) and *R. radiobacter*. The ACC deaminase genes from *R. leguminosarum* bv. *viciae* 128C53K and 99A1 showed 64% similarities with the gene reported in plant growth-promoting bacterium, *Pseudomonas putida* UW4 (Shah et al. 1998). However, all these rhizobial strains had relatively low ACC deaminase activities compared to that expressed by *P. putida* UW4. Furthermore, the regulation study of ACC deaminase in strain 128C53K revealed its very low basal level of expression in the absence of ACC, which could be induced by ACC concentrations as low as 1 μM (Ma et al. 2003a), suggesting that ACC deaminase in strain 128C53K could lower the ethylene concentration in pea roots as a consequence of nodulation (Ma et al. 2003a). In fact, an ACC deaminase knockdown strain 128C53K was approximately 30% less efficient at nodulating pea plant roots than the wild-type. In contrast, introduction of the ACC deaminase gene and its upstream regulatory gene, a leucine responsive regulatory protein (LRP)-like gene (acdR) from strain 128C53K, into a strain of *S. meliloti*, which does not produce this enzyme, made it 35–40% more effective and competitive than the wild-type at nodulating *M. sativa* (Ma et al. 2004). ACC deaminase genes in *M. loti* strains MAFF303099 and R7A are preceded by a nifA-dependent (Owens et al. 1972) promoter and thus appear to be expressed only in the symbiotic state (Sullivan et al. 2002; Uchiumi et al. 2004). Indeed, the ACC deaminase genes of *M. loti* strains MAFF303099 were drastically upregulated in bacteroids compared with the level of its transcription in free-living cells (Uchiumi et al. 2004). In contrast, ACC deaminase genes in *B. japonicum* and *R. leguminosarum* possess a σ promoter (Peters and Crist-Estes 1989) with an LRP (leucine responsible regulatory protein) box (Kaneko et al. 2002). Since the promoter structure of σ/LRP was involved in induction of ACC deaminase gene in *R. leguminosarum* and *E. cloacae* (Ma et al. 2003a, b), ACC is probably an inducer of the gene in *B. japonicum* chromosome. The proteome analysis of *B. japonicum* bacteroids has shown that ACC deaminase is one of the major proteins in bacteroids

rather than the free-living cell, suggesting that ACC in host plants might induce the ACC deaminase in rhizobia (Hoa et al. 2004).

An important question is how a decreased level of ethylene enhances nodulation. To address this issue, several models have been proposed depicting the relationship between signal transduction, ethylene sensing and the development of nodulation (Stearns and Glick 2003). Thus, two strategies can be adopted: (1) construct transgenic legumes with altered ethylene sensitivities; the expression of ethylene receptors that cannot bind ethylene confers reduced ethylene sensitivity to heterologous plants in a genetically dominant manner (Bleecker 1999); and (2) engineer rhizobia to enhance nodulation competitiveness on host legumes rather than by single inoculation (Okazaki et al. 2003; Uchiumi et al. 2004). Nodulation competitiveness, the ability of certain rhizobial strain to form nodules in a multistrain environment, has practical importance in agriculture due to the fact that supernodulating strains often perform poorly under field conditions due to better competing populations of inferior rhizobia in soils. It is, therefore, possible that engineering rhizobia to exhibit high ACC deaminase activity is likely to delay the nodule senescence by reducing ethylene production, and thereby promoting nodule development and nitrogen fixation. Accordingly, nodules with high bacteroid ACC deaminase activity are expected to grow larger and contain more bacteroids that remain active for a longer period of time. For example, the ACC deaminase of *R. leguminosarum* bv. *viciae* 128C53K has been previously reported to be able to enhance nodulation of peas. The ACC deaminase structural gene (acdS) and its upstream regulatory gene, a leucine-responsive regulatory protein (LRP)-like gene (lrpL) from *R. leguminosarum* bv. *viciae* 128C53K, were introduced into *S. meliloti*, which does not produce this enzyme, in two different ways: through a plasmid vector and by in situ transposon replacement. The resulting ACC deaminase-producing transformed strain showed 35–40% increase in both nodule numbers and biomass in alfalfa (*M. sativa*) plants, likely by reducing ethylene production in the host plants. Furthermore, the genetically modified ACC deaminase-producing *S. meliloti* strain was more competitive in nodulation than the wild-type strain, and perhaps utilizes ACC as a nutrient within the infection threads (Ma et al. 2004). Thus, even if some rhizobial strains lack the ability to decrease ethylene levels in host legumes, the introduction of genes coding for ACC deaminase into these rhizobia may enhance the symbiotic interactions with the cognate host legumes and eventually reduce the ethylene level. This strategy of ethylene reduction in legumes is an interesting area of research that could lead to facilitating the productivity of legumes under stressed conditions. However, how the ACC deaminase activity affects nodulation competitiveness is unclear. The plausible explanation is that the ACC deaminase blocks ethylene biosynthesis locally at the infection sites and cancels the effects of ethylene on nodulation during the mitogenic process (Okazaki et al. 2004). Therefore, it has been suggested that the rhizobia employ more than one strategies, i.e., the ACC deaminase and rhizobiotoxine synthesis to reduce the extent of ethylene biosynthesis by the host legumes and enhance nodule formation.

## 4.6 Performance of ACC Deaminase Containing Transgenic Plants/Rhizobia

The exciting advances in molecular engineering facilitated the genetic manipulation of organisms for achieving a defined set of goals and objectives. An approach has been extended to understand the mechanism of reduction of ethylene levels in plants through genetic manipulation in genetically engineered legumes or rhizobia, for overexpression of ACC deaminase. Introduction of genes responsible for expression of ACC deaminase in plants has received more attention than rhizobia due to several reasons such as: (1) the ACC deaminase activity is widely reported among autochthonous PGPR species including rhizobia; (2) the degree of success in transforming the ACC deaminase genes into plants has been more successful; and (3) engineered organisms after inoculation face severe competition from indigenous ones, and therefore the chances of survival and proliferation of genetically modified transgenic bacteria in natural soil environments is quite often reduced. Because of all these factors, the plant molecular biologists opt for developing transgenic plants that express the ACC deaminase genes.

Many plant species have been genetically engineered with ACC deaminase expression to protect them against multiple biotic and abiotic stresses. For example, Grichko et al. (2000) expressed bacterial ACC deaminase in tomato (*Lycopersicum esculentum*) cv. Heinz 902 under the transcriptional control of two tandem 35S cauliflower mosaic virus promoters (constitutive expression), the rolD promoter from *Agrobacterium rhizogenes* (root-specific expression) or the pathogenesis-related prb-1b promoter from tobacco. Transgenic tomato plants expressing ACC deaminase particularly controlled by the prb-1b promoter accumulate larger amounts of metals within the plant tissues. However, because the tomato plants are unlikely to be used in the phytoremediation of contaminated sites, Nie et al. (2002) expressed ACC deaminase genes in canola (*Brassica napus*) plants and tested their potential to grow in the presence of high levels of arsenate in the soil for metal accumulation in plant tissues. Also, the ability of the plant growth-promoting bacterium *E. cloacae* CAL2 has been tested to facilitate the growth of both non-transformed and ACC deaminase-expressing canola plants for developing a successful phytoremediation strategy. In all cases, transgenic canola expressing ACC deaminase genes accumulated larger amounts of arsenate from the contaminated soil than nontransformed canola plants. Similarly, the expression of *Pseudomonas* sp. 6G5 *acd*S gene under the control of a 35S promoter in tomato plants led to decreased ethylene synthesis and delayed fruit ripening (Klee et al. 1991). Such heterologous expression studies provide a functional demonstration of the role of AcdS to lower ethylene level in plants. In addition, these transgenic plants showed higher tolerance to flooding, heavy metal stress and pathogen attack (Klee et al. 1991; Klee 1993; Stearns and Glick 2003), as expected from the involvement of ethylene in stress responses.

Ethylene inhibits the establishment of symbiosis between rhizobia and legumes. To examine how and when endogenous ethylene inhibits rhizobial infection and

nodulation, transgenic, *L. japonicus* carrying the mutated melon ethylene receptor gene *Cm-ERS1/H70A* that confers ethylene insensitivity and fixes the transgene in the $T_3$ generation were produced. The resultant transgenic plants showed reduced ethylene sensitivity because of ACC resistance and increased flowering duration, probably due to a dominant negative mechanism. When inoculated with *M. loti*, transgenic plants showed markedly higher numbers of infection threads and nodule primordia on their roots than did either wild-type or azygous plants during the early stage of cultivation period as well as during later stages, when the number of mature nodules had reached a steady state. In addition, transcripts of *NIN*, a gene governing infection thread formation, increased in the inoculated transgenic plants as compared to the wild-type plants. The infection responses of transgenic plants were similar to those of wild-type plants treated with ethylene inhibitors. These results suggest that the endogenous ethylene in *L. japonicus* roots inhibits rhizobial infection at the primary nodulation, probably via *NIN* gene, and suggest that ethylene perception assists negative feedback regulation of secondary nodule initiation (Nukui et al. 2004). Furthermore, in order to examine the regulation of the *acd*S gene encoding ACC deaminase in *M. loti* MAFF303099 during symbiosis with the host legume *L. japonicus*, Nukui et al. (2006) introduced the β-glucuronidase (GUS) gene into *acd*S so that GUS was expressed under control of the *acdS* promoter. The histochemical GUS assay demonstrated the exclusive expression of *acd*S in mature root nodules. Two homologous *nif*A genes, mll5857 and mll5837, were found in the symbiosis island of *M. loti* and were designated *nif*A1 and *nif*A2, respectively. Quantitative reverse transcription-PCR demonstrated that *nif*A2 disruption resulted in considerably diminished expression of *acdS*, *nif*H, and *nif*A1 in bacteroid cells. In contrast, *nif*A1 disruption slightly enhanced the expression of *acd*S transcripts and suppressed *nif*H to some extent. These observations indicate that the *acd*S gene and other symbiotic genes are positively regulated by the NifA2 protein, but not by the NifA1 protein, in *M. loti*. The mode of gene expression suggests that *M. loti acd*S participates in the establishment and/or maintenance of mature nodules by interfering with the production of ethylene, which induces negative regulation of nodulation (Nukui et al. 2006). Recently, the role of ACC deaminase of symbionts in nodulation and growth of *Leucaena leucocephala* has been studied (Tittabutr et al. 2008). The *acdS* genes encoding ACC deaminase were cloned from *Rhizobium* sp. strain TAL1145 and *Sinorhizobium* sp. BL3 in multicopy plasmids, and transferred to TAL1145. The BL3-*acd*S gene greatly enhanced ACC deaminase activity in TAL1145 compared to the native *acdS* gene. The transconjugants of TAL1145 containing the native or BL3 *acd*S gene could grow in minimal media containing 1.5 mM ACC, whereas BL3 could tolerate up to 3 mM ACC. The TAL1145 *acd*S gene is inducible by mimosine and not by ACC, while the BL3 *acdS* gene was highly inducible by ACC and not by mimosine. The transconjugants of TAL1145 containing the native- and BL3-*acd*S genes formed nodules in greater numbers and sizes, and produced higher root mass on *L. leucocephala* than did TAL1145. Introduction of multiple copies of the *acd*S gene increased ACC deaminase activities of TAL1145 and enhanced its

symbiotic efficiency on *L. leucocephala* (Tittabutr et al. 2008). The acdS::gus fusions in strains TAL1145:pUHR353::gus expressed in *Leucaena* nodules, although at low levels., which suggests the possibility of increasing ACC deaminase activity in the nodule through multiple copies of acdS in the symbiont. Thus, the inoculation with TAL1145 carrying multiple copies of acdS could increase nodule number, nodule dry weight and root dry weight of *Leucaena* seedlings after 16 weeks. The increases in nodule number and nodule dry weight due to inoculation with TAL1145:pUHR353 and TAL1145:pUHR354 were detectable even when the plants were harvested and analyzed after 8 weeks. Larger differences in the root dry weights due to high ACC deaminase activity in the symbionts may be observed when the plants are grown for longer periods of time under field conditions. It is difficult to assess the effects of increased ACC deaminase activities on nodule senescence and maintenance using *Leucaena*, which forms indeterminate type of nodules that continue to grow longitudinally producing new meristematic and bacteroid zones. The effects of increased symbiotic ACC deaminase activities may be more visible on plants like beans and soybeans, which produce determinate type of nodules that senesce entirely after a fixed period of nitrogen fixation. It is expected that increased ACC deaminase activities may delay senescence and prolong the nitrogen-fixing period in such nodules, leading to increased growth and yield of the plants. Thus, enhanced ACC deaminase activities in *Rhizobium* bacteroids inside *Leucaena* nodules reduce ethylene biosynthesis and consequently promote nodule development. Reducing ethylene synthesis may also help in nodule maintenance by delaying senescence. These properties, together with enhanced ACC deaminase activities in the nodule, are likely to result in increased $N_2$ fixation and higher growth of the plant. Besides synthesizing ACC deaminase, rhizobia also facilitate legume growth by providing the plants with other benefits such as siderophores (Wani et al. 2008), phytohormones (Wani et al. 2007; Ahmad et al. 2008; Bajgiran et al. 2008), P solubilization (Sridevi et al. 2007), and metal detoxification (Wani et al. 2007, 2008). Thus, by genetically engineering rhizobial strains to produce ACC deaminase, there is a real possibility of obtaining better strains for use as field inocula to increase the yield of leguminous crops.

## 4.7 Conclusion

Symbiotic nitrogen fixation process plays a significant role in improving the soil fertility and productivity of low-N soils. Hence, the symbiosis can relieve the requirements for added nitrogenous fertilizer during the growth of leguminous crops. Symbiotic nitrogen fixers in addition to nitrogen fixation, utilize a variety of mechanisms, both direct and indirect, to stimulate the growth of plants and/or to compete in nodulation. Ethylene is a gaseous phytohormone produced by the plants including legumes during normal growth conditions. However, under biotic and abiotic stresses, the synthesis of ethylene increases and adversely affects the legume–*Rhizobium* symbiosis. The rhizobia possessed with ACC deaminase

activity lowers the level of ethylene and consequently improves the overall performance of legumes. Interestingly, the rhizobia that do not contain ACC deaminase are unable to nodulate their cognate legumes to the same extent that they might be able to if they possessed this enzyme. Moreover, it is possible to genetically engineer strains that normally lack ACC deaminase with the gene encoding this enzyme, with the expectation that the transformed strain will nodulate its cognate legume to a greater extent than the nontransformed strain. In this regard, the studies demonstrated that *Rhizobium* (e.g., *Sinorhizobium meliloti* Rm1021), which does not have ACC deaminase activity, can nodulate its host legume (alfalfa) more efficiently when it is transformed with the *acd*S and *lrp*L genes from *R. leguminosarum* bv. *viciae* 128C53K. Thus, by genetically engineering rhizobial strains to produce ACC deaminase, there is a real possibility of obtaining better strains for use as field inocula to increase the yield of leguminous crops. Furthermore, ethylene production can be induced during many environmental stresses, such as infection by pathogens, flooding, heavy metal poisoning, and mechanical wounding. The ACC deaminase containing PGPR including symbiotic nitrogen fixers have been found to be able to help the plants resist the detrimental effects of stress ethylene on plant growth imposed by both biotic and abiotic stresses, besides providing other benefits to legumes. Thus, the ethylene decreasing ability of nodule forming bacteria is interesting and suggestive for further understanding of legume–rhizobia interactions and signaling events of *Rhizobium*–legume symbiosis.

## References

Abd-Alla MH (1994) Solubilization of rock phosphates by *Rhizobium* and *Bradyrhizobium*. Folia Microbiol 39:53–56

Abeles FB, Morgan PW, Saltveit ME Jr, Abeles FB, Morgan PW, Saltveit ME Jr (1992) Regulation of ethylene production by internal, environmental and stress factors. Ethylene in plant biology, 2nd edn. Academic, San Diego, pp 56–119

Ahmad F, Ahmad I, Khan MS (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol Res 163:173–181

Alikhani HA, Saleh-Rastin N, Antoun H (2006) Phosphate solubilization activity of rhizobia native to Iranian soils. Plant Soil 287:35–41

Arshad M, Shaharoona B, Mahmood T (2008) Inoculation with *Pseudomonas* spp. containing ACC-deaminase partially eliminates the effects of drought stress on growth, yield, and ripening of pea (*Pisum sativum* L.). Pedosphere 18:611–620

Bajgiran AR, Lakzian A, Rastin NS (2008) Elongation of shoot and root in wheat by ACC deaminase of *Rhizobium* Spp. indigenous to soils of Iran. Int J Agri Biol 10:481–486

Bartsev A, Kobayashi H, Broughton WJ (2004) Rhizobial signals convert pathogens to symbionts at the legume interface. In: Gillings M, Holmes A (eds) Plant microbiology. Garland Science, Abingdon, pp 19–31

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick BR (2005) Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biol Biochem 37:241–250

Belimov AA, Dodd IC, Safronova VI, Hontzeas N, Davies WJ (2007) *Pseudomonas brassicacearum* strain Am3 containing 1-aminocyclopropane-1-carboxylate deaminase can show

both pathogenic and growth promoting properties in its interaction with tomato. J Exp Bot 58:1485–1495

Blaha D, Prigent-Combaret C, Mirza MS, Moenne-Loccoz Y (2006) Phylogeny of the 1-aminocyclopropane-1-carboxylic acid deaminase-encoding gene acdS in phytobeneficial and pathogenic Proteobacteria and relation with strain biogeography. FEMS Microbiol Ecol 56:455–470

Bleecker A (1999) Ethylene perception and signaling: an evolutionary perspective. Trends Plant Sci 4:269–274

Bras CP, Joda MA, Wijfjes AAM, Hartveld M, Stuurman N, Thomas-Oates JE, Spaink HP (2000) A *Lotus japonicus* nodulation system based on heterologous expression of the fucosyl transferase NodZ and the acetyl transferase NoIL in *Rhizobium leguminosarum*. Mol Plant Microbe Interact 13:475–479

Cheng Z, Park E, Glick BR (2007) 1-aminocyclopropane-1-carboxylate deaminase from *Pseudomonas putida* UW4 facilitates the growth of canola in the presence of salt. Can J Microbiol 53:912–918

Cheng Z, Duncker BP, McConkey BJ, Glick BR (2008) Transcriptional regulation of ACC deaminase gene expression in *Pseudomonas putida* UW4. Can J Microbiol 54:128–136

Contesto C, Desbrosses G, Lefoulon C, Bena G, Borel F, Galland M, Gamet L, Varoquaux F, Touraine B (2008) Effects of rhizobacterial ACC deaminase activity on *Arabidopsis* indicate that ethylene mediates local root responses to plant growth-promoting rhizobacteria. Plant Sci 175:178–189

Duan J, Müller KM, Charles TC, Vesely S, Br G (2008) 1-Aminocyclopropane-1-carboxylate (ACC) deaminase genes in rhizobia from southern Saskatchewan. Microb Ecol . doi:10.1007/s00248-008-9407-6

Duodu S, Bhuvaneswari TV, Stokkermans TJW, Peters NK (1999) A positive role for rhizobitoxine in *Rhizobium*-legume symbiosis. Mol Plant Microbe Interact 12:1082–1089

Ecker JR (1995) The ethylene signal transduction pathway in plants. Science 268:667–675

Esashi Y, Esashi Y (1991) Ethylene and seed germination. In: Matoo AK, Shuttle JC (eds) The plant hormone ethylene. CRC, Boca Raton, Florida, pp 133–157

Farwell AJ, Vesely S, Nero V, Rodriguez H, McCormack K, Shah S, Dixon GD, Glick BR (2007) Tolerance of transgenic canola plants (*Brassica napus*) amended with plant growth-promoting bacteria to flooding stress at a metal-contaminated field site. Environ Pollut 147:540–545

Fernandez-Lopez M, Goormachtig S, Gao M, D'Haeze W, Van Montagu M, Holsters M (1998) Ethylene-mediated phenotypic plasticity in root nodule development on *Sesbania rostrata*. Proc Natl Acad Sci USA 95:12724–12728

Frankenberger WTJ, Arshad M (1995) Phytohormones in soil. Microbial production and function. Marcel Dekker, New York, p 503

Gage DJ, Bobo T, Long SR (1996) Use of green fluorescent protein to visualize the early events of symbiosis between *Rhizobium meliloti* and alfalfa (*Medicago sativa*). J Bacteriol 178: 7159–7166

Glick BR (1995) The enhancement of plant growth by free-living bacteria. Can J Microbiol 41:109–117

Glick BR (2005) Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiol Lett 251:1–7

Glick BR, Jacobson CB, Schwarze MMK, Pasternak JJ (1994) Does the enzyme 1-aminocyclopropane-lcarboaylate deaminase play a role in plant growth promotion by Pseudomonas putida GR12–2? In: Ryder MH, Stephens PM, Bowen GD (eds) Improving plant productivity with rhizosphere bacteria. CSIRO, Adelaide, pp 150–152

Glick BR, Penrose DM, Li J (1998) A model for the lowering of plant ethylene concentrations by plant growth-promoting bacteria. J Theor Biol 190:63–68

Glick BR, Patten CL, Holguin G, Penrose DM (1999) Biochemical and genetic mechanisms used by plant growth promoting bacteria. Imperial College Press, London, p 270

Glick BR, Cheng Z, Czarny J, Duan J (2007a) Promotion of plant growth by ACC deaminase-producing soil bacteria. Eur J Plant Pathol 119:329–339

Glick BR, Todorovic B, Czarny J, Cheng Z, Duan J, McConkey B (2007b) Promotion of plant growth by bacterial ACC deaminase. Crit Rev Plant Sci 26:227–242

Goodlass G, Smith KA (1979) Effects of ethylene on root extension and nodulation of pea (*Pisum sativum* L.) and white clover (*Trifolium repens* L.). Plant Soil 51:387–395

Grichko VP, Glick BR (2001) Amelioration of flooding stress by ACC deaminase-containing plant growth-promoting bacteria. Plant Physiol Biochem 39:11–17

Grichko VP, Filby B, Glick BR (2000) Increased ability of transgenic plants expressing the bacterial enzyme ACC deaminase to accumulate Cd, Co, Cu, Ni, Pb, and Zn. J Bacteriol 81:45–53

Grobbelaar N, Clarke B, Hough MC, Grobbelaar N, Clarke B, Hough MC (1971) The nodulation and nitrogen fixation of isolated roots of *Phaseolus vulgaris* L. III. The effect of carbon dioxide and ethylene. Plant Soil Spl Vol:216–223

Guinel FC, Geil RD (2002) A model for the development of the rhizobial and arbuscular mycorrhizal symbioses in legumes and its use to understand the roles of ethylene in the establishment of these two symbioses. Can J Bot 80:695–720

Guinel FC, Sloetjes LL (2000) Ethylene is involved in the nodulation phenotype of *Pisum sativum* R50 (sym 16). An pleiotropic mutant that nodulated poorly and has pale green leaves. J Exp Bot 51:885–894

Hall J, Peirson AD, Ghosh S, Glick BR (1996) Root elongation in various agronomic crops by the plant growth promoting rhizobacterium Pseudomonas putida GR12–2. Israel J Plant Sci 44:37–42

Hameeda B, Rupela OP, Reddy G, Satyavani K (2006) Application of plant growth-promoting bacteria associated with composts and macrofauna for growth promotion of Pearl millet (*Pennisetum glaucum* L.). Biol Fertil Soil 43:221–227

Heidstra RW, Yang WC, Yalcin Y, Peck S, Emons AM, van Kammen A, Bisseling T (1997) Ethylene provides positional information on cortical cell division but is not involved in Nod factor-induced root hair tip growth in *Rhizobium*-legume interaction. Development 124:1781–1787

Herder JD, Goormachtig S, Holsters M (2006) Ethylene in the *Rhizobium*-legume symbiosis. In: Khan NA (ed) Ethylene action in plants. Springer , Berlin, pp 119–134

Hirsch AM, Fang Y (1994) Plant hormones and nodulation: what's the connection? Plant Mol Biol 26:5–9

Hoa LTP, Nomura M, Tajima S (2004) Characterization of bacteroid proteins in soybean nodules formed with *Bradyrhizobium japonicum* USDA110. Microb Environ 19:71–75

Honma M (1993) Stereospecific reaction of 1-aminocyclopropane-1-carboxylate deaminase. In: Pech JC, Latche A, Balague C (eds) Cellular and molecular aspects of the plant hormone ethylene. Kluwer, Dordrecht, The Netherlands, pp 111–116

Honma M, Shimomura T (1978) Metabolism of 1-aminocyclopropane- 1-carboxylic acid. Agric Biol Chem 42:1825–1831

Hontzeas N, Richardson AO, Belimov A, Safronova V, Abu-Omar MM, Glick BR (2005) Evidence for horizontal transfer of 1- aminocyclopropane-1-carboxylate deaminase genes. Appl Environ Microbiol 71:7556–7558

Jackson MB (1991) Ethylene in root growth and development. In: Matto AK, Suttle JC (eds) The plant hormone ethylene. CRC, Boca Raton, Florida, pp 159–181

Jalili F, Kazem Khavazi K, Pazira E, Nejati A, Rahmani HA, Sadaghiani HR, Miransari M (2008) Isolation and characterization of ACC deaminase producing fluorescent pseudomonads, to alleviate salinity stress on canola (*Brassica napus* L.) growth. J Plant Physiol . doi:doi:10.1016/j.jplph.2008.08.004

Jeroen DH, Sofie G, Marcelle H (2006) Ethylene in the *Rhizobium*-legume symbiosis. In: Khan NA, Khan NA (eds) Ethylene action in plants. Springer, Berlin Heidelberg, pp 119–134

Kaneko T, Nakamura Y, Sato S, Asamizu E, Kato T, Sasamoto S, Watanabe A, Idesawa K, Ishikawa A, Kawashima K, Kimura T, Kishida Y, Kiyokawa C, Kohara M, Matsumoto M, Matsuno A, Mochizuki Y, Nakayama S, Nakazaki N, Shimpo S, Sugimoto M, Takeuchi C, Yamada M, Tabeta S (2000) Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. DNA Res 7:331–338

Kaneko T, Nakamura Y, Sato S, Minamisawa K, Uchiumi T, Sasamoto S, Watanabe A, Idesawa K, Iriguchi M, Kawashima K, Kohara M, Matsumoto M, Shimpo S, Tsuruoka H, Wada T, Yamada M, Tabata S (2002) Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. DNA Res 9:189–197

Klee HJ (1993) Ripening physiology of fruit from transgenic tomato (*Lycopersicon esculentum*) plants with reduced ethylene synthesis. Plant Physiol 102:911–916

Klee HJ, Hayford MB, Kretzmer KA, Barry GF, Kishore GM (1991) Control of ethylene synthesis by expression of a bacterial enzyme in transgenic tomato plants. Plant Cell 3:1187–1193

Kloepper JW, Leong J, Teintze M, Schroth MN (1980) Pseudomonas siderophores: a mechanism explaining disease suppressive soils. Curr Microbiol 4:317–320

Krishnan HB, Kang BR, Krishnan AH, Kim KY, Young Cheol Kim YC (2007) *Rhizobium etli* USDA9032 engineered to produce a phenazine antibiotic inhibits the growth of fungal pathogens but is impaired in symbiotic performance. Appl Environ Microbiol 73:327–330

Kucey RM, Hynes MF (1989) Populations of *Rhizobium leguminosarum* bv. *phaseoli* and *viciae* in fields of bean and pea in rotation with nonlegumes. Can J Microbiol 35:661–667

Lee KH, LaRue TA (1992) Exogenous ethylene inhibits nodulation of *Pisum sativum* L. cv. Sparkle. Plant Physiol 100:1759–1763

Ligero F, Liuch C, Olivares J (1986) Evolution of ethylene from roots of *Medicago sativa* plants inoculated with *Rhizobium meliloti*. J Plant Physiol 125:361–365

Ligero F, Caba JM, Lluch C, Olivares J (1991) Nitrate inhibition of nodulation can be overcome by the ethylene inhibitor aminoethoxyvinylglycine. Plant Physiol 97:1221–1225

Long SR (1989) *Rhizobium*–legume nodulation: life together in the underground. Cell 56:203–214

Long SR, Staskawicz BJ (1993) Prokaryotic plant parasites. Cell 73:921–935

Lorteau MA, Ferguson BJ, Guinel FC (2001) Effects of cytokinin on ethylene production and nodulation in pea (*Pisum sativum*) cv Sparkle. Physiol Plant 112:421–428

Lynch JM, Whipps JM (1991) Substrate flow in the rhizosphere. In: Keister DL, Cregan PB (eds) The rhizosphere and plant growth. Kluwer, Dordrecht, The Netherlands, pp 15–24

Ma W, Guinel FC, Glick BR (2003a) The *Rhizobium leguminosarum* bv. *viciae* ACC deaminase protein promotes the nodulation of pea plants. Appl Environ Microbiol 69:4396–4402

Ma W, Sebestianova S, Sebestian J, Burd GI, Guinel F, Glick BR (2003b) Prevalence of 1-aminocyclopropaqne-1-carboxylate in deaminase in *Rhizobia* spp. Anton Van Leeuwenhoek 83:285–291

Ma W, Charles TC, Glick BR (2004) Expression of an exogenous 1-aminocyclopropane-1-carboxylate deaminase gene in *Sinorhizobium meliloti* increases its ability to nodulate alfalfa. Appl Environ Microbiol 70:5891–5897

Mandal SM, Mondal KC, Dey S, Pati BR (2007) Optimization of cultural and nutritional conditions for indole-3-acetic acid (IAA) production by a *Rhizobium* sp. isolated from root nodules of *Vigna mungo* (L.) Hepper. Res J Microbiol 2:239–246

Mayak S, Tivosh T, Glick BR (1999) Effect of wild type and mutant plant growth promoting rhizobacteria on the rooting of mungbeen cuttings. J Plant Growth Regul 18:49–53

Minami R, Uchiyama K, Murakami T, Kawai J, Mikami K, Yamada T, Yokoi D, Ito H, Matsui H, Honma M (1998) Properties, sequence, and synthesis in *Escherichia coli* of 1- aminocyclopropane-1-carboxylate deaminase from *Hansenula saturnus*. J Biochem 123:1112–1118

Naveed M, Za Z, Khalid M, Asghar HN, Akhtar MJ, Arshad M (2008) Rhizobacteria containing Acc-deaminase for improving growth and yield of wheat under fertilized conditions. Pak J Bot 40:1231–1241

Nelson LM (2004) Plant growth promoting rhizobacteria (PGPR): prospects for new inoculants. Plant Manage Netw . doi:10.1094/CM-2004-0301-05-RV

Nie L, Shah S, Burd GI, Dixon DG, Glick BR (2002) Phytoremediation of arsenate contaminated soil by transgenic canola and the plant growth-promoting bacterium *Enterobacter cloacae* CAL2. Plant Physiol Biochem 40:355–361

Nukui N, Ezura H, Yuhashi K, Yasuta T, Minamisawa K (2000) Effects of ethylene precursor and inhibitors for ethylene biosynthesis and perception on nodulation in *Lotus japonicus* and *Macroptilium atropurpureum*. Plant Cell Physiol 41:893–897

Nukui N, Ezura H, Minamisawa K (2004) Transgenic *Lotus japonicus* with an ethylene receptor gene Cm-ERS1/H70A enhances formation of infection threads and nodule primodia. Plant Cell Physiol 45:427–435

Nukui N, Minamisawa K, Ayabe SI, Aoki T (2006) Expression of the 1-aminocyclopropane-1-carboxylic acid deaminase gene requires symbiotic nitrogen-fixing regulator gene nifA2 in *Mesorhizobium loti* MAFF303099. Appl Environ Microbiol 72:4964–4969

Okazaki S, Yuhashi K, Minamisawa K (2003) Quantitative and time-course evaluation of nodulation competitiveness of rhizobitoxine-producing *Bradyrhizobium elkanii*. FEMS Microbiol Ecol 45:155–160

Okazaki S, Nukui N, Sugawara M, Minamisawa K (2004) Rhizobial strategies to enhance symbiotic interactions: rhizobiotoxine and 1-aminocyclopropane-1-carboxylate deaminase. Microb Environ 19:99–111

Okazaki S, Sugawara M, Yuhashi KI, Minamisawa K (2007) Rhizobitoxine-induced chlorosis occurs in coincidence with methionine deficiency in soybeans. Ann Bot 100:55–59

Oldroyd GE, Engstrom EM, Long SR (2001) Ethylene inhibits the Nod factor signal transduction pathway of *Medicago truncatula*. Plant cell 13:1835–1849

Owens LD (1973) Herbicidal potential of rhizobitoxine. Weed Sci 21:63–66

Owens LD, Wright DA (1965) Rhizobial-induced chlorosis in soybeans: isolation, production in nodules, and varietal specificity of the toxin. Plant Physiol 40:927–930

Owens LD, Thompson JF, Fennessy PV (1972) Dihydrorhizobitoxine, a new ether amino-acid from *Rhizobium japonicum*. J Chem Soc Chem Commun 1972:715

Pawlowski K, Bisseling T (1996) Rhizobial and actiorhizal symbioses: what are the shared featrues? Plant Cell 8:1899–1913

Penmetsa RV, Cook DR (1997) A legume ethylene-insensitive mutant hyper infected by its rhizobial symbiont. Science 275:527–530

Penrose DM, Glick BR (2001) Levels of 1-aminocyclopropane-1-carboxylic acid (ACC) in exudates and extracts of canola seeds treated with plant growth-promoting bacteria. Can J Microbiol 47:368–372

Perret X, Staehelin C, Broughton WJ (2000) Molecular basis of symbiotic promiscuity. Microbiol Mol Biol Rev 64:180–201

Peters NK, Crist-Estes DK (1989) Nodule formation is stimulated by the ethylene inhibitor aminoethoxyvinylglycine. Plant Physiol 91:690–693

Pitts RJ, Cernac A, Estelle M (1998) Auxin and ethylene promote root hair elongation in *Arabidopsis*. Plant J 16:553–560

Prayitno J, Rolfe BG, Mathesius U (2006) The ethylene insensitive sickle mutant of *Medicago truncatula* shows altered auxin transport regulation during nodulation. Plant Physiol 142: 168–180

Reed MLE, Glick BR (2005) Growth of canola (*Brassica napus*) in the presence of plant growth-promoting bacteria and either copper or polycyclic aromatic hydrocarbons. Can J Microbiol 51:1061–1069

Safronova VI, Stepanok VV, Engqvist GL, Alekseyev YV, Belimov AA (2006) Root-associated bacteria containing 1-aminocyclopropane-1-carboxylate deaminase improve growth and nutrient uptake by pea genotypes cultivated in cadmium supplemented soil. Biol Fertil Soils 42:267–272

Schmidt JS, Harper JE, Hoffman TK, Bent AF (1999) Regulation of soybean nodulation independent of ethylene signaling. Plant Physiol 119:951–959

Shah S, Li J, Moffat BA, Glick BR (1998) Isolation and characterization of ACC deaminase genes from two different plant growth promoting rhizobacteria. Can J Microbiol 44:833–843

Shaharoona B, Jamro GM, Zahir ZA, Arshad M, Memon KS (2007) Effectiveness of various *Pseudomonas* spp. and *Burkholderia caryophylli* containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.). J Microbiol Biotechnol 17:1300–1307

Spaink HP (1997) Ethylene as a regulator of *Rhizobium* infection. Trends Plant Sci 2:203–204

Sridevi M, Mallaiah KV (2007) Production of indole-3-acetic acid by *Rhizobium* isolates from *Sesbania* species. Afr J Microbiol Res 1:125–128

Sridevi M, Mallaiah KV, Yadav NCS (2007) Phosphate solubilization by *Rhizobium* isolates from *Crotalaria* species. J Plant Sci 2:635–639

Stearns J, Glick BR (2003) Transgenic plants with altered ethylene biosynthesis or perception. Biotechnol Adv 21:193–210

Storey EP, Boghozian R, Little James L, Lowman DW, Chakraborty R (2006) Characterization of 'Schizokinen'; a dihydroxamate-type siderophore produced by *Rhizobium leguminosarum* LARI 917. Biometals 19:637–649

Sturz AV, Christie BR, Nowak J (2000) Bacterial endophytes: potential role in developing sustainable systems of crop production. Crit Rev Plant Sci 19:1–30

Suganuma N, Yamauchi H, Yamamoto K (1995) Enhanced production of ethylene by soybean roots after inoculation with *Bradyrhizobium elkanii*. Plant Sci 111:163–168

Sugawara M, Shin O, Noriyuki N, Hiroshi E, Hisayuki M, Kiwamu M (2006) Rhizobitoxine modulates plant–microbe interactions by ethylene inhibition. Biotech Adv 24:384–388

Sullivan JT, Trzebiatowski JR, Cruickshank RW, Guozy J, Brown SD, Elliot RM, Fleetwood DJ, McCallum NG, Rossbach U, Stuart GS, Weaver JE, Webby RJ, DeBruijn FJ, Ronson CW (2002) Comparative sequence analysis of the symbiosis island of *Mesorhizobium loti* strain R7A. J Bacteriol 184:3086–3095

Tamimi SM, Timko MP (2003) Effects of ehtylene and inhibitors of ethylene synthesis and action on nodulation in common bean (*Phaseolus vulgaris* L.). Plant Soil 257:125–131

Tanimoto M, Roberts K, Dolan L (1995) Ethylene is a positive regulator of root hair development in *Arabidopsis thaliana*. Plant J 8:943–948

Timmers ACJ, Auriac M-C, Truchet G (1999) Refined analysis of early symbiotic steps of the *Rhizobium-Medicago* interaction in relationship with microtubular cytoskeletion re- arrangements. Development 126:3617–3628

Tittabutr P, Jonathan D, Awaya QXL, Dulal B (2008) The cloned 1-aminocyclopropane-1-carboxylate (ACC) deaminase gene from *Sinorhizobium* sp. strain BL3 in *Rhizobium* sp. strain TAL1145 promotes nodulation and growth of *Leucaena leucocephala*. Syst Appl Microbiol 31:141–150

Uchiumi T, Ohwada T, Itakura M, Mitsui H, Nukui N, Dawadi P, Kaneko T, Tabata S, Yokoyama T, Tejima K, Saeki K, Omori H, Hayashi M, Maekawa T, Sriprang R, Murooka Y, Sioya K, Abe M, Minamisawa K (2004) Expression islands clustered on the symbiosis island of the *Mesorhizobium loti* genome. J Bacteriol 186:2439–2448

van Spronsen PC, van Brussel AAN, Kijne JW (1995) Nod factors produced by *Rhizobium leguminosarum* biovar *viciae* induce ethylene-related changes in root cortical cells of *Vicia sativa* ssp. *nigra*. Eur J Cell Biol 68:463–469

VanLoon LC, Glick BR (2004) Increased plant fitness by rhizobacteria. In: Sandermann H (ed) Molecular ecotoxicology of plants. Springer, Berlin, pp 177–205

VanLoon LC, Geraats PBJ, Linthorst HJM (2006) Ethylene as a modulator of disease resistance in plants. Trends Plant Sci 11:184–190

Venkadasamy G, Senthilkumar M, Kishore G, Kannepalli A (2008) Isolation and characterization of ACC deaminase gene from two plant growth-promoting rhizobacteria. Curr Microbiol 57:312–317

Vernié T, Sandra M, de Françoise B, Julie P, Jean-Philippe C, Christian Rogers GO, Florian FAN, Pascal G (2008) EFD is an ERF transcription factor involved in the control of nodule number and differentiation in *Medicago truncatula*. Plant Cell 20:2696–2713

Wang C, Knill E, Glick BR, Défago G (2000) Effect of transferring 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHA0 and its *gacA* derivative CHA96 on their growth-promoting and disease-suppressive capacities. Can J Microbiol 46:898–907

Wani PA, Khan MS, Zaidi A (2007) Synergistic effects of the inoculation with nitrogen fixing and phosphate solubilizing rhizobacteria on the performance of field grown chickpea. J Plant Nutr Soil Sci 170:283–287

Wani PA, Khan MS, Zaidi A (2008) Chromium reducing and plant growth promoting *Mesorhizobium* improves chickpea growth in chromium amended soil. Biotechnol Lett 30:159–163

Xie ZP, Staehelin C, Wiemken A, Boller T (1996) Ethylene responsiveness of soybean cultivars characterized by leaf senescence, chitinase induction and nodulation. J. Plant Physiol 149: 690–694

Yang J, Kloepper JW, Ryu CM (2008) Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci . doi:10.1016/j.tplants.2008.10.004

Yuhashi K, Ichikawa N, Ezura H, Akao S, Minakawa Y, Nukui N, Yasuta T, Minamisawa K (2000) Rhizobitoxine production by *Bradyrhizobium elkanii* enhances nodulation and competitiveness on *Macroptilium atropurpureum*. Appl Environ Microbiol 66:2658–2663

Zahir ZA, Arshad M, Frankenberger WT Jr (2004) Plant growth promoting rhizobacteria: applications and perspectives in agriculture. Adv Agron 81:97–168

Zahir ZA, Munir A, Asghar HN, Shaharoona B, Arshad M (2008) Effectiveness of rhizobacteria containing ACC deaminase for growth promotion of peas (*Pisum sativum*) under drought conditions. J Microbiol Biotechnol 18:958–963

Zhang Y, Zhao L, Wang Y, Yang B, Chen S (2008) Enhancement of heavy metal accumulation by tissue specific co-expression of iaaM and ACC deaminase genes in plants. Chemosphere 72:564–571

# Chapter 5
# Strategies for Crop Improvement in Contaminated Soils Using Metal-Tolerant Bioinoculants

**Anju Rani and Reeta Goel**

**Abstract** Heavy metal contamination due to natural and anthropogenic sources is a global environmental concern. Release of heavy metals without proper treatment poses a serious threat to public health because of its persistence, biomagnification and accumulation in food chain. Nonbiodegradability and sludge production are the two major constraints of metal treatment. The bioremediation of soil, sludge, sediments and wastes polluted with heavy metals generally involves the active microbiological processes of biosorption, bioaccumulation, sequestration and efflux. Bioremediation using microbes well adapted to diverse physiological conditions could be utilized for remediation of heavy metal-contaminated sites. The application of proteomics in environmental bioremediation program provides a global view of the protein compositions of the microbial cells and offers a promising approach to understand the molecular mechanisms of bioremediation. In this chapter, attention is paid to highlighting the strategies for crop improvement using metal-tolerant microbes in soils contaminated with heavy metals.

## 5.1 Introduction

Metals have been an important constituent of the earth's crust from the time it was evolved. Thus, even the early life has arisen in the presence of abundance of metals. Over the ages, all living systems have evolved to use some metals as vital constituents, while they have learned to grapple with some others which are toxic (Choudhury and Srivastava 2001). Environmental pollution by metals became evident when mining and industrial activities increased in the late nineteenth and twentieth centuries. Potentially hazardous levels of heavy metals are being

R. Goel (✉) and A. Rani
Department of Microbiology, Govind Ballabh Pant University of Agriculture and Technology, Pantnagar, Uttaranchal, 263145, India
e-mail: rg55@rediffmail.com

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement,*
DOI: 10.1007/978-3-642-01979-1_5, 

dispersed into subsurface sediments and groundwater in a number of metal-contaminated sites and represent a challenge for environment restoration.

## 5.2 Heavy Metals

The term "heavy metals" refers to metals and metalloids having densities greater than 5 g $cm^{-3}$ and is usually associated with pollution and toxicity, although some of these elements (essential metals) are required by organisms at low concentrations (Adriano 2001). For example, zinc (Zn) is the component of a variety of enzymes (dehydrogenases, proteinases, peptidases) but is also involved in the metabolism of carbohydrates, proteins, phosphate, auxins, in RNA and ribosome formation in plants (Kabata-Pendias and Pendias 2001). Copper (Cu) contributes to several physiological processes (photosynthesis, respiration, carbohydrate distribution, nitrogen and cell wall metabolism, seed production) in plants including disease resistance (Kabata-Pendias and Pendias 2001). The effective functioning of the metabolisms of humans and bacteria is also dependent on these metals (Adriano 2001; Blencowe and Morby 2003; Cavet et al. 2003). However, at high concentrations, these metals exhibit toxic effects on cells (Baker and Walker 1989). In contrast, cadmium (Cd) is not involved in any known biological processes (nonessential metal) and may be quite toxic as it is accumulated by organisms. It is known to: (1) disturb enzyme activities, (2) inhibit the DNA-mediated transformation in microorganisms, (3) interfere in the symbiosis between microbes and plants, and (4) increase plant predisposition to fungal invasion (Kabata-Pendias and Pendias 2001). In humans, it may promote several disorders in the metabolism of Ca and vitamin D leading to bone degeneration and kidney damage (itai-itai disease) (Adriano 2001). The excessive uptake of heavy metals by animals and humans is the result of the successive accumulation of these elements in the food chain, the starting point being the contamination of the soil.

## 5.3 Origin of the Contamination in Soils

The main problem with heavy metals (such as Cu, Zn and Cd) in soils is that, unlike organic pollutants, they cannot be biologically degraded and, therefore, persist in the environment for longer periods of time. Their presence in soils may be from natural or anthropogenic origins. Natural sources include atmospheric emissions from volcanoes, the transport of continental dusts, and the weathering of metal-enriched rocks (Ernst 1998). However, the major source of contamination is from anthropogenic origin: the exploitation of mines and smelters, the application of metal-based pesticides and metal-enriched sewage sludges in agriculture, combustion of fossil fuel, metallurgical industries and electronics (manufacture, use and disposal), military training, etc., contribute to an increased input of heavy metals in soils (Alloway 1995). Whereas the industrial emissions of metals may be controlled

by the installation of adequate air filters, the main source of contamination for humans remains the ingestion of plants growing in derelict soils. The use of intensive farm management practices, like application of phosphatic fertilizers, sewage sludge input and pesticide treatment, are responsible for the pollution of conventional agricultural soils. Although these practices significantly increase the yields by protecting plants from deleterious pathogens and providing them with all the nutrients necessary for a rapid and sustained growth, they may also add large amounts of heavy metals and organic pollutants to soil which, in turn, may accumulate in plants. For instance, Hamon et al. (1998) have shown that the addition of phosphatic fertilizers increased Cd uptake of wheat (*Triticum aestivum*). However, the risk emerging from heavy metals largely depends on their bioavailability (Adriano 2001).

Mineral rock weathering and anthropogenic sources provide two of the main types of metal to soils. According to Ross (1994), the anthropogenic sources of metal contamination can be divided to five main groups: (1) metalliferous mining and smelting (e.g., arsenic, cadmium, lead, mercury), (2) industry (e.g., arsenic, cadmium, chromium, cobalt, copper, mercury, nickel, zinc), (3) atmospheric deposition (arsenic, cadmium, chromium, copper, lead, mercury, uranium), (4) agriculture (e.g., arsenic, cadmium, copper, lead, selenium, uranium, zinc), and (5) waste disposal (e.g., arsenic, cadmium, chromium, copper, lead, mercury, zinc).

## 5.4 Heavy Metal Toxicity to Plants

Exposure of plants even to minute concentrations of toxic heavy metals may lead to the alteration of many cellular processes and structures (Hall 2000). One of the characteristic effects of metal poisoning, observable at an early stage, is a reduction in cell proliferation and growth (Schützendübel et al. 2001). It has also been associated with the appearance of oxidative stress (Schützendübel and Polle 2002). Accumulation of reactive oxygen species (ROS), leading to an oxidative burst, is thought to increase cellular damage through oxidation of several macromolecules (Hall 2000), such as lipids (Sandalio et al. 2001) and proteins (Romero-Puertas et al. 2002). Some metals, such as $Fe^{2+}$ and $Cu^{+}$, might induce oxidative cell damage coupled to their autooxidation, through Fenton-type reactions. However, this type of reaction has not been described for either $Cd^{2+}$ or $Hg^{2+}$ in plants (Schützendübel and Polle 2002), despite the evidence of oxidative stress induction in different plants after exposure to Cd (Lozano-Rodriguez et al. 1997; Dixit et al. 2001) and to Hg (Cho and Park 2000). Other cellular responses observed after addition of heavy metals are changes in thiol-peptide metabolism (Rauser 1991).

Although lead has no defined biological function, it can be accumulated in plant organs (such as roots and shoots) and exhibit toxicity leading to a decrease in biomass and inhibition of chlorophyll biosynthesis (Koeppe 1981). Heavy metals disrupt the physiological process by binding to protein sulfhydryl groups or cause deficiency/substitution of essential metals (Van Assche and Clijsters 1990). Lead,

a strong reactant to protein N and S ligands, was shown to inhibit chlorophyll synthesis (Sengar and Pandey 1996; Tripathi et al. 2005; Rani et al. 2008) as well as electron transport and Rubisco activity (Stiborova et al. 1986) in vitro.

The ability of plants to bioaccumulate metals and possibly other contaminants varies with both the genotypes of plant and the nature of metal contaminants (Naidu et al. 2003). For instance, cadmium, when taken up in excess by plants, inhibits directly or indirectly the physiological processes, such as respiration, photosynthesis, cell elongation, plant water relationships, nitrogen metabolism and mineral nutrition, resulting in poor growth and low biomass (Di et al. 1999; Rani et al. 2008). In follow-up studies with other metals, the higher concentrations of different metals (e.g., cadmium, copper, zinc, lead, etc.), when used either alone or as mixtures, have been shown to significantly reduce the dry matter accumulation, symbiosis and yield of greengram (*Vigna radiata* (L.) Wilczek) (Wani et al. 2007a), chickpea (*Cicer arietinum*) (Wani et al. 2007b) and pea (*Pisum sativum*) (Wani et al. 2008a), grown in metal-treated sandy clay loam soils.

## 5.5 Mechanisms of Metal Toxicity in Microorganisms

Metal species exert toxicity through numerous biochemical pathways that can be divided into five mechanistic categories. First, toxic metal species can bind to proteins in lieu of essential inorganic ions, thereby altering the biological function of the target molecule. An example is the replacement of Ni for Mg in some redox-active metalloproteins or in DNA, which destroys their function and/or may lead to DNA damage, respectively. Second, toxic metal species can participate in an array of reactions with thiols and disulphides, thereby destroying the biological function of proteins that contain sensitive S groups (Stohs and Bagchi 1995; Zannoni et al. 2007). These reactions frequently require and produce ROS, which are by-products of normal metabolism (Fig. 5.1). Thiol groups are often involved in the binding of

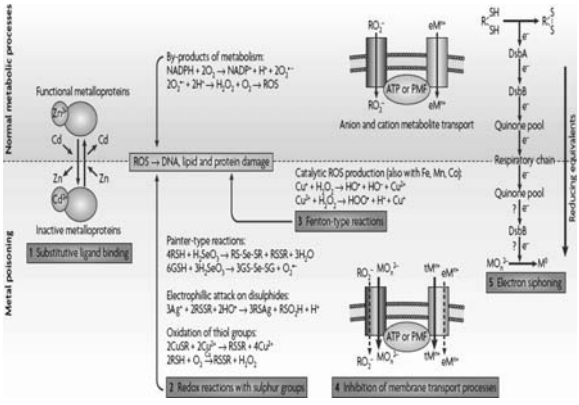



**Fig. 5.1** Biochemical mechanisms of microbiological metal toxicity

substrates to specific carriers whose transport mechanism can be impaired by toxic metal species. The destruction of sensitive thiol groups on nascent proteins by metals may also impair protein folding or binding of apoenzymes by cofactors, thereby destructing the normal biological activity of the protein (Harrison et al. 2007). Third, certain transition metals can participate in catalytic reactions, known as Fenton-type reactions, that produce ROS. Collectively, these reactions place the cell in a state of oxidative stress, and increased levels of ROS damage DNA, lipids and proteins through a range of biochemical routes (Geslin et al. 2001). Fourth, toxic metal species must gain entry into cells through transporters or by binding lipophilic carriers, as cell membranes are nonpermeable to these compounds. The transporter-mediated uptake of toxic metals might interfere with the normal transport of essential substrates owing to competitive inhibition. This transport process gains energy from the proton motive force (PMF) or ATP pool (Foulkes 1998). Fifth some metal oxy-anions are reduced by the oxidoreductase DsbB, which draws electrons from the bacterial transport chain through the quinone pool (Borsetti et al. 2007). In effect, certain toxic metal species starve microbial cells by indirectly siphoning electrons from the respiratory chain (Lohmeier-Vogel et al. 2004). The formation of ROS that damage DNA, proteins and lipids also occur in normal metabolic processes; however, the production of ROS is enhanced during metal poisoning which may mediate additional cellular damage.

## 5.6 Influence of Bacteria on Heavy Metal Bioavailability

Overall toxic effects of heavy metals to soil microorganisms depend on their bioavailability. Although heavy metal bioavailability is mainly dependent on the soil properties (pH and organic matter), bacteria can also directly influence the solubility of heavy metals by altering their chemical properties (Fig. 5.2).

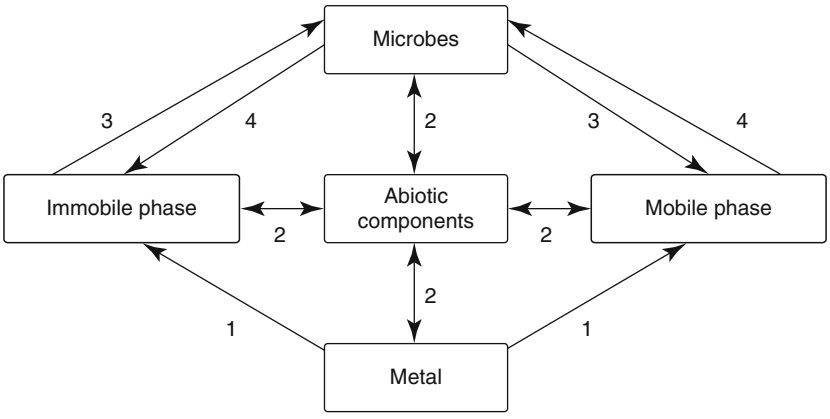



**Fig. 5.2** Microbial roles in the environmental mobility of metals

Microorganisms have evolved several mechanisms which can immobilize, mobilize or transform heavy metals. The exploitation of these bacterial properties for the remediation of heavy metal-contaminated sites has been shown to be a promising bioremediation alternative (Lovely and Coates 1997; Lloyd and Lovley 2001).

## 5.7 Heavy Metal Resistance Systems in Bacteria

Bacteria have developed several efficient systems to detoxify the metals. These mechanisms include: (1) intracellular sequestration, (2) export, (3) reduced permeability, (4) extracellular sequestration, and (5) extracellular detoxification (Rough et al. 1995). Almost all known bacterial resistance mechanisms are encoded on plasmids and transposons (Silver and Walderhaug 1992), and it is probably by gene transfer or spontaneous mutation that bacteria acquire their resistance to heavy metals (Osborn et al. 1997). In Gram-negative bacteria (e.g., *Ralstonia eutropha*), the *czc* system is responsible for the resistance to Cd, Zn and Co. The *czc*-genes encode for a cation–proton antiporter (CzcABC) which exports Cd, Zn and Co (Nies 1995). A similar mechanism, called *ncc* system, has been found in *Alcaligenes xylosoxidans* which is resistant to Ni, Cd and Co. In contrast, the Cd resistance mechanism in Gram-positive bacteria (e.g., *Staphylococcus*, *Bacillus* or *Listeria)* is a Cd-efflux ATPase. The two most well-studied Cu resistance systems are *cop* from *Pseudomonas syringae* pv. *tomato* and *pco* from *Escherichia coli*. The *cop* genes encode for different Cu-binding proteins which allow the sequestration of Cu in the periplasm or in the outer membrane. In contrast, the *pco* system is expected to be an ion-dependent Cu antiporter (Kunito et al. 1998). The bacterial resistance properties can be used for different purposes: in the case of mercury pollution, the insertion of the microbial mercury reductase in a transgenic plant significantly improved the phytoextraction process (Heaton et al. 1998). Another example was the inoculation of heavy metal-resistant bacteria in a contaminated soil which seemed to protect the plants from metal toxicity.

## 5.8 Heavy Metal Remediation

The contamination of agricultural land and groundwater by heavy metals is essentially linked to human activities. Depending on the extension, depth and kind of the contamination, different remediation approaches have been proposed (Mulligan et al. 2001). In general, three strategies are possible: the containment of the contaminants, their removal from the environment, or their in situ stabilization. Physical containment is the least expensive approach but this leaves the contaminant in place without treatment. As ex situ techniques are expensive, environmentally invasive and labor intensive, in situ approaches are generally preferred. One of these in situ techniques, phytoremediation, uses plants to remove pollutants from

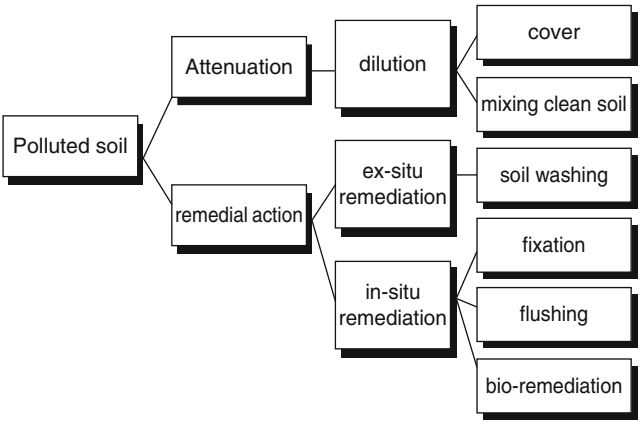



**Fig. 5.3** Option for remediation for polluted sites

the environment or to render them harmless (Salt et al. 1995; Flathman and Lanza 1998). These processes either "decontaminate" the soil, or "stabilize" the pollutant within it (Fig. 5.3). Decontamination reduces the amount of pollutants within the soil by removing them, while stabilization does not reduce the quantity of pollutant at a site, but makes use of soil amendments to alter the soil chemistry and sequester or absorb the pollutant into the matrix so as to reduce or eliminate environmental risks (Cunningham et al. 1995). Furthermore, these traditional techniques present significant disadvantages, such as energy requirements, and are very expensive.

## 5.9 Bioremediation

Many novel approaches to environmental clean-up are being developed or are already in use as alternatives to costly physical methods, such as incineration. Bio-remediation in its different forms is perceived as a relatively economical and nonintrusive approach to remediate polluted environments. The biological treatment utilizes the natural reactions of microorganisms living in the environments and, hence, provides a crucial key technology in the remediation of heavy metal-contaminated soils and waters (Khan et al. 2009). In addition, recent developments in molecular biology have provided insight for enhancing the microorganism's natural remediation capability as well as improving the current biological treatment (Fig. 5.4).

Several key microbial processes may affect mobilization or immobilization of toxic elements by one or more of the following mechanisms: (1) chelation of elements by metabolites, (2) oxidation–reduction of metals which affect the solubility or their valency, (3) changes in pH which affect the ionic state, (4) biosorption by functional groups on the cell surface, (5) bioaccumulation by an energy-dependent

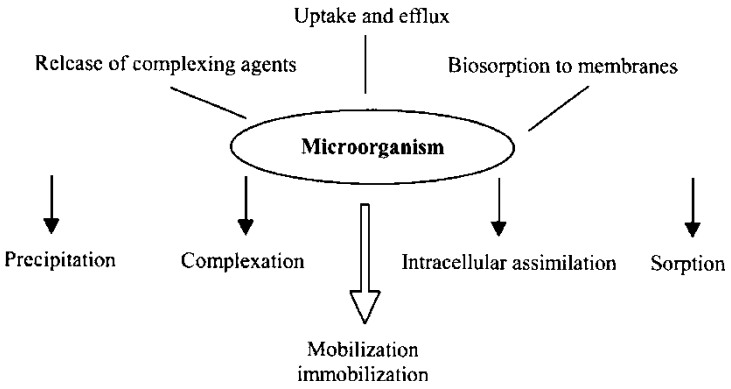



**Fig. 5.4** Metal processing mechanisms of microorganisms

**Table 5.1** Examples of toxic heavy metals accumulating microorganisms

| Organism | Element |
|---|---|
| *Citrobacter* sp. | Lead, cadmium |
| *Thiobacillus ferrooxidans* | Silver |
| *Bacillus cereus* | Cadmium |
| *Bacillus subtilis* | Chromium |
| *Pseudomonas aeruginosa* | Uranium |
| *Micrococcus luteus* | Strontium |
| *Rhizopus arrhizus* | Mercury |
| *Aspergillus niger* | Thorium |
| *Saccharomyces cerevisiae* | Uranium |

transport system, (6) immobilization due to formation of stable materials, (7) biomethylation, and (8) biodegradation of organic complex of metals. A wide variety of microorganisms including plant growth-promoting rhizobacteria, fungi, yeast, and algae are known that can interact with metals employing several mechanisms and can transform them to nontoxic or less toxic forms (Poole and Gadd 1989; Wani et al. 2007c, 2008b) (Table 5.1).

### *5.9.1 Biosorption*

Biosorption of toxic metals is based on nonenzymatic processes such as adsorption. Adsorption is due to the nonspecific binding of ionic species to cell surface-associated or extracellular polysaccharides and proteins (Mullen et al. 1989; Volesky 1990). Bacterial cell walls and envelopes, and the walls of fungi, yeasts, and algae, are efficient metal biosorbents that bind charged groups. The cell walls of Gram-positive bacteria bind larger quantities of toxic metals than the envelopes of the Gram-negative bacteria. Biosorbents may be regenerated by treatment with acid

or with certain chelating agents. Besides bacteria, waste fungal biomass derived from several industrial fermentations may also provide an economical source of biosorptive materials. Many species have high cell wall chitin contents which act as an effective biosorbent as do the chitosan and glucans.

Biosorption is defined as a property of certain inactive or dead microbial biomass to bind and concentrate heavy metals from even very dilute aqueous solutions (Vasudevan et al. 2001). The uptake of metal could be active, passive or both in nature. Passive uptake is independent of cellular metabolisms, and metal binds to polyionic cell walls through ion exchange as the process is not affected by physical conditions such as pH and ionic strength. It is a rapid process taking only 5–10 min to complete. The process is also reversible and can involve both living and dead cells. The active process on the other hand is slow and depends on cellular metabolism. It is affected by metabolic inhibitors, uncouplers and temperature. In the active process, the metal complex with specific proteins like metallothionins are contained in vacuole. Both the active and passive mode may occur simultaneously. The passive process is relatively nonspecific with respect to the metal (Pandey et al. 2001).

#### 5.9.1.1 Biosorption by Fungi

Polysaccharides in association with lipids and proteins form the main constituent of fungal cell wall. In filamentous fungi, the outer cell wall layers mainly contain natural polysaccharides (glycans and mannans) while the inner layers contain more of glucosamines (chitin and chitosan) in a microfibrillar structure, which are found to be associated with metal-binding (Vasudevan et al. 2001). Mahesh et al. (2001) investigated $Cu^{2+}$ sorption behavior of three isolated native forms of fungi in a laboratory-scale batch reactor. *Fusarium solani* fungal species tend to remove 90% $Cu^{2+}$ from solution at alkaline pH (8.0–10.0). Metal uptake capacities of some fungi have also been observed by Bagdwal et al. (2003).

#### 5.9.1.2 Biosorption by Algae

Special polysaccharides are present in the algal cell wall. The number and nature of binding sites depends on the chemical composition of the cell wall. In pheophycean members, algin is present which contributes significantly to metal-binding.

#### 5.9.1.3 Biosorption by Bacteria

The anionic nature of bacterial surface enables them to bind metal cations through electrostatic interactions. Because of their thickness and anionic character (which is mainly due to peptidoglycan, teichoic acid and teichuronic acids), the cell walls of Gram-positive bacteria have a high capacity for metal-binding. It has been reported

by Beveridge and Murray (1980) that peptidoglycan is the major cell wall component responsible for metal-binding by *Bacillus subtilis*. In contrast, metal-binding by *Bacillus licheniformis* is predominantly due to teichuronic acid. Three strains of thermotolerant polymer-producing bacteria, *B. subtilis* WD90, *B. subtilis* SH29, and *Enterobacter agglomerans* 5M 38, as well as their bioflocculants, were capable of nickel and cadmium removal. However, the bioflocculant of the three isolates gave higher metal adsorption than the cells due to synthesis of extracellular polysaccharide and other cell wall components (Kaewchai and Praseptsan 2002). In another study, *Pseudomonas aeruginosa* (CW-96-1) tolerated cadmium up to a concentration of 5 mM, and, 140 h after inoculation, metal-tolerant strain (CW-96-1) removed >99% of the cadmium from solution. Electron micrographs and energy dispersive microanalysis indicated that cadmium was bound to the cell wall as a sulfur complex with a 1:1 stoichiometry (Wang et al. 1997). *Pseudomonas putida* actively accumulates cadmium from the medium, and the resistance mechanism involves both polyphosphate and a series of low molecular weight cysteine-rich cadmium proteins that are induced during different growth phases. Nuclear magnetic resonance (NMR) study on the major native cadmium protein ($CdBP_1$) establishes a definite relationship to cadmium metallothioneins (Higham et al. 1984).

### 5.9.2 Bioaccumulation

Bioaccumulation has been described for such metals as mercury, lead, silver, cadmium and nickel. Intracellular accumulation of toxic elements is carried out by an energy-dependent transport system (Gadd 1988). Potential mechanisms of toxic metal flux across membranes can be ion pumps, ion channels, carrier mediated transport, endocytosis, complex permeation, and lipid permeation. Permeabilization of cell membranes to toxic elements can result in further exposure of intracellular metal-binding sites and increase passive accumulation. Assessment of heavy metal accumulation in the microbial cells can be done by transmission electron microscopy (TEM). For example, TEM analysis of *P. putida* 62BN demonstrated intracellular and periplasmic accumulation of cadmium (Rani et al. 2009). A high metal concentration may lead to intracellular precipitation of metal (Hughes and Poole 1989). Additionally, cadmium transport via the $Mn^{2+}$ transport system has been reported in many bacteria (Laddaga and Silver 1985; Perry and Silver 1982) and may also contribute to cadmium transport in 62BN. The intracellular and periplasmic cadmium accumulation in *P.putida* 62BN suggested the presence of metal-binding and/or efflux mechanisms inside the cells mediating resistance against metal toxicity. Cytoplasmic (Yoshida et al. 2002) and periplasmic (Naz et al. 2005; Pazirandeh et al. 1998) accumulation of heavy metal ions as a result of metallothioneins expression has been reported in *E. coli*.

Microbial biofilms, natural or engineered, could be used to remediate heavy metal pollution by biochemical modification and/or the accumulation of toxic metal ions (Munoz et al. 2006; Chang et al. 2006). An understanding of metal toxicity in biofilms is crucial to the successful design of bioreactors that are used for biomining (Rawlings and Johnson 2007), as well as those reactors that are used for biodegrading organic contaminants that are frequently intermingled with metals (Singh et al. 2006). Moreover, this information might provide insights into the observed vulnerabilities, physiological shifts and species changes of natural aquatic biofilm communities that have been exposed to toxic heavy metals (Lawrence et al. 2004; Vilchez et al. 2007). Multimetal resistance and tolerance in microbial biofilms is a multifactorial property of the adherent population. In general, the decreased susceptibility of biofilms to toxic metal species can be viewed as an emergent property that arises from several interrelated physiological and chemical parameters. Many of these parameters arise from the natural process of phenotypic diversification that is ongoing during biofilm growth. Biofilm formation is a process that gives rise to multiple cell types – each with a range of expression patterns that correspond to the diversity of biofilm microniches – which allows the population to withstand a diverse range of environmental stresses, including metal toxicity.

### *5.9.3 Siderophores*

When microorganisms are grown in an iron-deficient medium, they produce specific iron chelators, so-called siderophores, in the medium. They play an important role in the complexation of toxic metals and increase their solubility (Neilands 1983). Siderophores are compounds that possess catecholate, phenolate or hydroxamate as their binding groups. Over the past few years, many siderophore or siderophore-like compounds have been identified from various biological systems. Although siderophores are primarily specific for Fe (III), they can also complex other metals and radionuclides. One way to relieve heavy metals toxicity to plants might involve the use of growth-promoting bacteria. Free-living rhizobacteria exert some beneficial effects on plant development when they are either applied to seeds or incorporated into the soil (Kloepper et al. 1989; Glick et al. 1999). Of the several mechanisms used to facilitate plant growth, siderophore synthesized by microbes including rhizobia (Wani et al. 2007d, 2008c) and species of *Bacillus*, *Pseudomonas* and *Azotobacter* (Wani et al. 2007c; Ahmad et al. 2008) is well documented due to their iron sequestration ability from the soil. Microbial iron siderophore complex can be taken up by plants as an iron source (Wang et al. 1993). The most appropriate approach to prevent plant chlorosis due to high levels of heavy metals should be to provide them with an associated siderophore-producing bacterium that supplements sufficient amounts of iron to the plant (Tripathi et al. 2005; Wani et al. 2008c).

## 5.10 Tracking the Insights of Bioremediation Using Proteomics/ Genomics

Environmental pollutants in the soil are a major concern worldwide. Bioremediation mediated by microorganisms is a highly promising technology as it is environmentally friendly, safe, effective and inexpensive. However, incomplete biological information regarding the cellular responses in many microbial communities restricts progress in the site-specific mineralization process. The application of proteomics in environmental bioremediation research provides a global view of the protein compositions of the microbial cells and offers a promising approach to understanding the molecular basis of bioremediation. With the combination of proteomics, functional genomics provide an insight into global metabolic and regulatory networks that can enhance the understanding of gene functions (Fig. 5.5).

### *5.10.1 Proteomic Studies for the Cellular Responses to $Cd^{2+}$ in Microorganism*

Compared with the molecular/cellular biological studies of individual genes or proteins one at a time, as has traditionally been done, the global analysis either at

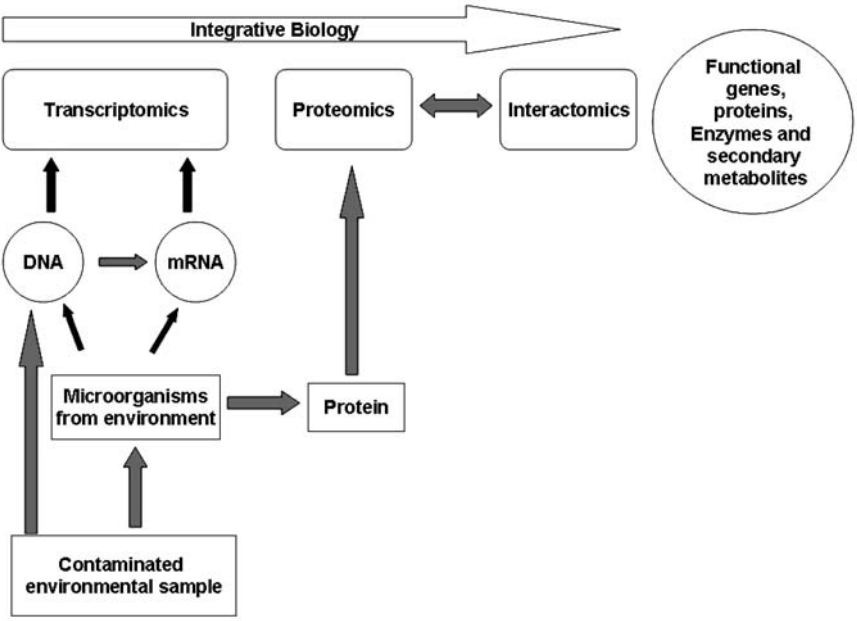



**Fig. 5.5** Post-genomic technologies using a systematic biology approach to track the insights of bioremediation

a genomic or at a proteomic level allows for a systematic overview of thousands of genes or their products in a species at the same time (Tyers and Mann 2003; Pandey and Mann 2000; Dutt and Lee 2000; Humphery-Smith et al. 1997). Proteomics can produce more accurate and comprehensive information than genomic studies can provide because protein expressions are regulated not only at transcriptional but also at translational levels, resulting in more details about mature proteins and their interactions than genome-based predictions (Humphery-Smith et al. 1997). Therefore, significant discrepancies between mRNA and protein levels have also been found in several studies (Gygi et al. 1999; Chen et al. 2002; Griffin et al. 2002). It is inevitable that study of the cellular responses to different stresses at the proteomic level will inform us what gene products are actually expressed and their changes (Abram et al. 2008). In this regard, proteomics complements other functional genomics approaches such as microarray-based expression profiles, systematic phenotypic profiles at the cell and organism level, and small-molecule-based arrays. An understanding of the growth conditions governing the expression of the proteome (for example, enzymes and regulatory proteins of heavy metal resistance, energy generation pathways, transport and stress-related proteins) in a specific environment is essential for developing rational strategies for successful bioremediation.

Cadmium ($Cd^{2+}$) is one of the well-known toxic heavy metal ions. To gain a global understanding of how $Cd^{2+}$ affects cells at the molecular level, various studies were performed. Proteome-wide investigation unequivocally identified 1133 *Shizosaccharomyces pombe* proteins, of which the AACT-based quantitative analysis revealed 106 upregulated and 55 downregulated proteins on the $Cd^{2+}$ exposure. In the most prevalent functional class in the upregulated proteins, 28% of proteins were involved in protein biosynthesis, showing a time-dependent biphasic expression pattern characteristic with rapid initial induction and later repression. Most significantly, 27 proteins, functionally classified as cell rescue and defense, were upregulated for oxygen and radical detoxification, heat-shock response, and other stress responses (Bae and Chen 2004).

Moreover, Pukárová et al. (2001) reported that during cadmium-induced growth arrest of *E. coli* cells, DNA and RNA synthesis is rapidly inhibited while the rates of protein synthesis and proteolysis increase transiently (Ferianc et al. 2000). The protein synthesis includes de novo synthesis of cadmium-induced proteins (CDPs) (Ferianc et al. 2000), which together make up the cadmium stress stimulon (Ferianc et al. 1998). Some CDPs belong to global regulatory networks, which include the SOS, oxidative stress, heat-shock and stringent response networks (Ferianc et al. 1998; VanBogelen et al. 1987). However, only a limited number of the proteins in these regulons are induced during cadmium exposure and the synthesis of these CDPs constitutes a minor fraction of the overall cellular response (VanBogelen et al. 1987). In addition, these general stress responses are only transiently activated during cadmium-induced growth inhibition. When proliferation resumes, these regulons are downregulated and expression reaches a new steady-state level (Ferianc et al. 1998). Other CDPs, however, are specific to cadmium stress. These CDPs retain an elevated production level, relative to the production level of general stress proteins, even in accommodated cells that have resumed growth during

prolonged cadmium exposure. One of these proteins has been identified as the product of ORF *o216* (Ferianc et al. 1998), later renamed *yodA*, a 216 amino acid residue protein, identified on two-dimensional polyacrylamide gels (VanBogelen et al. 1996; Ferianc et al. 1998). N-terminal sequencing has demonstrated that the protein is processed and contains a 24 amino acid signal sequence, suggesting that the mature product is exported from the cytoplasm. YodA, together with two other putative proteins, YrpE of *B. subtilis* (Sorokin et al. 1997) and pXO1-130 of *Bacillus anthracis* (Okinaka et al. 1999), may constitute a new family of stress proteins based on sequence similarity (44.6% identity) and size (YodA, 216; YrpE, 251; pXO1-130, 237 aa). These three proteins were found to also exhibit sequence similarity with the 200 aa residue C-terminal part of the streptococcal adhesin, AdcA (Pukárová et al. 2001), which is a lipoprotein containing a putative metal-binding site (Dintilhac and Claverys 1997). In addition, YodA exhibits weak sequence similarity with the N-terminal part (about 200 aa) of the copper-binding protein amine oxidase, encoded by *maoA* (Ferianc et al. 1998), of both *E. coli* (Azakami et al. 1994) and *Klebsiella aerogenes* (Sugino et al. 1992).

Further, Rab et al. (2006) showed that the bacterium *Rhodobacter capsulatus* B10 in the presence of 150 μM $CdCl_2$ induced heat-shock proteins (GroEL and Dnak), *S*-adenosylmethinine synthetase, ribosomal protein S1, aspartate aminotransferase, and phosphoglycerate kinase. Ribosomal protein S1 appeared to be involve in the repair of cadmium-mediated cellular damage. Five cadmium binding proteins, including 2-methylcitrate dehydratase, phosphate periplasmic binding protein, inosine-5′- monophosphate dehydrogenase/guanosine-5′- monophosphate reductase, inositol monophosphatase, and lytic murein transglycosylase, were also identified.

### *5.10.2 Differential Gene Expression Under Cadmium Stress*

In *Caulobacter crescentus* (6M cadmium sulfate), 144 genes were upregulated by at least twofold under cadmium stress. Several groups of annotated genes are given in Table 5.2 (Hu et al. 2005).

## 5.11 Conclusion

Generally, the concentration of heavy metals in soil has increased during the last few years posing a serious threat to the environment and human health worldwide. However, due to the fact that large areas of land contaminated with metals cannot be economically decontaminated by applying conventional chemical approaches, microbe/plant-mediated approaches can prove to be the best viable and inexpensive alternative for remediation of heavy metal-polluted sites. Remediation of metal-polluted soils using biological systems (both plants and

**Table 5.2** Selected genes (and description) upregulated under cadmium stress

| Gene | Fold change | Annotation |
|---|---|---|
| Efflux pumps (Cluster I) | | |
| CC2721 | 21.3 | Outer membrane efflux protein |
| CC2722 | 36 | Metal ion efflux membrane fusion protein, contains HlyD domain |
| CC2723 | 20 | Hypothetical protein |
| CC2724 | 22.8 | Homologous to nccA and czcA |
| CC2725 | 8 | Conserved hypothetical protein |
| CC2726 | 12.4 | Cation transporting P-type ATPase |
| CC2727 | 6 | Conserved hypothetical protein |
| Efflux pumps (Cluster II) | | |
| CC3195 | 3.4 | Outer membrane efflux protein |
| CC3196 | 2.6 | Contains HlyD domain |
| CC3197 | 3 | Cation/multidrug efflux pump, with AcrB/ AcrD/ AcrF domain |
| Protect against oxidative stress | | |
| CC1777 | 18.9 | Superoxide dismutase (cofactor, $Mn^{2+}$) (sod A) |
| CC3557 | 2.2 | Superoxide dismutase (cofactor, $Fe^{2+}$) (sod B) |
| CC1316 | 3.2 | Glutathione *S*-transferase |
| CC2434 | 2.2 | Glutathione *S*-transferase |
| CC0062 | 2.4 | Thioredoxin-like protein |
| CC0110 | 2 | Thioredoxin |
| CC3539 | 2.3 | Thioredoxin |
| CC2505 | 2.3 | Glutaredoxin-related protein |
| CC0994 | 2.5 | Peptide methinine sulfoxide reductase |
| CC1039 | 2.3 | Peptide methinine sulfoxide reductase |
| CC0141 | 2.3 | Glutathione synthetase |
| CC0885 | 3.6 | Riboflavin biosynthesis protein (rib D) |
| CC0886 | 4.3 | Riboflavin synthase, alpha subunit (rib E) |
| CC0887 | 4 | GTP cyclohydrolase II (rib AB) |
| CC0888 | 3.6 | Riboflavin synthase, alpha subunit (rib E) |
| CC0459 | 4.1 | GTP cyclohydrolase I (tetrahydrofolate biosynthesis pathway) |
| Arsenic resistance | | |
| CC1503 | 4.8 | Arsenic reductase (arsC) |
| CC1504 | 4.4 | Transmembrane channel protein |
| CC1505 | 4.4 | Transcriptional regulator (arsR) |
| CC1506 | 9.9 | Arsenic resistant protein |
| DNA Repair | | |
| CC1428 | 6 | Deoxyribodipyrimidine photolyase, removes cyclobutane – type pyrimidine dimmers in DNA |
| CC2590 | 2 | Excinuclease ABC, subunit A |
| Others | | |
| CC0260 | 2.7 | Ribonucleotide reductase, alpha subunit |
| CC3492 | 2.2 | Ribonucleotide reductase, beta subunit |
| CC2129 | 4.5 | NADH: flavin oxidoreductase |

microbes) is an emerging area of interest and has shown a substantial progress in situ, which needs to be further consolidated through field trials under different agro-climatic zones of the world. Furthermore, with progress in genomics and proteomic studies in many laboratories around the world, we may expect this research area to develop and expand further in the near future, delivering more robust technologies for the bioremediation of metal-contaminated soils. And, hence, the molecular engineering of both microbes and plants with desired genes is likely to help immensely to enhance the efficiency of microbe-mediated or plant-based remediation of contaminated soils. However, to make remediation a successful option for detoxifying contaminated sites, some of the problems, like how the remediation effects will change under field conditions, and how mobilization and transfer of metal to different organs of plants is effected, need to be critically addressed by the scientists before the potential of bioremediation in decontaminating polluted sites or reducing the toxicity of metals can be appreciated.

## References

Abram F, Su WL, Wiedmann M, Boor KJ, Coote P, Botting C, Karatzas KAJ, O' Bryne CP (2008) Proteomic analysis of a *Listeria monocytogenes* mutant lacking $\sigma^B$ identify new components of the $\sigma^B$ regulon and highlight a role for $\sigma^B$ in the utilization of Glycerol. Appl Environ Microbiol 74:594–604

Adriano DC (2001) Trace elements in terrestrial environments: biochemistry, bioavailability and risks of metals. Springer, New York

Ahmad F, Ahmad I, Khan MS (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol Res 163:173–181

Alloway BJ (1995) Soil processes and the behaviour of heavy metals. In: Alloway B (ed) Heavy metals in soils. Chapman and Hall, New York, pp 11–37

Azakami H, Yamashita M, Roh JH, Suzuki H, Kumagai H, Murooka Y (1994) Nucleotide sequence of the gene for monoamine oxidase (*maoA)* from *Escherichia coli*. J Ferment Bioeng 77:315–319

Bae W, Chen X (2004) Proteomic study for the cellular responses to $Cd^{2+}$ in *Schizosaccharomyces pombe* through amino acid-coded mass tagging and liquid chromatography tandem mass spectrometry. Mol Cell Proteomics 3(6):596–607

Bagdwal N, Gupta A, Goel R (2003) Metal resistant growth promotry fluorescent *Pseudomonads*. In: Trivedi PC (ed) Microbial biotechnology. Aavishkar, Jaipur, India, pp 126–161

Baker AJM, Walker PL (1989) Ecophysiology of metal uptake by tolerant plants. In: Shaw A (ed) Heavy metal tolerance in plants - evolutionary aspects. CRC, Boca Raton, pp 155–177

Beveridge TJ, Murray RGE (1980) Sites of metal deposition in the cell wall of *Bacillus subtilis*. J Bacteriol 141:876–887

Blencowe DK, Morby AP (2003) Zn(II) metabolism in prokaryotes. FEMS Microbiol Rev 27:291–311

Borsetti F, Francia F, Turner RJ, Zannoni D (2007) The thiol:disulfide oxidoreductase DsbB mediates the oxidizing effects of the toxic metalloid tellurite ($TeO3^{2-}$) on the plasma membrane redox system of the facultative phototroph *Rhodobacter capsulatus*. J Bacteriol 189:851–857

Cavet JS, Borrelly GPM, Robinson NJ (2003) Zn, Cu and Co in cyanobacteria: selective control of metal availability. FEMS Microbiol Rev 27:165–181
Chang WC, Hsu GS, Chiang SM, Su MC (2006) Heavy metal removal from aqueous solution by wasted biomass from a combined AS-biofilm process. Bioresource Technol 97:1503–1508
Chen G, Gharib TG, Huang CC, Taylor JM, Misek DE, Kardia SL, Giordano TJ, Iannettoni MD, Orringer MB, Hanash SM, Beer DG (2002) Discordant protein and mRNA expression in lungadenocarcinomas. Mol Cell Proteomics 1:304–313
Cho UH, Park JO (2000) Mercury-induced oxidative stress in tomato seedlings. Plant Sci 156:1–9
Choudhury R, Srivastava S (2001) Zinc resistance mechanism in bacteria. Curr Sci 81:768–775
Cunningham SD, Berti WR, Huang JW (1995) Phytoremediation of contaminated soils. Trends Biotechnol 13:393–397
Di S, Toppi L, Gabbrielli R (1999) Response to cadmium in higher plants. Environ Exp Bot 41:105–130
Dintilhac A, Claverys JP (1997) The *adc* locus, which affects competence for genetic transformation in *Streptococcus pneumoniae*, encodes an ABC transporter with a putative lipoprotein homologous to a family of streptococcal adhesins. Res Microbiol 148:119–131
Dixit V, Pandey V, Shyam R (2001) Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). J Exp Bot 52:1101–1109
Dutt MJ, Lee KH (2000) Proteomic analysis. Curr Opin Biotechnol 11:176–179
Ernst WHO (1998) The origin and ecology of contaminated, stabilized and non-pristine soils. In: Vangronsveld J, Cunningham SD (eds) Metal-contaminated soil. Springer, New York, pp 17–29
Ferianc P, Farewell A, Nyström T (1998) The cadmium-stress stimulon of *Escherichia coli* K-12. Microbiology 144:1045–1050
Ferianc P, Pukárová A, Godoíková J, Polek B, Tóth D (2000) The effect of cadmium on culturability, macromolecule synthesis and protein degradation in a marine *Vibrio* sp. Biologia 55:653–659
Flathman PE, Lanza GR (1998) Phytoremediation: current views on an emerging green technology. J Soil Contam 7:415–432
Foulkes EC (1998) Biological membranes in toxicology. Taylor and Francis, Philadelphia
Gadd GM (1988) Accumulation of metals by microorganisms and algae. In: Rehm HJ (ed) Biotechnology: a comprehensive treatise. VCH Verlagsgesellschaft, Weinheim, pp 401–433
Geslin C, Llanos J, Prieur D, Jeanthon C (2001) The manganese and iron superoxide dismutases protect *Escherichia coli* from heavy metal toxicity. Res Microbiol 152:901–905
Glick BR, Pattern CL, Holguin G, Penrose DM (1999) Biochemical and genetic mechanism used by plant growth promoting bacteria. Imperial College Press, London
Griffin TJ, Gygi SP, Ideker T, Rist B, Eng J, Hood L, Aebersold R (2002) Complementary profiling of gene expression at the transcriptome and proteome levels in *Saccharomyces cerevisiae*. Mol Cell Proteomics 1:323–333
Gygi SP, Rochon Y, Franza BR, Aebersold R (1999) Correlation between protein and mRNA abundance in yeast. Mol Cell Biol 19:1720–1730
Hall JL (2000) Cellular mechanisms for heavy metal detoxification and tolerance. J Exp Bot 53:1–11
Hamon RE, Mclaughlin MJ, Naidu R, Correll R (1998) Long-term changes in cadmium bioavailability in soil. Envion Sci Technol 32:3699–3703
Harrison JJ, Ceri H, Turner RJ (2007) Multimetal resistance and tolerance in microbial biofilms. Nature 5:928–938
Heaton ACP, Rugh CL, Wang NJ, Meagher RB (1998) Phytoremediation of mercury- and methylmercury-polluted soils using genetically engineered plants. J Soil Contam 7:497–509
Higham DP, Sadler PJ, Scawen MD (1984) Cadmium resistance in *Pseudomonas putida*: growth and uptake of cadmium. J Gen Microbiol 131:2539–2544
Hu P, Brodie EL, Sujuki Y, McAdams HH, Anderson GL (2005) Whole-genome transcriptional analysis of heavy metal stresses in *Caulobacter crescentus*. J Bacteriol 187:8437–8449

Hughes MN, Poole RK (1989) Metals and micro-organisms. Chapman and Hall, New York, NY, p 290

Humphery-Smith I, Cordwell SJ, Blackstock WP (1997) Proteome research: complementarity and limitations with respect to the RNA and DNA worlds. Electrophoresis 18:1217–1242

Kabata-Pendias A, Pendias H (2001) Trace elements in soils and plants. CRC, London

Kaewchai S, Praseptsan P (2002) Biosorption of heavy metal by thermotolerant polymer producing bacterial cells and the bioflocculant. Songklanakarin J Sci Technol 24:421–430

Khan MS, Zaidi A, Wani PA, Oves M (2009) Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. Environ Chem lett 7:1–19

Kloepper JW, Litshitz R, Zablotowicz RM (1989) Free living bacterial inocula for enhancing crop producitivity. Trends Biotechnol 7:39–43

Koeppe DE (1981) Lead: understanding the minimal toxicity of lead in plants. In: Lepp NW (ed) Effect of heavy metal pollution on plants, vol 2. Applied Science, London and New Jersey, pp 55–76

Kunito T, Oyaizu H, Matsumoto S (1998) Ecology of soil heavy metal-resistant bacteria and perspective of bioremediation of heavy metal-contaminated soils. Rec Res Dev Agric Biol Chem 2:185–206

Laddaga RA, Silver S (1985) Cadmium uptake in *Escherichia coli* K-12. J Bacteriol 162: 1100–1105

Lawrence JR, Chenies MR, Roy R, Beaumier D, Fortin N, Swerhone GDW, Neu TR, Greer CW (2004) Microscale and molecular assessment of impacts of nickel, nutrients, and oxygen level on structure and function of river biofilm communities. Appl Environ Microbiol 70:4326–4339

Lloyd JR, Lovley DR (2001) Microbial detoxification of metals and radionuclides. Curr Opin Biotechnol 12:248–253

Lohmeier-Vogel EM, Ung S, Turner RJ (2004) *In vivo* 31P nuclear magnetic resonance investigation of tellurite toxicity in *Escherichia coli*. Appl Environ Microbiol 70:7342–7347

Lovely DR, Coates JD (1997) Bioremediation of metal contamination. Curr Opin Biotechnol 8:285–289

Lozano-Rodriguez E, Hernandez LE, Bonay P, Carpena-Ruiz RO (1997) Distribution of cadmium in shoot and root tissues of maize and pea plants: physiological disturbances. J Exp Bot 306:123–128

Mahesh S, Ramesh HS, Sudhir HS, Kumar BR (2001) Microbial scavengers-fungi for $Cu^{+2}$ removal from industrial waste water. J Ind Pollut Control 17:135–140

Mullen MD, Wolf DC, Ferris FC, Beveridge TJ, Flemming CA, Bailey FW (1989) Bacterial sorption of heavy metals. Appl Environ Microbiol 55:3143–3149

Mulligan CN, Yong RN, Gibbs BF (2001) Remediation technologies for metal contaminated soils and groundwater: an evaluation. Eng Geol 60:193–207

Munoz R, Alvarez MT, Munoz A, Terrazas E, Guieysse B, Mattiasson B (2006) Sequential removal of heavy metal ions and organic pollutants using an algal-bacterial consortium. Chemosphere 63:903–911

Naidu R, Oliver D, McConnell S (2003) Heavy metal phytoxicity in soils. In: Proceedings of the Fifth National Workshop on the assessment of site contamination. Adelaide, May 2002

Naz N, Young HK, Ahmed N, Gadd GM (2005) Cadmium accumulation and DNA homology with metal resistance genes in sulfate-reducing bacteria. Appl Environ Microbiol 71:4610–4618

Neilands JB (1983) Siderophores. In: Eichhorn L, Marzilli LG (eds) Advances in inorganic biochemistry, vol 5. Elsevier, Amsterdam, pp 137–166

Nies DH (1995) The cobalt, zinc, and cadmium efflux system CzcABC from *Alcaligenes eutrophus* functions as a cation-proton antiporter in *Escherichia coli*. J Bacteriol 177:2707–2712

Okinaka RT, Cloud K, Hampton O et al (1999) Sequence and organization of pXO1, the large *Bacillus anthracis* plasmid harboring the anthrax toxin genes. J Bacteriol 181:6509–6515

Osborn AM, Bruce KD, Strike P, Ritchie DA (1997) Distribution, diversity and evolution of the bacterial mercury resistance (mer) operon). FEMS Microbiol Rev 19:239–262

Pandey A, Mann M (2000) Proteomics to study genes and genomes. Nature 405:837–846

Pandey A, Nigam P, Singh D (2001) Biotechnological treatment of pollutants. Chem Ind Digest 14:93–95

Pazirandeh M, Wells BM, Ryan RL (1998) Development of bacterium-based heavy metal biosorbents: enhanced uptake of cadmium and mercury by *Escherichia coli* expressing a metal binding motif. Appl Environ Microbiol 64:4068–4072

Perry RD, Silver S (1982) Cadmium and manganese transport in *Staphylococcus aureus* membrane vesicles. J Bacteriol 150:973–976

Poole RK, Gadd GM (1989) Metal-microbe interaction. IRL, Oxford

Pukárová A, Janeek S, Ferianc P, Polek B (2001) Putative Cd-stress proteins YodA, YrpE and pXO1–130 share sequence similarity with adhesin AdcA. Biologia 56:337–339

Rab MFGS, Abdel FSA, Fukumori Y (2006) Effects of cadmium stress on growth, morphology and protein expression in *Rhodobacter capsulatus* B10. pp. Biosci Biotechnol Biochem 70:2394–2402

Rani A, Shouche Y, Goel R (2008) Declination of copper toxicity in pigeon pea and soil system by growth promoting *Proteus vulgaris* KNP3 strain. Curr Microbiol 57:78–82

Rani A, Shouche Y, Goel R (2009) Comparative assessment for *in situ* bioremediation potential of cadmium resistant acidophilic *Pseudomonas putida* 62BN and alkalophilic *Pseudomonas monteilli* 97AN strains on soybean. Int J Biodet Biodegrad 63:62–66

Rauser WE (1991) Cadmium-binding peptides from plants. Methods Enzymol 205:319–333

Rawlings DE, Johnson DB (2007) The microbiology of biomining: development and optimization of mineral oxidizing microbial consortia. Microbiology 153:315–324

Romero-Puertas MC, Palma JM, Gomez M, Del Ro LA, Sandalio LM (2002) Cadmium causes the oxidative modification of proteins in pea plants. Plant Cell Environ 25:677–686

Ross S (1994) Toxic metals in soil-plant systems. Wiley, Chichester, UK

Rough DA, Lee BTO, Morby AP (1995) Understanding celllular responses to toxic agents: a model for mechanism-choice in bacterial metal resistance. J Ind Microbiol 14:132–141

Salt DE, Blaylock M, Kumar NPBA, Dushenkov V, Ensley BD, Chet I, Raskin I (1995) Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Biotechnology 13:468–474

Sandalio LM, Dalurzo HC, Gomez M, Romero-Puertas MC, Del Ro LA (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. J Exp Bot 52:2115–2126

Schützendübel A, Polle A (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. J Exp Bot 53:1351–1365

Schützendübel A, Schwanz P, Teichmann T, Gross K, Langenfeld-Heyser R, Goldbold DL, Polle A (2001) Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in Scots pine roots. Plant Physiol 127:887–898

Sengar RS, Pandey M (1996) Inhibition of chlorophyll biosynthesis by lead in greening *Pisum sativum* leaf segments. Biol Plant 38:459–462

Silver S, Walderhaug M (1992) Gene-regulation of plasmid-determined and chromosome-determined inorganic ion transport in bacteria. Microbiol Rev 56:195–228

Singh R, Paul D, Jain R (2006) Biofilms: implications in bioremediation. Trends Microbiol 14:389–397

Sorokin A, Bolotin A, Purnelle B, Hilbert H, Lauber J, Dusterhoft A, Ehrlich SD (1997) Sequence of the *Bacillus subtilis* genome region in the vicinity of the *lev* operon reveals two new extracytoplasmic function RNA polymerase sigma factors SigV and SigZ. Microbiology 143:2939–2943

Stiborova M, Doubravova M, Leblova SC (1986) Comparative study of the effect of heavy metal ions on ribulose-1, 1-biphosphate carboxylase and phosphoenolpyruvete carboxylase. Biochem Physiol Pflanzen 181:373–379

Stohs SJ, Bagchi D (1995) Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med 18:321–336

Sugino H, Sasaki M, Azakami H, Yamashita M, Murooka Y (1992) A monoamine-regulated *Klebsiella aerogenes* operon containing the monoamine oxidase structural gene (*maoA)* and the *maoC* gene. J Bacteriol 174:2485–2492

Tripathi M, Munot HP, Shouche Y, Meyer JM, Goel R (2005) Isolation and functional characterization of siderophore producing lead and cadmium resistant *Pseudomonas putida* KNP9. Curr Microbiol 50:233–237

Tyers M, Mann M (2003) From genomics to proteomics. Nature 422:193–197

Van Assche F, Clijsters H (1990) Effects of metals on enzyme activity in plants. Plant Cell Environ 13:19–206

VanBogelen RA, Kelley PM, Neidhardt FC (1987) Differential induction of heat shock, SOS, and oxidation stress regulons and accumulation of nucleotides in *Escherichia coli*. J Bacteriol 169:26–32

VanBogelen RA, Abshire KZ, Pertsemlidis A, Clark RL, Neidhardt FC (1996) Gene-protein database of *Escherichia coli* K-12. In: Neidhardt FC (ed) *Escherichia coli* and *Salmonella*. American Society for Microbiology, Washington, DC, pp 2067–2117

Vasudevan P, Padmavathy V, Tewari N, Dhingra SC (2001) Biosorption of heavy metal ions. J Sci Ind Res 60:112–120

Vilchez R, Pozo C, Gomez MA, Rodelas B, Gonzalez-Lopez J (2007) Dominance of sphingomonads in a copper exposed biofilm community for groundwater treatment. Microbiology 153:325–337

Volesky B (1990) Biosorption of heavy metals. CRC, Boca Raton

Wang V, Brown HN, Crowley DE, Szaniszlo PJ (1993) Evidence for direct utilization of siderophore, ferroxamine B in axenically grown cucumber. Plant Cell Environ 16:579–585

Wang CL, Michel PC, Dawson SC, Kitisakkul S, Baross JA, Keasling JD, Clark DS (1997) Cadmium removal by a new strain of *Pseudomonas aeruginosa* in aerobic culture. Appl Environ Microbiol 63:4075–4078

Wani PA, Khan MS, Zaidi A (2007a) Cadmium, chromium and copper in greengram plants. Agron Sustain Dev 27:145–153

Wani PA, Khan MS, Zaidi A (2007b) Impact of heavy metal toxicity on plant growth, symbiosis, seed yield and nitrogen and metal uptake in chickpea. Aust J Exp Agric 47:712–720

Wani PA, Khan MS, Zaidi A (2007c) Chromium reduction, plant growth-promoting potentials, and metal solubilization by *Bacillus* sp. isolated from alluvial soil. Curr Microbiol 54:237–243

Wani PA, Khan MS, Zaidi A (2007d) Effect of metal tolerant plant growth promoting *Bradyrhizobium* sp. (*vigna*) on growth, symbiosis, seed yield and metal uptake by greengram plants. Chemosphere 70:36–45

Wani PA, Khan MS, Zaidi A (2008a) Effects of heavy metal toxicity on growth, symbiosis, seed yield and metal uptake in pea grown in metal amended soil. Bull Environ Contam Toxicol 81:152–158

Wani PA, Khan MS, Zaidi A (2008b) Chromium reducing and plant growth promoting *Mesorhizobium* improves chickpea growth in chromium amended soil. Biotechnol Lett 30:159–163

Wani PA, Khan MS, Zaidi A (2008c) Effect of metal tolerant plant growth promoting *Rhizobium* on the performance of pea grown in metal amended soil. Arch Environ Contam Toxicol 55: 33–42

Yoshida N, Kato T, Yoshida T, Ogawa K, Yamashita M, Murooka Y (2002) Bacterium-based heavy metal biosorbents: enhanced uptake of cadmium by *Escherichia coli* expressing a metallothionein fused to β-galactosidase. Biotechniques 32:551–558

Zannoni D, Borsetti F, Harrison JJ, Turner RJ (2007) The bacterial response to the chalcogen metalloids Se and Te. Adv Microb Physiol 53:1–71

# Chapter 6
# Functional Diversity Among Plant Growth-Promoting Rhizobacteria: Current Status

**Mohammad Saghir Khan, Almas Zaidi, P. A. Wani, Munees Ahemad, and Mohammad Oves**

**Abstract** Root-colonizing bacteria (rhizobacteria) that exert beneficial effects on plant development via direct or indirect mechanisms have been defined as plant growth-promoting rhizobacteria (PGPR). These natural bioresources provide essential nutrients to plants and improve growth, competitiveness, and responses to external stress factors by an array of mechanisms under different agro-ecosystems. The PGPR facilitate plant growth by synthesizing or altering: the concentration of phytohormones: asymbiotic and symbiotic $N_2$ fixation, antagonism against phytopathogenic microorganisms by producing siderophores, antibiotics and cyanide; solubilization of mineral phosphates and other nutrients; and by synthesizing 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which helps to reduce the inhibitory effects of ethylene on plants. And hence, use of such PGPR may be a viable alternative to chemical fertilizers for increasing the productivity of various crops. However, despite their proven ability of growth promotion, PGPR have yet to fulfil their promise and potential as commercial bioinoculants. Understanding functional diversity of PGPR is vital for low-input sustainable production. Recent progress focusing on the principles and mechanisms of action of PGPR is reviewed and discussed.

## 6.1 Introduction

Growth of plants is influenced by a myriad of abiotic and biotic factors. In agricultural practices, in order to manage the soil environment and consequently to improve crop yields, growers routinely adopt physical and chemical approaches. However, these approaches are not only expensive but their excessive and repeated

M.S. Khan (✉), A. Zaidi, P.A. Wani, M. Ahemad, and M. Oves
Faculty of Agricultural Sciences, Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, U.P., India
e-mail: khanms17@rediffmail.com

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_6, 

use may also deplete soil fertility. An alternative to these physico-chemical methods is the exploitation of naturally occurring microbes whose application, however, is inexpensive but less common. These microbes thrive well in the region around the root (the rhizosphere) which is relatively rich in nutrients, due to the accumulation of plant photosynthates, released from the roots of different plants. These photosynthates support microbial communities of soils which may exhibit beneficial, neutral, or deleterious effects on plant growth. Beneficial rhizobacteria capable of aggressively colonizing the rhizosphere and facilitating plant growth are often termed plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth 1978) or plant health-promoting rhizobacteria (PHPR) according to their mode of action (Sikora 1992). In contrast, deleterious rhizobacteria are presumed to adversely affect plant growth and development through the production of undesirable metabolites (phytotoxins) or through competition for nutrients or inhibition of the other beneficial effects (Sturz and Christie 2003). In order to exert their effect on plants, PGPR must (1) be able to colonize the root, (2) survive and multiply in the rhizosphere or within plant tissues, and (3) facilitate plant growth (Barea et al. 2005). These PGPR have been found to facilitate plant growth in laboratory and greenhouse environments, but responses have been variable in the field. Although many studies have been conducted to identify the specific traits by which PGPR promote plant growth, the majority of them have focused on one or two of these traits. Moreover, the presence of a PGPR trait in vitro does not guarantee that a particular isolate is a PGPR. The mechanisms by which PGPR enhance plant growth are not fully understood, but are believed to include: the ability to produce or change the concentration of the plant hormones, such as indoleacetic acid (Ahmad et al. 2008), gibberellic acid (Mahmoud et al. 1984), and cytokinins (Frankenberger and Arshad 1995) and ethylene (Glick 1995; Garcia de Salamon et al. 2001); $N_2$ fixation (Zaidi 1999; Wani et al. 2007a); antagonism against phytopathogenic microorganisms (Khan et al. 2002) by production of siderophores (Wani et al. 2007b), β-1,3-glucanase (Flaishman et al. 1996), chitinases (Renwick et al. 1991), antibiotics (Shanahan et al. 1992), and cyanide (Wani et al. 2007a); and solubilization of inorganic phosphates and toxic metals (Wani et al. 2007b, 2007c). Due to these properties, these beneficial microbes have become a potential component in management practices to achieve the optimum yield. This chapter surveys the developments in the functional diversity among PGPR that could help to develop a bioinoculant possessing multifaceted activity for use in diverse agro-ecological regions of the world.

## 6.2 Rhizosphere and Plant Growth Promoting Rhizobacteria

The rhizosphere can be defined as any volume of soil specifically influenced by plant roots and/or in association with root hairs and plant-produced materials (Bringhurst et al. 2001). This space includes soil bound by plant roots, often extending a few millimeters from the root surface, and can include the plant root

epidermal layer (Mahaffee and Kloepper 1997). Plant exudates in the rhizosphere, such as amino acids and sugars, provide a rich source of energy and nutrients for rhizosphere microbes including PGPR, resulting in bacterial populations greater in this area than outside the rhizosphere. Broadly, PGPR can be divided into two major groups according to their relationship with the host plants: (1) symbiotic bacteria, and (2) free-living rhizobacteria (Khan 2005). Somers et al. (2004) have, however, classified PGPR into the following functional groups depending on their inherent activities: (1) biofertilizers (capable of accelerating the accessibility of nutrients to the plant); (2) phytostimulators (capable of facilitating the plant growth usually by synthesizing phytohormones); (3) rhizoremediators (involved in the degradation of organic pollutants); and (4) biopesticides (capable of managing plant diseases by the production of antimicrobial metabolites). Furthermore, based on their localization, PGPR can be: (1) intracellular PGPR (iPGPR) which are bacteria residing inside plant cells, producing nodules and being localized inside those specialized structures (e.g., nodules); and (2) extracellular PGPR (ePGPR) which are those bacteria living outside plant cells and not producing nodules, but enhancing plant growth through production of signal compounds that directly stimulate growth, improve disease resistance, or nutrient status of soil. The ePGPR has further been subdivided into three types, based on the degree of association with plant roots: (1) those living near, but not in contact with the roots; (2) those colonizing the root surface; and (3) those living in the spaces between cells of the root cortex. Of these PGPR, iPGPR are mostly Gram-negative and rod-shaped, with a few bacterial populations being Gram-positive rods, cocci and pleomorphic forms. Generally, iPGPR include the members of rhizobiace, capable of forming nodules on the root systems of leguminous plants. In contrast, some of the agronomically important ePGPR include genera such as *Bacillus* (Ryder et al. 1999), *Pseudomonas* (De Freitas and Germida 1991) *Erwinia* (Nelson 1998), *Enterobacter* (Tannii et al. 1990), *Caulobacter*, *Serratia* (Zhang et al. 1996), *Flavobacterium* (Tannii et al. 1990), *Actinobacter* sp. (Tannii et al. 1990), *Aeromonas* (Inbar and Chet 1991), *Agrobacterium* (Ryder and Jones 1990), *Alcaligenes* sp. (Yuen et al. 1985), *Phyllobacterium* sp. (Lambert et al. 1990), and *Bacillus* (Bai et al. 2002), *Hyphomycrobium, Azotobacter*, *Azospirillum*, and *Acetobacter* (Prithiviraj et al. 2003).

## 6.3 Search for Plant Growth-Promoting Rhizobacteria

Microbial communities in general, and PGPR in particular, form an important component of soil and help in predicting the changes in soils, as they affect the physico-chemical properties of soil. These microbes can be identified using physiological and biochemical tests, which require a regular subculturing. The community structure of PGPR in general can be characterized using phenotypic and genotypic approaches. Of these, phenotypic methods include standard plating methods on selective media, community level physiological profiles (CLPP)

using the BIOLOG system (Garland 1996), and phospholipid fatty acid (PLFA) and fatty acid methyl ester (FAME) profiling (Germida et al. 1998). Moreover, the culture-independent molecular techniques that are based on direct extraction of DNA from soil, and 16S-rRNA gene sequence analysis, bacterial artificial chromosome, or expression cloning systems, (Rondon et al. 1999) have allowed the easier identification and determination of the potentials they possess. These approaches can also be used to determine the impact of inoculation of PGPR on the rhizosphere community (Steddom et al. 2002). Yet one of the challenges in developing PGPR for commercial application is ensuring its effective selection and screening procedure, so that the most promising PGPR with multiple traits are identified and raised. However, no efficient high-throughput assays to select the best PGPR are currently available. Various approaches for initial selection and screening of rhizobacterial isolates include host plant specificity, adaptation to a particular soil, and screening assays (Bowen and Rovira 1999). Furthermore, the functional properties associated with PGPR, like root colonization, synthesis of IAA, solubilization of insoluble P, synthesis of ACC deaminase, and antibiotics and siderophores, have also been used to characterize these PGPR. Moreover, the impact of pollutants including heavy metals on these rhizosphere microbes in metal-stressed soils can be assessed using the signature biomarkers such as nucleic acid and fatty acids. The development of methods for direct extraction of nucleic acids and fatty acids from both contaminated and nonpolluted soil samples in combination with recombinant DNA and molecular phylogeny methods have provided a new insight into the identification of specific bacterial strains.

## 6.4 Mechanism of Growth Promotion by Plant Growth-Promoting Rhizobacteria

PGPR can affect plant growth either indirectly or directly (Glick et al. 1999; Antoun and Prévost 2006). The indirect promotion of plant growth occurs when PGPR lessen or prevent the deleterious effects of one or more phytopathogenic organisms by: synthesizing antibiotics; depletion of iron from the rhizosphere; induced systemic resistance; synthesis of antifungal metabolites; production of fungal cell wall lysing enzymes; competition for sites on the root; stimulation of other beneficial symbioses; and by decreasing the toxicity of hazardous substances in contaminated soils. While using direct mechanisms, PGPR promotes the growth of plants by either providing plants with a compound synthesized by the bacterium or facilitating the uptake of certain nutrients from the environment, iron sequestration by siderophores, the production of bacterial volatiles and phytohormones, and lowering the ethylene level in plants. Thus, PGPR functions in three different ways: (1) synthesizing particular compounds for uptake by plants (Zaidi et al. 2003; Zaidi and Khan 2006; Khan et al. 2007); (2) facilitating the uptake of certain nutrients from environment (Lucas García et al. 2004; Çakmakçi et al. 2006); and

(3) protecting plants from diseases (Guo et al. 2004; Pandey et al. 2006; Trivedi et al. 2008). Regardless of the mechanisms of plant growth promotion, PGPR must colonize the rhizosphere around the roots, the rhizoplane or the root itself (Glick 1995). In general, PGPR improves plant growth by synthesizing phytohormones precursors (Wani et al. 2007a, b; Ahmad et al. 2008), vitamins, enzymes, and siderophores (Wani et al. 2008a), as well as antibiotics (Burd et al. 2000; Glick 2001), and by inhibiting ethylene synthesis (Glick et al. 2007), in addition to their ability to fix atmospheric N ($N_2$ fixers) and to solubilize inorganic P (Khan et al. 2007), and to make these elements accessible to plants (Perveen et al. 2002; Wani et al. 2007a, b; Khan and Zaidi 2007), to mineralize organic phosphate (Ponmurugan and Gopi 2006), and to improve plant stress tolerance to drought, salinity and metal toxicity (Wani et al. 2008b). Biochemical and molecular approaches are providing new insights into the genetic basis of these traits, the biosynthetic pathways involved, their regulation, and their importance for biological control in laboratory and field studies. An overview of the plant growth promotion by PGPR is presented in Fig. 6.1, while the growth-promoting substances synthesized by various PGPR are summarized in Table 6.1.

Use of such microbes possessing multiple traits, including their role in metal resistance/reduction and ability to promote plant growth in metal-contaminated soils also make them one of the most suitable choices for bioremediation. Among other PGPR, the symbiotic nitrogen fixers enhance the growth of legumes by: (1) biological $N_2$ fixation, (2) increasing the availability of nutrients in the rhizosphere, (3) inducing increases in root surface area, (4) enhancing other beneficial symbioses of the host, (5) reducing or preventing the deleterious effects of phytopathogenic

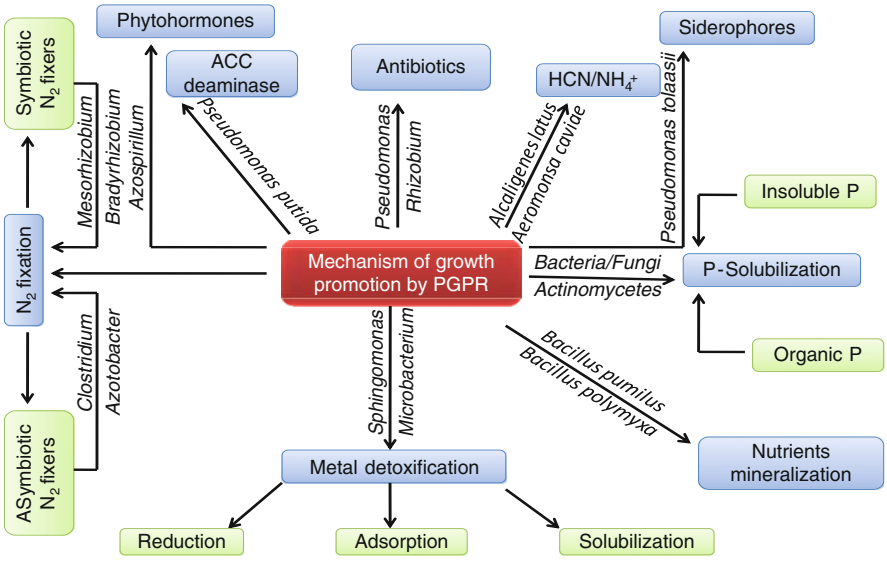



**Fig. 6.1** Functional diversity among plant growth-promoting rhizobacteria

**Table 6.1** Growth-promoting substances produced by plant growth-promoting rhizobacteria

| Organisms | Growth regulators | Reference |
|---|---|---|
| *Azotobacter,* fluorescent *Pseudomonas, Bacillus* | IAA, siderophore, ammonia, HCN, P-solubilization | Ahmad et al. (2008) |
| *Pantoea dispers* strain 1A | P-solubilization, IAA, siderophores, HCN | Selvakumar et al. (2008) |
| *Bacillus* spp. | IAA, siderophore, HCN | Wani et al. (2007b) |
| *Pseudomonas*, *Bacillus* | Siderophores, IAA, P-solubilization | Rajkumar et al. (2006) |
| *Brevibacillus* sp. | IAA | Vivas et al. (2006) |
| *Xanthomonas* sp. RJ3, *Azomonas* sp. RJ4, *Pseudomonas* sp. RJ10, *Bacillus* sp. RJ31 | IAA | Sheng and Xia (2006) |
| *Bacillus* sp. | P-solubilization | Canbolat et al. (2006) |
| *Brevibacterium* sp. | Siderophore | Noordman et al. (2006) |
| *Bacillus subtilis* | IAA, P-solubilization | Zaidi et al. (2006) |
| *Variovorax paradoxus*, *Rhodococcus* sp. and *Flavobacterium* (Cd tolerant) | IAA, siderophores | Belimov et al. (2005) |
| *Pseudomonas fluorescens* | IAA, siderophore, P-solubilization | Gupta et al. (2005) |
| *Pseudomonas putida* | Siderophore | Tripathi et al. (2005) |
| *Azotobacter, Pseudomonas fluorescens* | IAA | Ahmad et al. (2005) |
| *Bacillus*, *Azospirillum* sp. | IAA, P-solubilization | Yasmin et al. (2004) |
| *Pseudomonas aeruginosa* | IAA, siderophore, HCN | Bano and Musarrat (2003) |
| *Bacillus, Pseudomons, Azotobacter, Azospirillum* | P-solubilization, IAA | Tank and Saraf (2003) |
| *Pseudomonas* sp. | Siderophore | Sharma et al. (2003) |
| *Pseudomonas* sp. | IAA, siderophore, P-solubilization | Gupta et al. (2002) |
| *Pseudomonas fluorescens* | Siderophore | Khan et al. (2002) |
| *Azotobacter chroococcum* | Gibberellin, kinetin, IAA | Verma et al. (2001) |
| *Kluyvera ascorbata* | Siderophore | Burd et al. (2000) |

organisms (Khan et al. 2002), and (6) combination of modes of action. As an example of plant growth promoter, IAA, phytohormone of the auxin series produced by many rhizobia (Abd-Alla 1994; Wani et al., 2007a, 2007b, 2008a, 2008b, 2008c), and its metabolically related precursor, anthranilic acid, can reductively solubilize soil Fe (III), and increase its availability via a mechanism different from that involving siderophores. Further plant growth-promoting substances, siderophores, are specific Fe (III)-chelating agents that make the chelated iron unavailable to pathogenic organisms thereby leading to an increase in plant health. Microbial siderophores are known to regulate the availability of Fe in the plant rhizosphere, and it has been found that competition for iron in the rhizosphere is controlled by the affinity of the siderophores for iron. Interestingly, the binding affinity of

phyto-siderophores for iron is less than the affinity of microbial siderophores, but plants require a lower iron concentration for normal growth than do microbes. Although several rhizobial species are known to produce growth-promoting substances under metal-free environment, the syntheses of these compounds by metal-tolerant rhizobia have been limited. Nevertheless, there has been certain evidence where metals at lower concentrations exert no harmful effect and rather stimulate plant growth promoting activities of rhizobia. For instance, *Bradyrhizobium* (RM8), tolerant to nickel and zinc, *Rhizobium* sp. (RL9), tolerant to zinc and *Rhizobium* sp. (RP5), tolerant to zinc and nickel, produced substantial amounts of IAA (Wani et al. 2008a, c) under metal-stressed conditions. The production of growth promoting substances by metal-tolerant and natural rhizobial strains are presented in Table 6.2.

### 6.4.1 Plant Growth Regulators

Plant growth regulators (PGRs) are the substances that influence physiological processes of plant at very low concentrations and modify or control one or more specific metabolic events of a plant. According to the Environmental Protection Agency (EPA), the plant regulators have been defined as "any substance or mixture of substances intended, through physiological action, to accelerate or retard the rate of growth or maturation, or otherwise alter the behavior of plants or their produce." Such compounds produced by the plant or by PGPR are called plant hormones

**Table 6.2** Plant growth-promoting substances synthesized by symbiotic nitrogen fixers

| Symbiotic $N_2$ fixers | Plant growth-promoting substances | References |
|---|---|---|
| *Mesorhizobium* | IAA, siderophore, ammonia, HCN, P-solubilization | Ahmad et al. (2008) |
| *Bradyrhizobium* | IAA, siderophores, HCN | Wani et al. (2008a) |
| *Mesorhizobium* | IAA, siderophores | Wani et al. (2008c) |
| *Rhizobium* spp. | IAA, siderophores | Sridevi et al. (2008a, b) |
| *Rhizobium* spp. | P-solubilization | Sridevi et al. (2007) |
| *Bradyrhizobium japonicum* | IAA | Shaharoona et al. (2006) |
| *Rhizobium* | HCN, siderophore | Deshwal et al. (2003) |
| *Bradyrhizobium (Arachis)* | Siderophore, IAA, P-solubilization | Deshwal et al. (2003) |
| *Rhizobium* | P-solubilization, IAA | Tank and Saraf (2003) |
| *Mesorhizobium, Bradyrhizobium* sp. (*vigna*) | Siderophore | Khan et al. (2002) |
| *Rhizobium meliloti* | Siderophore | Arora et al. (2001) |
| *Bradyrhizobium, Rhizobium* | IAA, HCN, siderophore | Antoun et al. (1998) |
| *Bradyrhizobium, Rhizobium* | Siderophore | Duhan et al. (1998) |
| *Rhizobium ciceri* | Siderophore | Berraho et al. (1997) |
| *Bradyrhizobium japonicum* | Siderophore | Wittenberg et al. (1996) |
| *Rhizobium*, *Bradyrhizobium* | P-solubilization | Abd-Alla (1994) |

(Davies 1995; Karadeniz et al. 2006). The phytohormones and other compounds synthesized by PGPR are now reviewed and discussed.

#### 6.4.1.1 Phytohormones

Indoleacetic acid is commonly produced by PGPR by using the rich supplies of substrates exuded from the roots and it releases auxin in the rhizospheres as secondary metabolites. Among the PGPR, $N_2$-fixing bacteria in general are known exclusively for their $N_2$-fixing ability, yet they are also reported to produce IAA (Table 6.3). For example, species of *Bradyrhizobium*, *Rhizobium* and *Mesorhizobium* produced a substantial amount of IAA under in vitro conditions (Antoun et al. 1998; Wani et al. 2008a, 2008b, 2008c; Ahmad et al. 2008). Among other PGPR strains, *Pseudomonas*, *Bacillus*, *Agrobacterium* sp., *Alcaligenes piechaudii* and two strains of *Comamonas acidovorans* secreted IAA at lower levels as compared to deleterious bacteria (Barazani and Friedman 1999; Rajkumar et al. 2006). Bacteria associated with the roots of greenhouse tropical orchids have also been shown to produce IAA as demonstrated by thin layer chromatopraphy (TLC) and by biotests (Tsavkelova et al. 2005). In another study, numerous bacterial isolates recovered from wheat (*Triticum aestivum*) rhizosphere demonstrated the production of auxins (ranging from 1.1 to 12.1 mg $l^{-1}$) under in vitro conditions. However, when the medium was supplemented exogenously with tryptophan, it significantly enhanced the auxin biosynthesis which was confirmed by high performance liquid chromatography (HPLC) analysis (Khalid et al. 2004).

#### 6.4.1.2 Biosynthesis of Indole Acetic Acid

Indole-3-acetic acid and its analog are the primary active auxin in most plants. They are synthesized from tryptophan, primarily in leaf primordial, young leaves and developing seeds. Auxin plays an important role in the development of roots including root initiation, cell enlargement and cell division (Fig. 6.2). It has been shown that free IAA is easily converted into esterified IAA with sugar or amide-linked IAA, and that such conjugated forms are the forms in which IAA is stored in plants. Two kinds of genes that are involved in the formulation of conjugated IAA and the hydrolysis of IAA have been identified (Bartel and Fink 1995). However, the biosynthetic process of IAA in plants at the molecular level have not yet been characterized due to several reasons: (1) levels of IAA in intact cells are low, (2) indole compounds are nonenzymatically degraded, (3) bacterial contamination can complicate assays of enzymatic activity, and (4) compartmentalization of cells is disrupted under in vitro conditions. Indoleacetic acid is also important for the microbes that interact with plants, and the biosynthesis of IAA has been assayed mainly for plant-associated bacteria. At the molecular level, two pathways of IAA production have been identified: (1) the indole-3-pyruvic acid pathway, reported in PGPR, *Enterobacter cloacae*, *Rhizobium* and *Bradyrhizobium*, and the (2) indole

acetamide (IAM) pathway, which is often found in tumor-forming bacteria, such as *Pseudomonas syringae* pv. *savastanoi* and *Agrobacterium*, for which genes are plasmid-borne.

*E. cloacae*, isolated from the cucumber (*Cucumis sativus)* rhizosphere, has shown the secretion of IAA in the culture medium by the indole-3-pyruvic acid pathway (Koga et al. 1991). Interestingly, the cloned *E. coli* possessing IAA genes of *E. cloacae* produced large amounts of IAA, even though three different enzymes in the indole-3-pyruvic acid pathway are involved. Moreover, the gene (*ipdc*) transformed from *E. cloacae* did not encode tryptophan aminotransferase, regarded as the enzyme that catalyzes the rate-limiting step in the IAA synthesis. The gene encoded indole pyruvate decarboxylase, whose detection has been very difficult. The IAM pathway was first reported in *P. syringae* pv. *savastanoi*, which induces the production of tumorous outgrowths on olive (*Olea europaea)* and oleander (*Nerium oleander)* plants. The pathway depends on the products of two genes, *iaaM* and *iaaH*. The *iaaM* gene encodes tryptophan 2-monooxygenase, which catalyzes the conversion of L-tryptophan to IAM, while *iaaH* encodes IAM hydrolase, which catalyzes the conversion of IAM to IAA. Induction of tumor formation by *P. syringae* pv. *savastanoi* on its host plants requires the overproduction of IAA. This pathway has also been reported for other tumor-forming bacteria and *Erwinia herbicola* pv. *gypsophilae* (Clark et al. 1993).

### 6.4.2 Siderophores

Iron plays an important role in various biochemical and physiological processes, such as respiration, photosynthetic transport, nitrate reduction, chlorophyll synthesis, and $N_2$ fixation (Robinson and Postgate 1980). Iron also acts as a cofactor or is required for proper functioning of enzymes and proteins (e.g., peroxidase, POX, superoxide dismutase, nitrogenase, glutamate synthase, ribonucleotide, diphosphate reductase, aconitase, cytochromes, ferrodoxin, and flavoproteins) that facilitate electron transport, oxygen transport and other life-sustaining process. It exists in aerobic soil and water environments in the $Fe^{3+}$ state, most insoluble at physiological pH (Crowley et al. 1987). A level of at least 1 M iron is required for optimum growth, and if greater than 1 M, it is an iron-stressed condition (Ownley et al. 2003). The limitation of iron can inhibit growth, decrease genetic materials and inhibit sporulation, and can also change the cell morphology. The environmental restrictions and biological imperatives, therefore, require that microorganisms form this specific nutrient, which is, though, abundant but essentially unavailable (Leeman et al. 1996). Generally, all aerobic and facultative anaerobic prokaryotes and some plants produce low molecular weight compounds to provide themselves with iron. The low molecular mass (0.5–1.5 kDa) ferric-specific iron-chelator compounds, often called siderophores (iron bearers) (Nielsen and Sorensen 2003), are produced by PGPR. More than 500 different siderophores have been identified from microorganisms, and some bacteria produce more than one type of siderophores.

**Table 6.3** Plant growth-promoting rhizobacteria used in bioremediation

| Bacteria | Plant | Heavy metals | Conditions | Role of PGPR | References |
|---|---|---|---|---|---|
| *Methylobacterium oryzae, Berknolderia* sp. | *Lycopersicon esculentom* | Ni, Cd | Gnotobiotic and pot culture experiments | | Madhaiyan et al. (2007) |
| *Bacillus megaterium* HKP-1 | *Brassica Juncea* | Pb, Zn | Experiments in greenhouse | Protected plant from metal toxicity | Wu et al. (2006a) |
| *Bacillus subtilis* SJ-101 | *Brassica Juncea* | Ni | Experiments in growth chamber | Facilitated Ni accumulation | Zaidi et al. (2006) |
| *Xanthomonas* sp. RJ3, *Azomonas* sp. RJ4, *Pseudomonas* sp. RJ10, *Bacillus* sp. RJ31 | *Brassica napus* | Cd | Experiment in pots | Stimulated plant growth and increased cadmium accumulation | Sheng and Xia (2006) |
| *Pseudomonas* sp, *Bacillus* sp. | Mustard | Cr (VI) | Pot experiment | Stimulated plant growth and decreased Cr (VI) content | Rajkumar et al. (2006) |
| *Ochrobactrum, Bacillus cereus* | Mungbean | Cr (VI) | Experiment in pots | Lowers the toxicity of chromium to seedlings by reducing Cr (VI) to Cr (III) | Faisal and Hasnain (2006) |
| *Brevibacillus* | *Trifolium repens* | Zn | Pot experiment | Enhanced plant growth and nutrition of plants and decreased zinc concentration in plant tissues | Vivas et al. (2006) |
| *Variovox paradoxus, Rhodococcus* sp, *Flavobacterium* | *Brassica juncea* | Cd | Experiment in Petri dishes | Stimulating root elongation | Belimov et al. (2005) |
| *Pseudomas fluorescens* | Soybean | Hg | Experiment in greenhouse | Increased plant growth | Gupta et al. (2005) |
| *Ochrobactrum intermedium* | Sunflower | Cr (VI) | Experiment in pots | Increased plant growth and decreased Cr (VI) uptake | Faisal and Hasnain (2005) |
| *Kluyvera ascorbata* SUD165 | Indian mustard | Ni, Pb, Zn | Experiments in growth chamber | Both strains decresed some plant growth inhibition by | Burd et al. (2000) |
| *K. ascorbata* SUD165 | Canola, tomato | | | | |

*(continued)*

**Table 6.3** (continued)

| Bacteria | Plant | Heavy metals | Conditions | Role of PGPR | References |
|---|---|---|---|---|---|
| | | | | heavy metals, No increase of metal uptake with either strain over noninoculated plants | |
| *Brevundimonas* Kro13 | None | Cd | Culture media | Sequestered cadmium directly from solution | Robinson et al. (2001) |
| *Pseudomonas* sp. | Soybean, mungbean, wheat | Ni, Cd, Cr | Experiment in pots | Promotes growth of plants | Gupta et al. (2002) |

*(continued)*

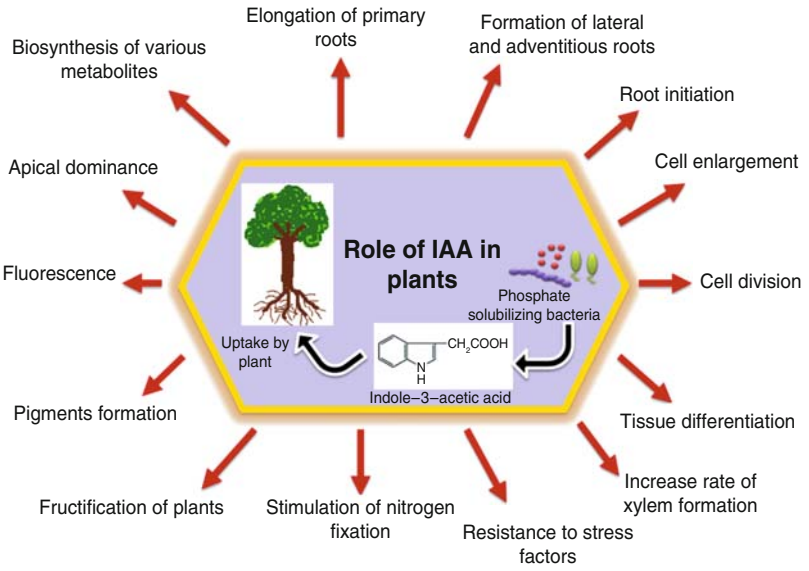



**Fig. 6.2** Role of IAA in plant growth promotion

When grown under iron-deficient conditions, many microbes will synthesize and excrete siderophores in excess of their own dry cell weight to sequester and solubilize iron. Most of the siderophores are water-soluble and can be secreted extracellularly or produced inside (intracellularly). Generally, most siderophore transport systems are highly specific for certain siderophores, although some broad-range siderophore-recognition systems have been described based on ligand-exchange mechanisms (Bultreys et al. 2003).

#### 6.4.2.1 Siderophore Production by Microorganisms

The siderophore production in iron-stress conditions confers upon these organisms an added advantage, resulting in exclusion of pathogens due to iron starvation. Siderophore production by rhizobial strains has been considered as a potential way to improve nodulation and $N_2$ fixation in iron-deficient conditions (O' Hara et al. 1988; Khan et al. 2002), and, hence, favors the persistence of rhizobia in iron-deficient soils (Lesueur et al. 1993). In a study, strains of *Mesorhizobium*, showed the production of siderophores in Chrome Azurol S (CAS) agar medium while the supernatants of this strain yielded salicylic acid and 2,3-dihydroxybenzoic acid (DHBA) as phenolate-type siderophores. Addition of ferric iron to the culture medium, though, increased growth yield, but decreased the synthesis of siderophores (Berraho et al. 1997). Similarly, other bradyrhizobial and rhizobial strains infecting greengram (*Vigna radiata* L. Wilczek), pigeonpea (*Cajanus cajans*) and pea (*Pisum sativum*) have shown the production of siderophores using CAS agar plate and CAS solution assay (Wani et al. 2008a, 2008b). The predominant form of siderophores secreted by these strains included hydroxymate and catechol. Similarly, nitrogen-fixing iron-stressed *A. vinelandii* in continuous culture formed DHBA, 2-*N*,6-*N*-di-(2,3-dihydroxybenzoyl)-L-lysine (DHBL) and a chromophoric yellow-green fluorescent peptide (YGFP) (Fekete et al. 1983).

#### 6.4.2.2 Chemical and Biological Properties of Siderophores

Broadly, siderophores have been classified into four groups: (1) hydroxamate, (2) phenol catecholates, (3) carboxylate, and (4) salicylic acid (2-hydroxy benzoic acid). Generally, PGPR produce both hydroxamate and catecholate siderophores (Witter and Luther 1998). Different types of siderophores produced by PGPR strains are presented in Fig. 6.3. Of these, hydroxamate siderophores are generally referred to as pseudobactin- (Fig. 6.4) or pyoverdine-type siderophores (Meyer et al. 1997). Each pyoverdin has three Fe binding ligands, one of which is always a α-dihydroxy aromatic group derived from quinoline located in the chromophore. The other two are located in the peptide chain and are hydroxamic acids derived from ornithine either acylated *N*-hydroxyornithine or cyclized *N*-hydroxyornithine, or one hydroxamic acid derived from ornithine plus a β-hydroxyaspartic acid residual. A catecholate siderophore complex consists of three catecholamide groups ligating the metal ion by six oxygen atoms. The catecholamide groups are linked to a trilactone ring or they are connected by a backbone of alkyl chain beginning at a tertiary carbon or a nitrogen atom. The oxygen atoms possess a high electron density, which exhibit a high affinity for protons when deprotonated at pH values above 6.5. In addition, because Fe (III) is a strong Lewis acid it readily donates protons to other atoms, such as the polarizable oxygen atoms of the catechol moiety. This electrostatic interaction gives catecholate siderophores a greater affinity for Fe (III) compared to their hydroxamate counterparts.

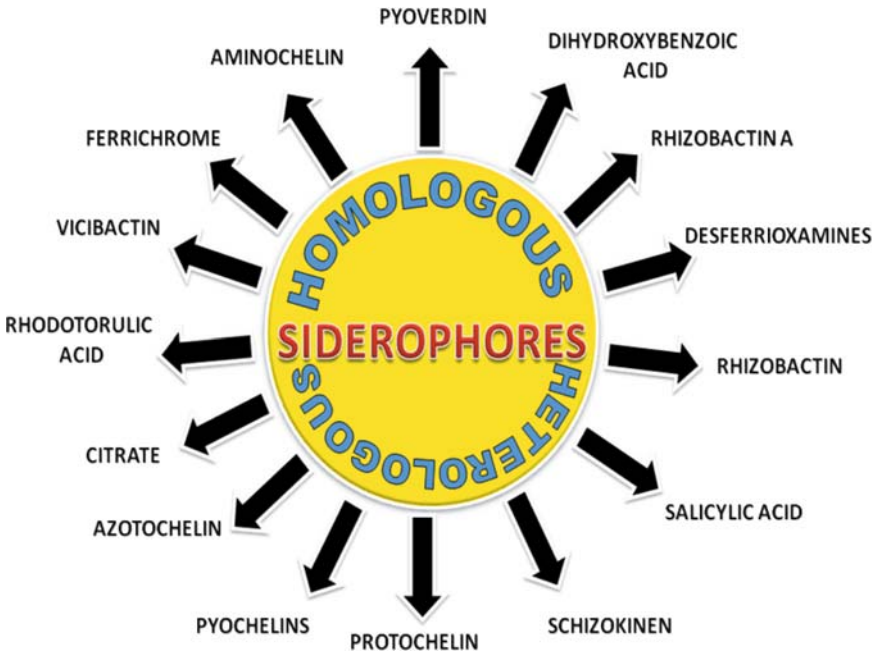



**Fig. 6.3** Types of siderophores

Even though the major function of siderophores is to obtain iron from insoluble hydroxides or from iron adsorbed onto solid surfaces, they can also extract iron from various other soluble and insoluble iron compounds. For instance, they can extract iron from ferric citrate, ferric phosphate, Fe-transferrin, ferritin or iron bound to sugars, plant flavone pigments and glycosides, or even from artificial chelators like EDTA and nitrilotriacetate by Fe (III)/ligand-exchange reactions. Siderophores are thus not only directly involved in iron solubilization, but can indirectly make iron available to both microbes and plants. The efficiency of siderophores in microbial metabolism is based mainly on three facts: (1) siderophores consisting of hydroxamate, catecholate or α-hydroxycarboxylate ligands contain the most efficient iron-binding ligand types in nature and satisfy the six coordination sites on ferric ions; siderophores also increase the stability due to its chelating effects; (2) regulation of siderophore biosynthesis is an economic means of spending metabolic energy, but it also allows the production of high local concentrations of siderophores in the vicinity of microbial cells during iron limitation, while over-production of siderophores by host-adapted bacterial strains leads to increased virulence; and (3) besides their ability to solubilize iron and to function as external iron carriers, siderophores exhibit structural and conformational specificities to fit into membrane receptors and/or transporters (Stintzi et al. 2000) and are involved in various biological processes (Fig. 6.5). Moreover, the siderophore-producing strains stimulate the $N_2$-fixing efficiency of the rhizobial strains (Duhan

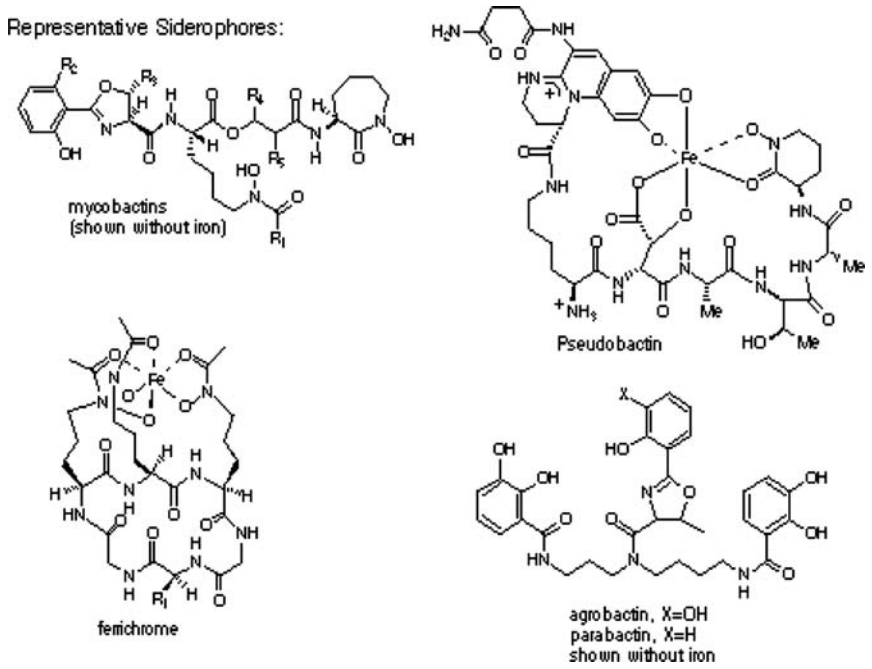



**Fig. 6.4** Representative siderophores

et al. 1998). Furthermore, of the 12 isolates of *Rhizobium meliloti* isolated from the medicinal plant, *Mucuna pruriens*, only two isolates (RMP3 and RMP5) inhibited the growth of phytopathogens (*Macrophomonia phaseolina*). Further, a marked enhancement in percentage seed germination, seedling biomass, nodule number and nodule weight of *M. phaseolina*-infected groundnut *(Arachis hypogaea)* plants inoculated with the strains RMP3 and RMP was observed, suggesting the growth promoting activities of siderophores (Arora et al. 2001). Among other PGPR, *Pseudomonas aeruginosa* (GRC1), isolated from potato (*Solanum tuberosum)* rhizosphere, produced several plant growth-promoting substances, including a siderophore. The siderophore was identified as hydroxymate and, when *P. aeruginosa* was used in field trials, enhanced growth and yield of Indian mustard (*Brassica juncea*) (Pandey et al. 2005).

### *6.4.3 Mineral Phosphate Solubilizing Activity*

Phosphorus (P) is an essential plant nutrient whose deficiency restricts crop yields severely. Most tropical and some subtropical soils are acidic, and strong P-sorption combined with low inherent P stocks lead to widespread P deficiency (Gaume 2000). Even where inorganic and organic P-forms are abundant in soils, their

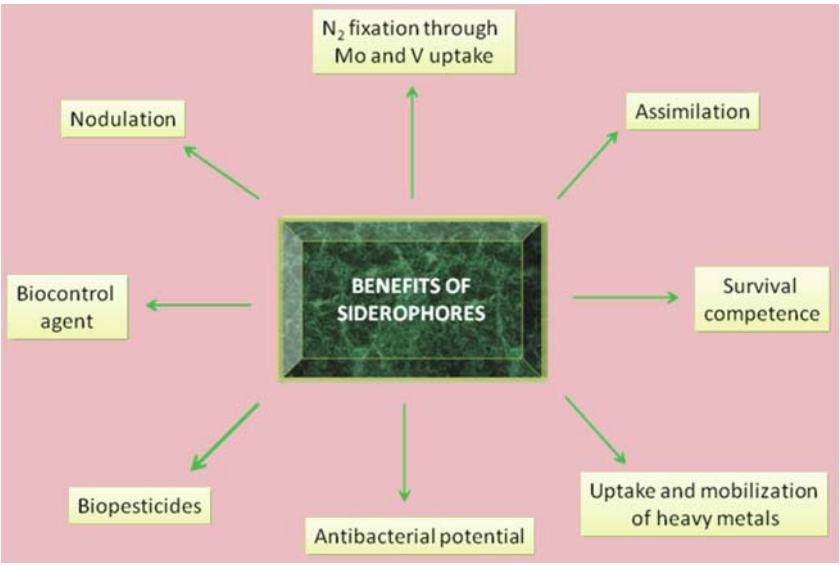



**Fig. 6.5** Biological functions of siderophores

concentration in the soil solution is in the micromolar range (Frossard et al. 2000). These low levels of P are mainly due to high reactivity of soluble P soil elements (Lindsay et al. 1989). Therefore, substantial amounts of manufactured water-soluble P (WSP) fertilizers such as superphosphate are commonly applied to correct P deficiencies. Most developing countries import these fertilizers, which are often in limited supply and represent a major outlay for resource-poor farmers. It is, therefore, imperative to explore alternative P sources. In this context, the PGPR-possessing mineral phosphate-solubilizing (MPS) activity provides an inexpensive and sustainable alternative to chemical P fertilizers (Pradhan and Sukla 2005; Khan et al. 2007). The microbial solubilization of soil P in liquid medium has often been due to the excretion of organic acids (Maliha et al. 2004) which can either directly dissolve the mineral P or can chelate both Fe and Al ions associated with P (Omar 1998). However, no definite correlation between the acids produced by PGPR and amounts of P solubilized are reported (Asea et al. 1988). Such PGPR strains possessed with mps activity, when used either alone or as composite cultures, have shown a substantial increase by supplying both P and other essential nutrients to plants.

### *6.4.4 Growth Modulation Enzyme*

The PGPR also increase the growth of plants through the synthesis of specific enzyme, 1-aminocyclopropane- 1-carboxylate (ACC) deaminase, which induce

physiological changes in plants. Ethylene is a plant hormone that is involved in the regulation of many physiological processes, such as leaf senescence, leaf abscission, epinasty, and fruit ripening (Arshad and Frankenberger 2002). Also, ethylene regulates nod factor signaling and nodule formation and has primary functions in plant defense systems. Besides its physiological role in different developmental stages of plants, ethylene is also considered as a stress hormone, whose synthesis in plants is increased substantially by a number of biotic and abiotic stresses. At higher concentrations, ethylene, however, inhibits growth and development of plants (Grichko and Glick 2001). However, ACC deaminase synthesized by PGPR (Belimov et al. 2005; Safronova et al. 2006; Madhaiyan et al. 2006; Rajkumar et al. 2006; Mellado et al. 2007) alleviates the stress induced by ethylene-mediated impact on plants by hydrolyzing ACC, the immediate precursor of ethylene in plants to $NH_3$ and α-ketobutyrate (Glick et al. 1998; Penrose and Glick 2001; Reed et al. 2005; Safronova et al. 2006), and consequently reduce the ethylene levels in plants. The bacteria utilize the $NH_3$ evolved from ACC as a source of N and thereby restrict the accumulation of ethylene within the plant, which otherwise inhibits plant growth (Belimov et al. 2002). Thus, the decreased levels of ethylene in turn allow the plants to grow better (Madhaiyan et al. 2007; Zahir et al. 2008). It has been observed that plants that are inoculated with PGPR containing ACC deaminase are dramatically more resistant to the deleterious effects of stress ethylene that is synthesized as a consequence of stressful conditions such as flooding (Grichko and Glick 2001), heavy metals (Burd et al. 1998; Grichko et al. 2000), presence of phytopathogens (Wang et al. 2000), drought, and high salt contents (Mayak et al. 2004a, b). In most of these cases, it has been reported that the PGPR-containing ACC deaminase significantly lowered the level of ACC in the stressed plants, thereby limiting the amount of stress ethylene synthesis and hence damage to the plant. Therefore, the use of such plant growth-promoting bacteria containing ACC deaminase may prove useful in developing strategies to facilitate plant growth in stressed soil environments. And, hence, it may be possible to productively cultivate a variety of crop plants under stressed conditions without genetically manipulating plants, provided these plants are grown in the presence of a suitable PGPR.

### *6.4.5 Antibiotics Production by Plant Growth Promoting Rhizobacteria*

PGPR also promotes the growth of plants by secreting antimicrobial compounds, induction of systemic resistance (ISR), and production of pathogen-related (PR) proteins (Compant et al. 2005). Antibiotic production by biocontrol PGPR is perhaps the most powerful mechanism against phytopathogens (Bashan and de-Bashan 2005), and the first clear-cut experiment demonstrating the role of PGPR in suppression of plant disease through antibiotic production was reported by Tomashow and Weller (1988). These antibiotics may be antitumor, antiviral, antimicrobial, antihelmenthic, and cytotoxic (Fernando et al. 2005). The antibiotics

can also contribute to microbial competitiveness besides their role in suppressing the growth of plant root pathogens. The PGPR strains that produce these compounds are, therefore, of considerable interest as biological control agents (Thomshow et al. 2003) and provide an alternative to chemical pesticides. Several antimicrobial compounds belonging to polypeptides, heterocyclic nitrogenous compounds, and lipopeptides groups active against phytopathogens have been reported (Thomshaw and Webler 1995). In addition, plants can acquire local and systemic resistance to diseases through various biological agents, including necrotizing pathogens, nonpathogens, and soil-borne rhizosphere bacteria and fungi (Van Loon et al. 1998). This type of resistance, known as induced systemic resistance, is mediated by a jasmonate/ethylene sensitive pathway (Van Loon et al. 1998). Induction of systemic resistance has been established as a new mechanism by which plants defend themselves against pathogen attachment. Various reports confirm the induction of systemic resistance by PGPR. For instance, PGPR strains, i.e., *P. putida* (strain 89B-27), *S. marcescens* (strain 90-166), *Flavomonas oryzihabitans* strain (INR-5), and *Bacillus pumilus* (strain INR-7), have significantly reduced populations of the striped cucumber (Zehnder et al. 1997). Furthermore, the combined inoculation of PGPR (*Bacillus* and *Pseudomonas*) and *Rhizobium* sp. increased the production of defense-related enzymes, i.e., L-phenylalanine ammonia lyase (PAL), POX and polyphenol oxidase (PPO), in coinoculated pigeonpea plants which in turn decreased the dry weight of mycelium and fusaric acid production by fusarial wilt of pigeonpea, suggesting that the combined use of PGPR and rhizobia for induction of systemic resistance against fusarial wilt in pigeon pea (Dutta et al. 2008).

Antibiosis and antagonistic activities of PGPR recovered from wheat (*T. aestivum*) and rice (*Oryza sativa*) seeds, corn (*Zea mays)* plants, and potato have been suggested as possible mechanisms of growth inhibition of various phytopathogens (Lodewyckx et al. 2002; Rosenblueth and Martínez-Romero 2006). For instance, *P. fluorescens* capable of synthesizing 2,4-diacetyl phloroglucinol (DAPG) has shown the production of antimicrobial compounds in planta conditions. Similarly, production of antibiotics phenazine, pyocyanine and DAPG by *Pseudomonas* spp. associated with induced systemic resistance (ISR) activity in sugarcane (*Saccharum officinarum)* against red rot disease has been reported (Viswanathan and Samiyappan 2004). However, the bacterial strains varied in their capability to produce the metabolites. The purified compounds tested for their antifungal activity completely arrested the conidial germination and mycelial growth of red rot pathogen *(Colletotrichum falcatum)*, suggesting that the metabolites played an important role in antagonism/ISR. Moreover, the suppressive ability of PGPR even against nematodes is reported (Sturz and Kimpinski 2004). Similarly, *Bacillus lentimorbus* and *Bacillus cereus* isolated from coffee (*Coffea canephora*) demonstrated inhibitory effects against coffee rust pathogen (*Hemileia vastatrix)* and significantly enhanced the coffee production. The pathogen suppression was suggested to be due possibly to the synthesis of a significant amount of fungal cell wall lysing enzymes, antibiosis, competition, and ISR in host (Shiomi et al. 2006). Recently, PGPR bioformulations (*Pseudomonas* and *Bacillus)*

were tested for their efficacy against blister blight (*Exobasidium vexans*) disease in tea (*Camellia sinensis*) under field conditions for two seasons. Among the bioformulations tested, foliar application of *Pseudomonas fluorescens* Pf1 at 7-day intervals consistently reduced the disease incidence of blister blight for two seasons, almost comparable with that of chemical fungicide. In addition to disease control, it also increased tea yield significantly compared to the untreated control. Defense enzymes, such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, chitinase, β-1,3-glucanase, and phenolics were found more in *P. fluorescens* Pf1-treated plants compared to control. This finding revealed the probable influence of plant growth promotion and induced systemic resistance (ISR) in enhancing the disease resistance in tea plants against blister disease by PGPR bioformulations (Saravanakumar et al. 2007).

### 6.4.6 Hydrogen Cyanide Production

Cyanide is yet another secondary metabolite produced during the early stationary growth phase (Knowles and Bunch 1986) by several PGPR, notably *Pseudomonas* spp. and *Bacillus* (Wani et al. 2007b; Ahmad et al. 2008), *Chromobacterium* (Faramarzi and Brand 2006), and *Rhizobium* spp. (Wani et al. 2008a, 2008b, 2008c) by oxidative decarboxylation pathway using glycine, glutamate, or methionine as precursors (Castric 1977; Curl and Truelove 1985). The cyanide so released by microbial communities in solution acts as a secondary metabolite and confers a selective advantage on the producer strains (Vining 1990). Although cyanide is a phytotoxic agent capable of disrupting enzyme activity involved in major metabolic processes, its role as a biocontrol substance is overwhelming (Voisard et al. 1989; Devi et al. 2007). Hydrogen cyanide (HCN) effectively blocks the cytochrome oxidase pathway and is highly toxic to all aerobic microorganisms at picomolar concentrations. However, producer microbes, mainly pseudomonads, are reported to be resistant (Bashan and de-Bashan 2005).

### 6.4.7 Production of Lytic Enzymes

A variety of other microbial compounds are involved in the suppression of phytopathogenic growth leading thereby to the reduction in damage to plants. These microbially synthesized compounds include defense enzymes, such as chitinase, β-1,3-glucanase, peroxidase, protease, and lipase (Bashan and de-Bashan 2005; Karthikeyan et al. 2006). Chitinase and β-1,3-glucanase degrade the fungal cell wall and cause lysis of fungal cell. Furthermore, chitin and glucan oligomers released during degradation of the fungal cell wall by the action of lytic enzymes act as elicitors that elicit various defense mechanisms in plants (Karthikeyan et al. 2005). Such enzymes produced by *Pseudomonas stutzeri* have

demonstrated the lysis of the pathogen *Fusarium* sp. (Bashan and de-Bashan 2005). Peroxidase represents another component of an early response in plants to pathogen attack and plays a key role in the biosynthesis of lignin which limits the extent of pathogen spread (Bruce and West 1989). In bean, rhizosphere colonized by various bacteria induced PO activity (Zdor and Anderson 1992). In a study, a rapid increase in PO activity was recorded in coconut (*Cocos nucifera* L.) treated with a mixture of *P. fluorescens*, *T. viride* and chitin which contributed to induced resistance against invasion by *Ganoderma lucidum*, the causal agent of *Ganoderma* disease (Karthikeyan et al. 2006). These findings suggest that PGPR possessing the ability to synthesize hydrolytic enzymes can effectively be utilized for managing the plant diseases and can help to reduce the pesticide usage.

## 6.5 Performance of Plant Growth-Promoting Rhizobacteria in Metal-Contaminated Soils

The PGPR have largely been used as growth-promoting agents in conventional agronomic practices; substantial emphasis is being placed on them in order to also exploit their bioremediation potential. Contaminated soils are often nutrient poor or sometimes nutrient deficient, due to the loss of beneficial microbes. However, such soils can be made nutrient rich by applying metal tolerant microbes, especially the PGPR, which would provide not only the essential nutrients to the plants growing in the derelict soils but also play a major role in detoxifying heavy metals (Mayak et al. 2004a) and thus helping plants to grow better (Glick 2003; Wani et al. 2008a, 2008b, 2008c). For example, PGPR *Kluyvera ascorbata* SUD165 isolated from metal-contaminated wetland near Sudbury, Ontario, Canada, when applied to soils amended with nickel, zinc, lead, and chromate, have shown to increase the growth of canola (*Brasica rapa*) while protecting the plants from nickel toxicity (Burd et al. 1998). Similarly, nickel-resistant *K. ascorbata* protected tomato (*Lycopersicon esculentum* L.), Indian mustard (*Brassica campestris*) and canola plants when grown in soils supplemented with nickel, lead and zinc (Burd et al. 2000). Moreover, the growth-promoting rhizobacteria, *Variovorax paradoxus*, *Rhodococcus* sp. and *Flavobacterium* sp., stimulated root elongation of Indian mustard seedlings either in the presence or absence of toxic cadmium (Belimov et al. 2005), suggesting that these bacterial strains could be developed as inoculants to improve growth of the metal-accumulating Indian mustard in the presence of toxic cadmium concentration, and for the development of plant inoculant systems useful for phytoremediation of polluted soils. Similarly, the canola plants inoculated with *Enterobacter cloacae*, when grown in the presence of arsenates, grew to a significantly greater extent than nontransformed canola plants (Nie et al. 2002). In yet other studies, *Ochrobacterium intermedium* and *Bacillus cereus* protected greengram plants against chromium toxicity (Faisal and Hasnain 2006), while inoculation of *Ochrobacterium intermedium* improved the overall growth of sunflower (*Helianthus annus*), when grown in metal-amended soils (Faisal and Hasnain

2005) as also reported for other crops (Chaudri et al. 2000; Wani et al. 2008b). The increase in the growth of plants grown in derelict soils by applying metal-tolerant rhizobacteria was attributed to the ability of rhizobacterial strains to mitigate the toxic effects of metals, besides providing plants with sufficient amounts of growth-promoting substances. Recent examples of PGPR affecting growth and development of plants in metal-affected soils are presented in Table 6.3. The remediation of heavy metal-contaminated sites using rhizobacteria is an exciting area of research, since these organisms can easily and inexpensively be mass produced. Therefore, the molecular engineering of both PGPR and plants with desired genes would help immensely to enhance the efficiency of growth-promoting rhizobacteria mediation or plant-based remediation of contaminated soils and, consequently, could lead to restoration of polluted soils for cultivation.

## 6.6 Conclusion and Future Prospects

Form the above discussion, it is evident that PGPR offer an environmentally sound and sustainable approach to increase soil fertility and, in turn, the crop productivity. In recent times, the understanding of the complex environment of the rhizosphere, functional diversity among PGPR, mechanisms of their action, and, of course, methods of development of the inoculants and their formulation and delivery system has increased considerably. We therefore hope to see new and exciting PGPR products with multiple traits in the commercial markets for ultimate transfer to the agrarian communities. However, there are several limitations to the use of PGPR for commercial use. Chief among them is inconsistent performance of PGPR under field conditions. Researchers, therefore, need to develop the PGPR inoculants with persistent plant growth-promoting activities and should also suggest ways as to how variations in soil type, management practices (e.g., agrochemical use, rotations), and indeed the effect of weather on the efficacy of PGPR could be minimized. With the advancement in the understanding of the mechanisms adopted by PGPR, it will become possible to enhance their capacity to stimulate plant growth by modifying/manipulating promising traits of PGPR by introducing genes responsible for the biosynthesis of desirable metabolites into other microbial communities. Such genetically engineered PGPR endowed with multiple growth-promoting traits could lead to improve colonization and growth-promoting efficiency, and in turn the sustainable plant productivity, while maintaining soil health and reducing the environmental pollution caused by the use of agro-chemicals. Further work focusing on the functional diversity, precise mode of action, and ecophysiology of these agronomically beneficial microbes would assist in unleashing their full promise as potential bio-inoculants for maintaining soil fertility and, consequently, the sustainability of crops in diverse agro-ecosystems.

## References

Abd-Alla MH (1994) Solubilization of rock phosphates by *Rhizobium* and *Bradyrhizobium*. Folia Microbiol 39:53–56

Ahmad F, Ahmad I, Khan MS (2005) Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. Turk J Biol 29:29–34

Ahmad F, Ahmad I, Khan MS (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol Res 163:173–181

Antoun H, Prévost D (2006) Ecology of plant growth promoting rhizobacteria. In: Siddiqui ZA (ed) PGPR: biocontrol and biofertilization. Springer, Heidelberg, pp 1–38

Antoun H, Beauchamp CJ, Goussard N, Chabot R, Llande R (1998) Potential of *Rhizobium* and *Bradyrhizobioum* species as plant growth promoting rhizobacteria on non-legumes: effect on radishes. Plant Soil 204:57–67

Arora NK, Kang SC, Maheshwari DK (2001) Isolation of siderophore producing strains of *Rhizobium meliloti* and their biocontrol potential against *Macrophomina phaseolina* that causes charcoal rot of groundnut. Curr Sci 81:673–677

Arshad M, Frankenberger WT Jr (2002) Ethylene: agricultural sources and applications. Kluwer, New York, p 342

Asea PEA, Kucey RMN, Stewart JWB (1988) Inorganic phosphate solubilization by two *Penicillium* species in solution culture and soil. Soil Biol Biochem 20:459–464

Bai Y, Souleimanov A, Smith DL (2002) An inducible activator produced by *Serratia proteamaculans* strain and its soybean growth promoting activity under greenhouse conditions. J Exp Bot 53:1495–1502

Bano N, Musarrat J (2003) Characterization of a new *Pseudomonas aeruginosa* strain NJ-15 as a potential biocontrol agent. Curr Microbiol 46:324–328

Barazani OZ, Friedman J (1999) Is IAA the major root growth factor secreted from plant growth mediating bacteria. J Chem Ecol 25:2397–2407

Barea JM, Pozo M, Azcón R, Azcón-Aguilar C (2005) Microbial co-operation in the rhizosphere. J Exp Bot 56:1761–1778

Bartel B, Fink GR (1995) Molecular cloning of the gene for indole pyruvate decarboxylase from *Enterobacter cloacae*. Science 268:1745–1748

Bashan Y, de-Bashan LE (2005) Bacteria. In: Hillel D, Hillel D (eds) Encyclopaedia of soils in the environment, vol 1. Elsevier, Oxford, UK, pp 103–115

Belimov AA, Safranova VI, Mimura T (2002) Response of spring rape (*Brassica napus*) to inoculation with PGPR containing ACC-deaminase depends on nutrient status of plant. Can J Microbiol 48:189–199

Belimov AA, Kunakova AM, Safronova VI, Stepanok VV, Yudkin LY, Alekseev YV, Kozhemyakov AP (2004) Employment of rhizobacteria for the inoculation of barley plants cultivated in soil contaminated with lead and cadmium. Microbiology 73:99–106

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick BR (2005) Cadmium-tolerant plant growth-promoting rhizobacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biol Biochem 37:241–250

Berraho EL, Lesueur D, Diem HG, Sasson A (1997) Iron requirement and siderophore production in *Rhizobium ciceri* during growth on an iron-deficient medium. World J Microbiol Biotechnol 13:501–510

Bowen GD, Rovira AD (1999) The rhizosphere and its management to improve plant growth. Adv Agron 66:1–102

Bringhurst RM, Cardon ZG, Gage DJ (2001) Galactosides in the rhizosphere: utilization by *Sinorhizobium meliloti* and development of a biosensor. Proc Natl Acad Sci USA 98:4540–4545

Bruce RJ, West CA (1989) Elicitation of lignin biosynthesis and isoperoxidase activity by pectic fragments in suspension cultures of cluster bean. Plant Physiol 91:889–897

Bultreys A, Gheysen I, Wathelet B, Maraite H, de Hoffman E (2003) High-performance liquid chromatography analyticalyses of pyoverdine siderophores differentiate among phytopathogenic fluorescent *Pseudomonas* species. Appl Environ Microbiol 69:1143–1153

Burd GI, Dixon DG, Glick BR (1998) A plant growth-promoting bacterium that decreases nickel toxicity in seedlings. Appl Environ Microbiol 64:3663–3668

Burd GI, Dixon DG, Glick BR (2000) Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. Can J Microbiol 46:237–245

Çakmakçi R, Dönmez F, Aydm A, Şahin F (2006) Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions. Soil Biol Biochem 38:1482–1487

Canbolat MY, Bilen S, Cakmakci R, Sahin F, Aydin A (2006) Effect of plant growth promoting bacteria and soil compaction on barley seedling growth, nutrient uptake, soil properties and rhizosphere microflora. Biol Fertil Soils 42:350–357

Castric PA (1977) Glycine metabolism by *Pseudomonas aeruginosa*: hydrogen cyanide biosynthesis. J Bacteriol 130:826–831

Chaudri AM, Allain CM, Barbosa-Jefferson VL, Nicholson FA, Chambers BJ, McGrath SP (2000) A study of the impacts of Zn and Cu on two rhizobial species in soils of a long term field experiment. Plant Soil 22:167–179

Clark E, Manulis S, Ophir Y, Barash I, Gafni Y (1993) ILRI, an amidohydrolase that releases active indole-3-acetic acid from conjugates. Phytopathology 83:234–240

Compant S, Reiter B, Sessitsch A, Nowak J, Clément C, Barka EA (2005) Endophytic colonization of *Vitis vinifera* L. by plant growth-promoting bacterium *Burkholderia* sp. strain PsJN. Appl Environ Microbiol 71:1685–1693

Crowley DE, Reidd CPP, Szaniszlo PJ (1987) Mirobial siderophores as iron sources for plants. In: Winkelmann G, Van der Helm D, Neilands JB (eds) Iron transport in animals, plants and microorganisms. VCH Chemie, Weinheim, Germany

Curl EA, Truelove B (1985) The rhizosphere. Springer, Berlin

Davies PJ (1995) Plant hormones. Kluwer, Dorderecht

De Freitas JR, Germida JJ (1991) *Pseudomonas cepacia* and *Pseudomonas putida* as winter wheat inoculants for biocontrol of *Rhizobium solani*. Can J Microbiol 37:780–789

Deshwal VK, Pandey P, Kang SC, Maheshwari DK (2003) Rhizobia as a biological control agent against soil borne plant pathogenic fungi. Ind J Exp Biol 41:1160–1164

Devi K, Nidhi S, Shalini K, David K (2007) Hydrogen cyanide producing rhizobacteria kill subterranean termite *Odontotermes obesus* (Rambur) by cyanide poisoning under *in vitro* conditions. Curr Microbiol 54:74–78

Duhan JS, Dudeja SS, Khurana AL (1998) Siderophore production in relation to $N_2$ fixation and iron uptake in pigeon pea–*Rhizobium* symbiosis. Folia Microbiol 43:421–426

Dutta S, Mishra AK, Dileep Kumar BS (2008) Induction of systemic resistance against fusarial wilt in pigeon pea through interaction of plant growth promoting rhizobacteria and rhizobia. Soil Biol Biochem 40:452–461

Faisal M, Hasnain S (2005) Bacterial Cr (VI) reduction concurrently improves sunflower (*Helianthus annuus* L. ) growth. Biotechnol Lett 27:943–947

Faisal M, Hasnain S (2006) Growth stimulatory effect of *Ochrobactrum intermedium* and *Bacillus cereus* on *Vigna radiata* plants. Lett Appl Microbiol 43:461–466

Faramarzi MA, Brand H (2006) Formation of water-soluble metal cyanide complexes from solid minerals by *Pseudomonas plecoglossicida*. FEMS Microbiol Lett 259:47–52

Fekete FA, Spence JT, Emery T (1983) Siderophore produced by nitrogen-fixing *Azotobacter vinelandii* OP in iron-limited continuous culture. Appl Environ Microbiol 46:1297–1300

Fernando WGD, Nakkeeran S, Zhang Y (2005) Biosynthesis of antibiotics by PGPR and its relation in biocontrol of plant diseases. In: Siddiqui ZA (ed) PGPR: biocontrol and biofertilization. Springer, Dordrecht, The Netherlands, pp 111–142

Flaishman MA, Eyal Z, Zilberstein A, Voisard C, Hass D (1996) Suppression of *Septoria tritici* blotch and leaf rust of wheat by recombinant cyanide-producing strains of *Pseudomonas putida*. Mol Plant Microbe Interact 9:642–645

Frankenberger WT Jr, Arshad M (1995) Phytohormones in soil: microbial production and function. Marcel Dekker, New York

Frossard E, Condron LM, Oberson A, Sena JS, Fardeu JC (2000) Progress governing phosphorus availability in temperate soils. J Environ Qual 29:12–53

Garcia de Salamon IE, Hynes RK, Nelson LM (2001) Cytokinin production by plant growth promoting rhizobacteria and selected mutants. Can J Microbiol 47:404–411

Garland JL (1996) Patterns of potential C source utilization by rhizosphere communities. Soil Biol Biochem 28:223–230

Gaume A (2000) Low P tolerance of various maize cultivars; the contribution of the root exudation. PhD dissertation, Swiss Federal institute of Technology, Zurich, Switzerland

Germida JJ, Siciliano SD, de Freitas JR, Seib AM (1998) Diversity of root-associated bacteria associated with field-grown canola (*Brassica napus* L.) and wheat (*Triticum aestivum*). FEMS Microbiol Ecol 26:43–50

Glick BR (1995) The enhancement of plant growth by free-living bacteria. Can J Microbiol 41:109–117

Glick BR (2001) Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnol Adv 21:383–395

Glick BR (2003) Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnol Adv 21:383–393

Glick BR, Penrose DM, Li J (1998) A model for the lowering of plant ethylene concentration by plant growth promoting bacteria. J Theor Biol 190:63–68

Glick BR, Patten CL, Holguin G, Penrose DM (1999) Biochemical and genetic mechanisms used by plant growth promoting bacteria. Imperial College Press, London

Glick BR, Cheng Z, Czarn YJ, Duan J (2007) Promotion of plant growth by ACC-deaminase producing soil bacteria. Eur J Plant Pathol 119:329–339

Grichko VP, Glick BR (2001) Amelioration of flooding stress by ACC deaminase containing plant growth-promoting bacteria. Plant Physiol Biochem 39:11–17

Grichko VP, Filby B, Glick BR (2000) Increased ability of transgenic plants expressing the bacterial enzyme ACC deaminase to accumulate Cd, Co, Cu, Ni, Pb, and Zn. J Biotechnol 81:45–53

Guo JH, Qi HY, Guo YH, Ge HL, Gong LY, Zhang LX (2004) Biocontrol of tomato wilt by plant growth-promoting rhizobacteria. Biol Control 29:66–72

Gupta A, Meyer JM, Goel R (2002) Development of heavy metal resistant mutants of phosphate solubilizing *Pseudomonas* sp. NBRI4014 and their characterization. Curr Microbiol 45:323–332

Gupta A, Rai V, Bagdwal N, Goel R (2005) *In situ* characterization of mercury resistant growth promoting fluorescent Pseudomonads. Microbiol Res 160:385–388

Inbar J, Chet I (1991) Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soil-borne plant pathogens by bacterium. Soil Biol Biochem 23:974–978

Karadeniz A, Topeuoglu SF, Inan S (2006) Auxin, gibberellin, cytokinin and abscisic acid production in some bacteria. World J Microbiol Biotechnol 22:1061–1064

Karthikeyan M, Jayakumar V, Radhika K, Bhaskaran R, Velazhahan R, Alice D (2005) Induction of resistance in host against the infection of leaf blight pathogen (*Alternaria palandui*) in onion (*Allium cepa* var *aggricatum*). Indian J Biochem Biophys 42:371–377

Karthikeyan M, Radhika K, Mathiyazhagan S, Bhaskaran R, Samiyappan R, Velazhahan R (2006) Induction of phenolics and defense-related enzymes in coconut (*Cocos nucifera* L.) roots treated with biocontrol agents. Brazil J Plant Physiol 18:367–377

Khalid A, Arshad M, Zahir ZA (2004) Screening of plant growth promoting rhizobacteria for improving growth and yield of wheat. J Appl Microbiol 96:473–480

Khan AG (2005) Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. J Trace Elem Med Biol 18:355–364

Khan MS, Zaidi A (2007) Synergicstic effects of the inoculation with plant growth promoting rhizobacteria and an arbuscular mycorrhizal fungus on the performance of wheat. Turk J Agric For 31:355–362

Khan MS, Zaidi A, Aamil M (2002) Biocontrol of fungal pathogens by the use of plant growth promoting rhizobacteria and nitrogen fixing microorganisms. Indian J Bot Soc 81:255–263

Khan MS, Zaidi A, Wani PA (2007) Role of phosphate solubilizing microorganisms in sustainable agriculture-A review. Agron Sustain Dev 27:29–43

Kloepper JW, Schroth MN (1978) Plant growth-promoting rhizobacteria on radishes. Fourth International Conference on Plant Pathogen Bacteria, Angers, France, vol 2, pp 879–882

Knowles CJ, Bunch AW (1986) Microbial cyanide metabolism. Adv Microb Physiol 27:73–111

Koga J, Adachi T, Hidaka H (1991) The cloning and characterization of iaaM and iaaH from *Erwinia herbicola* pathovar *gypsophilae*. Mol Gen Genet 226:10–16

Lambert B, Joos H, Dierick S, Vantomme R, Swings J, Kerters K, Van Montagu M (1990) Identification and plant interaction of *Phyllobacterium* sp., a predominant rhizobacterium of young sugar beat. Appl Environ Microbiol 56:1093–1102

Leeman M, Den OFM, Van PJA, Dirkx FPM, Steijl H, Bakker PAHM, Schippers B (1996) Iron availability affects induction of systemic resistance to *Fusarium* wilt of radish by *Pseudomonas fluorescens*. Phytopathology 86:149–155

Lesueur D, Diem HG, Meyer JM (1993) Iron requirement and siderophore production in *Bradyrhizobium* strains isolated from *Acacia mangium*. J Appl Bacteriol 74:675–682

Lindsay WL, Vlek PLG, Chien SH (1989) In: Dixon JB, Weed SB (eds) Phosphate minerals, soil environment, 2nd edn. Soil Science Society of America, Madison, pp 1089–1130

Lodewyckx CJ, Vangronsveld F, Porteous ERB, Moore ST, Mezgeay M, van der Lelie D (2002) Endophytic bacteria and their potential applications. Crit Rev Plant Sci 21:583–606

Lucas García JA, Domenech J, Santamaría C, Camacho M, Daza A, Gutierrez Mañero FJ (2004) Growth of forest plants (pine and holm-oak) inoculated with rhizobacteria: relationship with microbial community structure and biological activity of its rhizosphere. Environ Exp Bot 52:239–251

Madhaiyan M, Poonguzhali S, Ryu JH, Sa T (2006) Regulation of ethylene levels in canola (*Brassica campestris*) by 1-aminocyclopropane-1-carbxylate deaminase containing *Methylobacterium fujisawaense*. Planta 224:268–278

Madhaiyan M, Poonguzhali S, Sa T (2007) Metal tolerating methylotrophic bacteria reduces nickel and cadmium toxicity and promotes plant growth of tomato (*Lycopersicon esculentum* L.). Chemosphere 69:220–228

Mahaffee WF, Kloepper JW (1997) Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with fieldgrown cucumber (*Cucumis sativus* L.). Microb Ecol 34:210–223

Mahmoud SAZ, Ramadan EM, Thabet FM, Khater T (1984) Production of plant growth promoting substances by rhizosphere microorganisms. Zbl Mikrobiol 139:227–232

Maliha R, Samina K, Najma A, Sadia A, Farooq L (2004) Organic acid production and phosphate solubilisation by phosphate solubilising microorganisms under *in vitro* conditions. Pak J Biol Sci 7:187–196

Mayak S, Tirosh S, Glick BR (2004a) Plant growth promoting bacteria that confer resistance to water stress in tomatoes and peppers. Plant Physiol 166:525–530

Mayak S, Tirosh T, Glick BR (2004b) Plant growth promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiol Biochem 42:565–572

Mayak S, Tirosh T, Glick BR (2004c) Plant growth promoting bacteria that confer resistance to water stress in tomato and peppers. J Plant Sci 166:525–530

Bakker PAHM, Corné MJP L, van Loon LC (2007) Induced systemic resistance by fluorescent *Pseudomonas* spp. Phytopathology 97:239–243

Mellado JC, Onofre-Lemus J, Santos PE, Martinez-Aguilar L (2007) The tomato rhizosphere, an environment rich in nitrogen fixing *Burkholderia* species with capabilities of interest for agriculture and bioremediation. Appl Environ Microbiol 73:5308–5319

Meyer JM, Stintzi A, De Vos D, Cornelis P, Tappe R, Taraz K, Budzikiewicz H (1997) Use of siderohpores to type pseudomonads, three *Pseudomonas aereuginosa* pyoverdine systems. Microbiology 143:35–43

Nelson EB (1998) Biological control of *Pythium* seed rot and preemergence damping-off of cotton with *Enterobacter cloacae* and *Ervinis herbicola* applied as seed treatments. Plant Dis 72:140–142

Nie L, Shah S, Dixon BGI, DG GBR (2002) Phytoremediation of arsenate contaminated soil by transgenic canola and the plant growth-promoting bacterium *Enterobacter cloacae* CAL2. Plant Physiol Biochem 40:355–361

Nielsen TH, Sorensen J (2003) Production of cyclic lipopeptides by *Pseudomonas fluorescens* strains in bulk soil and in the sugarbeet rhizosphere. Appl Environ Microbiol 69:861–868

Noordman WH, Reissbrodt R, Bongers RS, Rademaker ILW, Bockelmann W, Smit G (2006) Growth stimulation of *Brevibacterium* sp. by siderophores. J Appl Microbiol 101:637–646

O' Hara GW, Dilworth MI, Boonkerd N, Parkpian P (1988) Iron deficiency specifically limits nodule development in pea nut inoculated with *Bradyrhizobium* sp. New Phytol 108:51–57

Omar SA (1998) The role of rock phosphate solubilizing fungi and vesicular arbuscular mycorrhiza (VAM) in growth of wheat plants fertilized with rock phosphate. World J Microbiol Biotechnol 14:211–219

Ownley BH, Duffy BK, Weller DM (2003) Identification and manipulation of soil properties to improve the biological control performance of phenazine-producing *Pseudomonas fluorescens*. Appl Environ Microbiol 69:3333–3343

Pandey P, Kang SC, Gupta CP, Maheshwari DK (2005) Rhizosphere competent *Pseudomonas aeruginosa* GRC1 produces characteristic siderophore and enhances growth of Indian mustard (*Brassica campestris*). Curr Microbiol 51:303–309

Pandey A, Trivedi P, Kumar B, Palni LMS (2006) Characterization of a phosphate solubilizing and antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-alpine location in the Indian Central Himalaya. Curr Microbiol 53:102–107

Penrose DM, Glick BR (2001) Levels of 1-aminocyclopropane-1-carboxylic acid (ACC) in exudates and extracts of canola seeds treated with plant growth promoting bacteria. Can J Microbiol 47:368–372

Persello-Cartieaux F, Nussaume L, Robaglia C (2003) Tales from the underground: molecular plant-rhizobacteria interactions. Plant Cell Environ 26:189–199

Perveen S, Khan MS, Zaidi A (2002) Effect of rhizospheric microorganisms on growth and yield of greengram (*Phaseolus radiatus*). Ind J Agric Sci 72:421–423

Ponmurugan P, Gopi C (2006) *In vitro* production of growth regulators and phosphatase activity by phosphate solubilizing bacteria. Afr J Biotechnol 5:340–350

Pradhan N, Sukla LB (2005) Solubilization of inorganic phosphate by fungi isolated from agriculture soil. Afr J Biotechnol 5:850–854

Prithiviraj B, Zhou X, Souleimanov A, Kahn W, Smith DL (2003) A host-specific bacteria to-plant signal molecule (Nod factor) enhances germination and early growth of diverse crop plants. Planta 216:437–445

Rajkumar M, Nagendran R, Kui Jae L, Wang Hyu L, Sung Zoo K (2006) Influence of plant growth promoting bacteria and Cr (vi) on the growth of Indian mustard. Chemosphere 62:741–748

Reed MLE, Warner B, Glick BR (2005) Plant growth-promoting bacteria facilitate the growth of the common reed *Phragmites australis* in the presence of copper or polycyclic aromatic hydrocarbons. Curr Microbiol 51:425–429

Renwick A, Campbell R, Coe S (1991) Assessment of *in vivo* screening systems for potential biocontrol agents of *Gaeumannomyces graminis*. Plant Pathol 40:524–532

Robinson RL, Postgate JR (1980) Oxygen and nitrogen in biological nitrogen fixation. Annu Rev Microbiol 34:182–207

Robinson B, Russell C, Hedley CB (2001) Cadmium adsorption by rhizobacteria: implications for New Zealand pastureland. Agric Ecosyst Environ 87:315–321

Rondon MR, Goodman RM, Handelsman J (1999) The earth's bounty: assessing and accessing soil microbial diversity. Trends Biotechnol 17:403–409

Rosenblueth M, Martínez-Romero E (2006) Bacterial endophytes and their interactions with hosts. Mol Plant Microbe Interact 19:827–837

Ryder MH, Jones DA (1990) Biological control of crown gall. In: Hornby D, Cook RJ, Henis Y (eds) Biological control of soil- borne plant pathogens. CAB International, Oxford, UK, pp 45–63

Ryder MH, Yan Z, Terrace TE, Rovira AD, Tang W, Correll RL (1999) Use of strains of *Bacillus* isolated in China to suppress take-all and rhizoctonia root rot, and promote seedling growth of glasshouse grown wheat in Australian soils. Soil Biol Biochem 31:19–29

Safronova VI, Stepanok VV, Engqvist GL, Alekseyev YV, Belimov AA (2006) Root associated bacteria containing1-aminocyclopropane-1-carboxylate deaminase improve growth and nutrient uptake by pea genotypes cultivated in cadmium supplemented soil. Biol Fertil Soils 42:267–272

Saravanakumar D, Vijayakumar C, Kumar N, Samiyappan R (2007) PGPR-induced defense responses in the tea plant against blister blight disease. Crop Prot 26:556–565

Selvakumar G, Kundu S, Joshi P, Nazim S, Gupta AD, Mishra PK, Gupta HS (2008) Characterization of a cold-tolerant plant growth-promoting bacterium *Pantoea dispersa* 1A isolated from a sub-alpine soil in the North Western Indian Himalayas. World J Microbiol Biotechnol 24:955–960

Shaharoona B, Arshad M, Zahir ZA (2006) Effect of plant growth promoting rhizobacteria containing ACC-deaminase on maize (*Zea mays* L.) growth under axenic conditions and on nodulation in mung bean. Lett Appl Microbiol 42:155–159

Shanahan P, O'Sullivan DJ, Simpson P, Glennon JD, O'Gara F (1992) Isolation of 2, 4-Diacetylphloroglucinol from a fluorescent pseudomonad and investigation of physiological parameters influencing its production. Appl Environ Microbiol 58:353–358

Sharma A, Johri BN, Sharma AK, Glick BR (2003) Plant growth-promoting bacterium *Pseudomonas* sp. strain GRP3 influences iron acquisition in mung bean (*Vigna radiata* L. Wilzeck). Soil Biol Biochem 35:887–894

Sheng XF, Xia JJ (2006) Improvement of rape (*Brassica napus*) plant growth and cadmium uptake by cadmium-resistant bacteria. Chemosphere 64:1036–1042

Shiomi HF, Silva HAS, de Melo IS, Nunes FV, Bettiol W (2006) Bioprospecting endophytic bacteria for biological control of coffee leaf rust. Sci Agric 63:32–39

Sikora RA (1992) Management of the antagonistic potential in agricultural ecosystems for the biological control of plant parasitic nematodes. Annu Rev Phytopathol 30:245–270

Somers E, Vanderleyden J, Srinivasan M (2004) Rhizosphere bacterial signalling: a love parade beneath our feet. Crit Rev Microbiol 30:205–240

Sridevi M, Mallaiah KV, Yadav NCS (2007) Phosphate solubilization by *Rhizobium* isolates from *Crotalaria* species. J Plant Sci 2:635–639

Sridevi M, Yadav NCS, Mallaiah KV (2008a) Production of Indole-acetic-acid by *Rhizobium* isolates from *Crotalaria* species. Res J Microbiol 3:276–281

Sridevi M, Kumar KG, Mallaiah KV (2008b) Production of catechol-type of siderophores by *Rhizobium* sp. isolated from stem nodules of *Sesbania procumbens* (Roxb.) W and A. Res J Microbiol 3:282–287

Steddom K, Menge JA, Crowley D, Borneman J (2002) Effect of repetititve applications of the biocontrol bacterium *Pseudomonas putida* 06909-rif/nal on citrus soil microbial communities. Phytopathology 92:857–862

Stintzi A, Barnes C, Xu J, Raymond KN (2000) Microbial iron transport via a siderophore shuttle: a membrane ion transport paradigm. Proc Natl Acad Sci USA 97:10691–10696

Sturz AV, Christie BR (2003) Beneficial microbial allelopathies in the root zone: the management of soil quality and plant disease with rhizobacteria. Soil Tillage Res 72:107–123

Sturz A, Kimpinski J (2004) Endo-root bacteria derived from marigolds (*Tagetes* spp.) can decrease soil population densities of root lesion nematodes in the potato root zone. Plant Soil 262:241–249

Tank N, Saraf M (2003) Phosphate solubilization, exopolysaccharide production and indole acetic acid secretion by rhizobacteria isolated from *Trigonella graecum*. Indian J Microbiol 43:37–40

Tannii A, Takeuchi T, Horita H (1990) Biological control of scab, black scruff and soft rot of potato by seed bacterization. In: Hornby D, Cook RJ, Henis Y (eds) Biological control of soil-borne plant pathogens. CAB International, Oxford UK, pp 143–146

Thomashow LS, Weller DM (1988) Role of a phenazine antibiotic from *Pseudomonas fluorescens* in biological control of *Gaeumannomyces graminis* var. *tritici*. J Bacteriol 170:3499–3508

Thomashow LS, Bonsal RF, Weller DM (2003) Detection of antibiotics production by soil and rhizosphere microbes *in situ*. Thomashow Lab Methods:1–13

Thomshaw LS, Webler DM (1995) Current concepts in the use of introduced bacteria for biological disease control of *Gaeumamomyces graminis* var. *tritici*. J Bacteriol 170:3499–3508

Tripathi M, Munot HP, Shouche Y, Meyer JM, Goel R (2005) Isolation and functional characterization of siderophore producing lead and cadmium resistant *Pseudomonas putida* KNP9. Curr Microbiol 50:233–237

Trivedi P, Pandey A, Palni LMS (2008) *In vitro* evaluation of antagonistic properties of *Pseudomonas corrugata*. Microbiol Res 163:329–336

Tsavkelova EA, Cherdyntseva TA, Netrusov AI (2005) Auxin production by bacteria associated with orchid roots. Microbiology 74:46–53

Van Loon LC, Bakker PAHM, Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36:453–483

Verma A, Kukreja K, Pathak DV, Suneja S, Narula N (2001) *In vitro* production of plant growth regulators (PGRs) by *Azorobacter chroococcum*. Indian J Microbiol 41:305–307

Vining LC (1990) Functions of secondary metabolites. Annu Rev Microbiol 44:395–427

Vivas A, Biro B, Ruiz-Lozano JM, Barea JM, Azcon R (2006) Two bacterial strains isolated from a Zn-polluted soil enhance plant growth and mycorrhizal efficiency under Zn toxicity. Chemosphere 52:1523–1533

Voisard C, Keel C, Haas D, Defago G (1989) Cyanide production by *Pseudomonas fluorescens* helps suppress black root of tobacco under gnotobiotic conditions. EMBO J 8:351–358

Wang C, Knill E, Glick BR, Defago G (2000) Effect of transferring 1-aminocyclopropane-l-carboxylic acid (ACC) deaminase genes into *Pseudomonas fluorescens* strain CHAO and its gac A derivative CHA96 on their growth promoting and disease-suppressive capacities. Can J Microbiol 46:898–907

Wani PA, Khan MS, Zaidi A (2007a) Synergistic effects of the inoculation with nitrogen fixing and phosphate-solubilizing rhizobacteria on the performance of field grown chickpea. J Plant Nutr Soil Sci 170:283–287

Wani PA, Khan MS, Zaidi A (2007b) Chromium reduction, plant growth promoting potentials and metal solubilization by *Bacillus* sp. isolated from alluvial soil. Curr Microbiol 54:237–243

Wani PA, Khan MS, Zaidi A (2007c) Co inoculation of nitrogen fixing and phosphate solubilizing bacteria to promote growth, yield and nutrient uptake in chickpea. Acta Agron Hung 55:315–323

Wani PA, Khan MS, Zaidi A (2008a) Chromium reducing and plant growth promoting *Mesorhizobium* improves chickpea growth in chromium amended soil. Biotechnol Lett 30:159–163

Wani PA, Khan MS, Zaidi A (2008b) Effect of metal tolerant plant growth promoting *Rhizobium* on the performance of pea grown in metal amended soil. Arch Environ Contam Toxicol 55: 33–42

Wani PA, Khan MS, Zaidi A (2008c) Impact of zinc-tolerant plant growth promoting rhizobacteria on lentil grown in zinc-amended soil. Agron Sustain Dev 28:449–455

Wittenberg JB, Wittenberg BA, Day DA, Udvardi MK, Appleby CA (1996) Siderophore bound iron in the peribacteroid space of soybean root nodules. Plant Soil 178:161–169

Witter AE, Luther WG (1998) Variation in Fe-organic complexation with depth in the Northwestern Atlantic Ocean as determined using a kinetic approach. Marine Chem 62:241–258

Wu CH, Wood TK, Mulchandani A, chen W (2006a) Engineering plant microbe symbioses for rhizoremediation of heavy metals. Appl Environ microbial 72:1129–1134

Yasmin S, Rahman M, Hafeez FY (2004) Isolation, characterization and beneficial effects of rice associated plant growth promoting bacteria from Zanzibar soils. J Basic Microbiol 44:241–252

Yuen GY, Schroth MN, McCain AH (1985) Reduction of *Fusarium* wilt of carnation with suppressive soils and antagonistic bacteria. Plant Dis 69:1071–1075

Zahir ZA, Munir A, Asghar HN, Shaharoona B, Arshad M (2008) Effectiveness of rhizobacteria containing ACC deaminase for growth promotion of peas (*Pisum sativum*) under drought conditions. J Microb Biotechnol 18:958–963

Zaidi A (1999) Synergistic interactions of nitrogen fixing microorganisms with phosphate mobilizing microorganisms. PhD Thesis, Aligarh Muslim University, Aligarh

Zaidi A, Khan MS (2006) Co-inoculation effects of phosphate solubilizing microorganisms and *Glomus fasciculatum* on greengram-*Bradyrhizobium* symbiosis. Turk J Agric For 30:223–230

Zaidi A, Khan MS, Amil M (2003) Interactive effect of rhizotrophic microorganisms on yield and nutrient uptake of chickpea (*Cicer arietinum* L.). Eur J Agron 19:15–21

Zaidi S, Usmani S, Singh BR, Musarrat J (2006) Significance of *Bacillus subtilis* strain SJ 101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. Chemosphere 64:991–997

Zdor RE, Anderson AJ (1992) Influence of root colonizing bacteria on the defense responses in bean. Plant Soil 140:99–107

Zehnder G, Klopper J, Yao C, Wei G (1997) Induction of systemic resistance in cucumber against cucumber beetles (Coleoptera Chrysomelidae) by plant growth promoting rhizobacteria. J Econ Entomol 90:391–396

Zhang F, Dhasti N, Hynes R, Smith DL (1996) Plant growth promoting rhizobacteria and soybean [*Glycine max* (L.) Merr.] nodulation and nitrogen fixation at sub-optimal root zone temperatures. Ann Bot 77:453–459

# Chapter 7
# Plant Growth Promoting Rhizobacteria and Sustainable Agriculture

**Azeem Khalid, Muhammad Arshad, Baby Shaharoona, and Tariq Mahmood**

**Abstract** The diverse groups of bacteria in close association with roots and capable of stimulating plant growth by any mechanism(s) of action are referred to as plant growth-promoting rhizobacteria (PGPR). They affect plant growth and development directly or indirectly either by releasing plant growth regulators (PGRs) or other biologically active substances, altering endogenous levels of PGRs, enhancing availability and uptake of nutrients through fixation and mobilization, reducing harmful effects of pathogenic microorganisms on plants and/or by employing multiple mechanisms of action. Recently, PGPR have received more attention for use as a biofertilizer for the sustainability of agro-ecosystems. Selection of efficient PGPR strains based on well-defined mechanism(s) for the formulation of biofertilizers is vital for achieving consistent and reproducible results under field conditions. Numerous studies have suggested that PGPR-based biofertilizers could be used as effective supplements to chemical fertilizers to promote crop yields on sustainable basis. Various aspects of PGPR biotechnology are reviewed and discussed.

## 7.1 Introduction

Sustainability in agricultural systems without compromising the environmental quality and conservation is one of the major concerns of today's world. The excessive use of agro-chemicals (fertilizers and pesticides) is posing serious threats

M. Arshad (✉) and B. Shaharoona
Institute of Soil and Environmental Sciences, University of Agriculture, Faisalabad 38040, Pakistan
e-mail: arshad_ises@yahoo.com; shaha_ss@yahoo.com
A. Khalid and T. Mahmood
Department of Environmental Sciences, PMAS Arid Agriculture University, Rawalpindi 46300, Pakistan
e-mail: azeemuaf@yahoo.com; qiratm@yahoo.com

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_7, 

to the environment. The gradual reduction in the use of chemicals in agriculture without affecting yield or quality of the crop produce can only be possible with a new generation of technologies. Furthermore, the long-term sustainability of agricultural systems depends most likely on effective handling of the internal/indigenous resources of agro-ecosystems. During the last couple of decades, the new biotechnologies have opened new vistas for enhancement of agriculture productivity in a sustainable manner. Advances in understanding of soil microbiology and biotechnology have made possible exploitation of soil microorganisms for improving crop productivity and, in turn, have offered an economically attractive and ecologically viable supplement to reduce external inputs to some extent.

The plant rhizosphere is a remarkable ecological environment as myriad microorganisms colonize in, on, and around the roots of growing plants. Distinct communities of beneficial soil microorganisms are associated with the root systems of all higher plants (Khalid et al. 2006). These rhizobacteria are considered as efficient microbial competitors in the root zone, and the net effect of plant–microbe associations on plant growth could be positive, neutral, or negative. Such bacteria inhabiting plant roots and influencing plant growth positively are often referred to as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al. 1986; Arshad and Frankenberger 1998; Zahir et al. 2004). These bacteria, after inoculation, rapidly colonize onto seeds and roots in response to exudation and, thus, affect plant growth.

A complex matrix of organic and inorganic constituents of soil, particularly rhizosphere, creates a unique and dynamic environment for the microorganisms which affect plants and other associative microorganisms. Many microorganisms are highly dependent for their survival on preformed substrates exuded by plant roots (Frankenberger and Arshad 1995; Glick et al. 1998; Khalid et al. 2006). In turn, the soil microflora inhabiting the rhizosphere can cause dramatic changes in plant growth and development by producing plant growth regulators (PGRs) or biologically active substances, or by altering endogenous levels of PGRs, and/or by facilitating the supply and uptake of nutrients and providing other benefits. A diverse array of bacteria, including species of *Rhizobium*, *Bradyrhizobium, Pseudomonas*, *Azospirillum*, *Azotobacter*, *Bacillus, Klebsiella*, *Enterobacter*, *Xanthomonas*, *Serratia* and many others, have been shown to facilitate plant growth by various mechanisms.

Over the years, the PGPR have received worldwide importance and acceptance for agricultural benefits. These microorganisms are the potential tools for sustainable agriculture because they not only ensure the availability of essential nutrients to plants but also enhance the nutrient use efficiency. Several authors have reported significant increases in growth and yield of agricultural crops in response to microbial (PGPR) inoculants both under greenhouse and field conditions (Kennedy et al., 2004; Khalid et al. 2004a, 2006; Gravel et al. 2007; Kumar et al. 2007; Zhuang et al. 2007; Arshad et al. 2008; Banchio et al. 2008; Contesto et al. 2008; Figueiredo et al. 2008; Mubeen et al. 2008; Naiman et al. 2009). Beside promoting plant growth, PGPR also enhance efficiency of fertilizers, mitigate abiotic stresses, manage plant pathogens, and cause the degradation of xenobiotic compounds (Glick 2003, 2004; Huang et al. 2004; Khan 2005; Arshad et al. 2007; Saleem

et al. 2007; Zhuang et al. 2007; Ahmad et al. 2008; Amor et al., 2008; Dell'Amico et al. 2008; Lebeau et al., 2008; Masoud and Abbas 2009; Kohler et al. 2009).

Many promising microorganisms have been isolated and marketed as biofertilizers; however, their effects on crop yields fluctuate from crop to crop, place to place, and from season to season, depending on the survival of the introduced microorganisms on seed, roots, and in soil (Poi and Kabi 1979; Chanway and Holl 1992; Nowak 1998; Khalid et al. 2004a; Hafeez et al. 2006). To make effective utilization of microbial inoculants, accurate and reliable methods for monitoring the fate of applied PGPR in the rhizosphere/rhizoplane are required to enhance their efficacy under field conditions. Scientists have used multidisciplinary approaches to understand the adaptation of PGPR to the rhizosphere, including their mechanisms of action and root colonization, production of determinants, and biodiversity, etc. They are trying to manipulate the rhizosphere so that PGPR perform better and help to increase food production for mankind on a sustainable basis. To achieve this successfully, we need to know the players and to understand their interactions with each other and with the growth substrate, in addition to abiotic factors which otherwise have drastic effects on PGPR as well as on plant growth under diverse field conditions. In this chapter, the potential mechanisms of action of PGPR, reasons for inconsistency in their performance, and formulation of effective biofertilizers to be used as the supplement to chemical fertilizers, are critically reviewed and discussed.

## 7.2 Mechanisms of Action

PGPR affect growth and development of plants by direct or indirect mechanisms (Table 7.1). The direct mechanisms include $N_2$-fixation, mobilization of nutrients via production of phosphatases, siderophores, or organic acids, and production of phytohormones and enzymes (Lucy et al. 2004; Khalid et al. 2004a, b; Gray and Smith 2005; Çakmakçi et al. 2006; Tsavkelova et al. 2007). Indirectly, the bacteria may exert a positive influence on plant growth by lessening certain deleterious effects of a pathogenic organism by inducing host resistance to the pathogen or by knocking out the pathogen from root surfaces or by producing chitinases or other pathogen-suppressing substances (Raj et al. 2003; Guo et al. 2004; Van Loon and Glick 2004; Van Loon 2007). Although scientists have reported both direct and indirect methods of growth stimulation by PGPR, but there is no clear separation between these two mechanisms. Certain bacteria posses multiple traits to affect plant growth where one trait may dominate the other one (Shaharoona et al. 2006a, Shaharoona et al. 2008; Hafeez et al. 2006). A bacterium influencing plant growth by releasing PGRs can also play a role in controlling plant pathogens and diseases, and vice versa. So, plant response to PGPR is a complex phenomenon, and recent advances in research at the molecular level have provided a sufficient basis to understand these mechanisms more precisely. The major mechanisms of PGPR action involved in the improvement of plant growth and development are discussed in the following sections.

**Table 7.1** Possible mechanism(s) of action of PGPR for plant growth promotion

| Plants | PGPR | Suggested mechanism(s) of action | References |
|---|---|---|---|
| *Lactuca sativa* L. cv. Tafalla | *Pseudomonas mendocina* Palleroni | ACC deaminase activity | Kohler et al. (2009) |
| *Oryza sativa* | *Methylobacterium* sp. strain NPFM-SB3 | Indole-3-acetic acid, cytokinins | Senthilkumar et al. (2009) |
| *Solanum tuberosum* | *Bacillus* sp. | Auxins | Ahmed and Hasnain (2008) |
| *Arabidopsis thaliana* | *Phyllobacterium brassicacearum* STM196, *Pseudomonas putida* UW4, *Rhizobium leguminosarum* bv. *viciae* 128C53K, *Mesorhizobium loti* MAFF303099 | ACC deaminase activity | Contesto et al. (2008) |
| *Phaseolus vulgaris* L. | *Rhizobiumtropici* (CIAT899), *Paenibacillus polymyxa* (DSM 36), *Rhizobium*, *P. polymyxa* strain Loutit (L), *Bacillus* sp. | Indole acetic acid, cytokinin, $N_2$-fixation | Figueiredo et al. (2008) |
| *Triticum aestivum* L. | *Pseudomonas* spp., *Burkholderia caryophylli* | ACC deaminase activity, chitinase | Shaharoona et al. (2007b, 2008) |
| *Cicer arietinum* L. | *Serratia oderifera* (J118), *Pantoea dispersa* (J112) and *Enterobactor gergoviae* (J107) | ACC deaminase, P-solubilization | Shahzad et al (2008) |
| *Malus domestica* Borkh | PGPR strains (OSU-142, OSU-7, BA-8 and M-3) | Indole acetic acid, cytokinin | Aslantas et al. (2007) |
| *Brassica rapa* | *Pseudomonas putida* UW4 | ACC deaminase activity | Cheng et al. (2007) |
| *Lycopersicon esculentum* | *Pseudomonas fluorescens*, *P. fluorescens* subgroup G strain 2, *P. marginalis*, *P. putida* subgroup B strain 1 and *P. syringae* strain1 | Indole acetic acid | Gravel et al. (2007) |
| *Apios americana* | *Pseudomonas fluorescens* TDK1 | ACC deaminase activity | Saravanakumar and Samiyappan (2007) |
| *Pisum sativum* | *Pseudomonas putida* biotype A, A7' *Acinetobacter calcoaceticus*, M9, *P. fluorescens*, AM3 | ACC deaminase, ethylene | Shaharoona et al. (2007a) |
| *Triticum aestivum* L. | *Bacillus pumilus* | Auxin, siderophore | Hafeez et al. (2006) |
| *Rubus niveus* | *Bacillus* sp. | $N_2$-fixation, P-solubilization | Orhan et al. (2006) |
| *Zea mays* L., *Vigna radiata* | *Pseudomonas* spp. | ACC deaminase activity, chitinase activity | Shaharoona et al. (2006a, b) |

(*continued*)

**Table 7.1** (continued)

| Plants | PGPR | Suggested mechanism(s) of action | References |
|---|---|---|---|
| *Zea mays* L. | *Azotobacter chroococcum, Bacillus megaterium,* Bacillus mucilaginous | $N_2$-fixation, P-solubilization, K-solubilization | Wu et al. (2005) |
| *Triticum aestivum* L. | PGPR | Indole-3-acetic acid | Khalid et al. (2004a, b) |
| *Lycopersicon esculentum, Capsicum annuum* | PGPR | Auxins | Garci'a et al. (2003) |
| *Brassica rapa, Vigna radiata* | *P. putida* GR12-2 and an IAA-deficient mutant | Indole-3-acetic acid | Patten and Glick (2002) |
| *Hordeum vulgare* | *Arthrobacter mysorens 7, Flavobacterium sp. L30, Klebsiella mobilis CIAM880* | Indole-3-acetic acid, ethylene | Pishchik et al. (2002) |
| *Pinus pinea* | *Bacillus* spp. | Gibberellins | Probanza et al. (2002) |
| *Oryza sativa* L. | *Rhizobium, Azospirillum* | Indole-3-acetic acid | Biswas et al. (2000) |
| *Oryza sativa* L. | *Rhizobium leguminosarum* (strain E11) | Indole-3-acetic acid | Dazzo et al. (2000) |
| *Vigna radiata* | *P. putida* GR 12-2 (wild type), GR12-2/acd36 (ACC deaminase minus mutant), GR 12-2/aux1 (IAA over producers) | Indole-3-acetic acid, ethylene | Mayak et al. (1999) |
| *Vigna radiata* | *Pseudomonas* sp. | Biocontrol | Sindhu et al. (1999) |
| – | *Paenibacillus polymyxa* | cytokinins | Timmusk et al. (1999) |
| *Cucumis sativus* | *P. putida, Serratia marcescens* | Biocontrol | Wei et al. (1996) |

### 7.2.1 Fixation, Mobilization and Uptake of Nutrients

Nutrients are one of the extremely important factors which influence growth, yield, and quality of different crops. Soil microorganisms can provide nutrients to plants either through the fixation of atmospheric $N_2$ or by enhancing nutrient mobilization/uptake through their biological activities, such as mineralization and through siderophore, organic acid and phosphatase production, etc.

Biological $N_2$ fixation by rhizobia and associative diazotrophic bacteria is a spontaneous process and one of the widely studied mechanisms by which plants benefit from the interacting partners. The bacteria benefit the plants by fixing $N_2$ in exchange for fixed carbon either provided directly to the bacteria or indirectly by

releasing carbon as root exudates. A range of bacteria participates in interactions with different plants, significantly increasing their vegetative growth and grain yields. However, obtaining maximum benefits on farms from diazotroph PGPR biofertilizer requires a systematic strategy designed to fully utilize all these beneficial factors, allowing crop yields to be maintained or even increased while fertilizer applications are reduced (Kennedy et al. 2004). Numerous studies have shown that different species of bacteria fix atmospheric $N_2$ and consequently affect growth and yield of various crops (Sindhu et al. 2002; Bai et al. 2003; Orhan et al. 2006; Afzal and Bano 2008; Khalequzaman and Hossain 2008; Figueiredo et al. 2008). In addition to biological $N_2$-fixation, PGPR are also known to affect the nutrient availability to the plant through acidification and redox changes or by producing iron chelators and siderophores, and/or mobilizing the metal phosphates (Burd et al. 2000; Römkens et al. 2002; Abou-Shanab et al. 2003). Several reports have suggested that PGPR can stimulate plant growth through their P-solubilizing activity (Khan et al. 2007; Wani et al. 2007; Afzal and Bano 2008). Furthermore, Wu et al. (2005) reported increased assimilation of nutrients, such as N, P, and K, in plants, in response to inoculation with P-solubilizer (*Bacillus megaterium*) and K-solubilizer (*Bacillus mucilaginous*). Likewise, Orhan et al. (2006) reported that inoculation with a phosphate-solubilizing *Bacillus* strain M3 significantly improved P, Fe, and Mn contents of the leaves of raspberry (*Rubus idaeus*), suggesting that *Bacillus* M3 alone or in combination with some other strains had the potential to increase the nutrition of raspberry plants, in addition to growth and yield.

### *7.2.2 Production of Plant Growth-Regulating Substances*

Plant growth-regulating substances are naturally occurring organic compounds that influence various physiological processes in plants, such as cell elongation and cell division. They perform these functions at concentrations far below the levels at which nutrients and vitamins normally affect plant processes. It is now well established that the majority of soil microorganisms can produce plant growth-regulating substances, including phytohormones (auxins, gibberellins, cytokinins, ethylene, and abscisic acid) and enzymes (Frankenberger and Arshad 1995; Glick 1995; Khalid et al. 2006), which is considered as one of the major mechanisms of plant growth promotion by PGPR. Production of PGRs by microorganisms is affected by the presence of suitable substrate(s)/precursor(s) as well as type and concentration of exudates. The inocula in the presence of a specific physiological precursor of a PGR and/or inocula that produce physiologically-active concentrations of a phytohormone can be highly effective in promoting plant growth and enhancing consistency and reproducibility (Frankenberger and Arshad 1995; Arshad and Frankenberger 1998, 2002).

Microflora capable of producing PGRs in vitro predominates in the rhizosphere of plants. However, the type and amount of growth-regulating substances released by such microorganisms are variable. The quantitative or qualitative variations in

plant growth-promoting substances in turn lead to the differences in plant responses to the PGPR inocula. Several studies have reported the ability of various PGPR to produce auxins in vitro and in vivo (Almonacid et al. 2000; Khalid et al. 2004a, b; Gravel et al. 2007). Similarly, plant-associated phototrophic purple bacterium (Serdyuk et al. 1995) and *Methylobacterium* sp. (Senthilkumar et al. 2009) have been reported to be capable of producing cytokinins in vitro. Different bacterial species such as *Proteus mirabilis*, *P. vulgaris*, *Klebsiella pneumoniae*, *Bacillus megaterium*, *B. cereus*, *Escherichia coli* and many more have been reported to synthesize plant growth-promoting substances, including auxin, gibberellin, cytokinin and abscisic acid (Tuomi and Rosenquist 1995; Karadeniz et al. 2006; Tsavkelova et al. 2007). Soil microbiota is also known to produce the gaseous phytohormone ethylene in vitro and in vivo (Weingart et al. 1999; Akhtar et al. 2005).

Some PGPR can influence plant growth by altering the synthesis of endogenous phytohormones through the production of specific enzymes. Among these enzymes, bacterial 1-aminocyclopropane-1-carboxylate (ACC) deaminase plays a significant role in the regulation of a plant hormone, ethylene, and thus the modification of the growth and development of plants (Arshad and Frankenberger 2002; Glick 2005). Bacterial strains with ACC deaminase can at least partially eliminate the stress-induced ethylene-mediated negative impact on plants by converting the germinating seed/root's ACC into α-ketobutyrate and ammonia (Glick et al. 1998). Since then, numerous species of Gram-negative and Gram-positive bacteria have been reported to produce ACC deaminase (Belimov et al. 2005; Pandey et al. 2005; Sessitsch et al. 2005; Blaha et al. 2006; Madhaiyan et al. 2006; Stiens et al. 2006; Shaharoona et al. 2006a, 2006b, 2007a, 2007b, 2008; Shahzad et al. 2008).

### *7.2.3 Biological Control*

Soil-borne pathogens are one of the major limiting factors for low crop productivity due to their inhibitory effects on plant health. In modern agronomic practices, a huge amount of chemicals (insecticide/fungicide) are used to offset various pathogens inflicting severe losses to crop yields. Although these chemicals are vital for controlling the pathogens on the one hand, on the other hand they can drastically affect the microbial diversity and functional properties of natural microbial communities of soils, leading thereby to imbalanced agro-ecosystems (Singh et al. 2005; Vig et al. 2006; Mubeen et al. 2008). It is therefore important to discover the most viable and economical means for effective disease management in an environment-friendly manner. Currently, biopesticides are receiving worldwide attention and considered important for the sustainability of the agricultural system. Furthermore, the WTO guideline that suggests that only residue-free agricultural produce can be exported has further created a great interest and demand for the use of biopesticides in crop protection systems. In recent times, PGPR have emerged as potential candidates with wide scope for inducing systemic resistance in crop plants

against many pathogens (Jeun et al. 2004; Nakkeeran et al. 2005; Khalequzaman and Hossain 2008; Mirik et al. 2008; Masoud and Abbas 2009). Various species of bacteria including *Pseudomonas fluorescens, P. putida, P. cepacia, P. aeruginosa, Bacillus* spp., *Rhizobium* and many other PGPR exhibit biological control activity and inhibit pathogens by synthesizing chitinase, and by production of hydrogen cyanide, protease, siderophores, and cellulase, and/or indirectly by promoting plant growth and health through any mode of action (Zahir et al. 2004; Hafeez et al. 2006; Narayanasamy 2008).

### *7.2.4 Multiple Mechanisms of Action*

Some PGPR can stimulate plant growth through multifarious activities. Although PGPR have been reported to influence plant growth by an array of mechanisms, the specific traits by which PGPR facilitate plant growth, yield, and nutrient uptake were limited to the expression of one or more of the traits expressed simultaneously in a given environment of plant–microbe interaction. A PGPR can promote plant growth by improving plant nutrition, modifying root growth architecture, and by plant responses to external stress factors, simultaneously (Egamberdiyeva and Hflich 2004; Dey et al. 2004; Saleem et al. 2007; Shaharoona et al. 2006a, b, 2007a, b, 2008). However, one trait may dominate the other one of the same PGPR when exposed to certain environmental conditions. For instance, Dey et al. (2004) reported more than one mechanism of PGPR responsible for growth promotion. They suggested that, besides ACC deaminase activity, expression of one or more of the traits such as suppression of phytopathogens, solubilization of tricalcium phosphate, production of siderophore and/or nodulation promotion by the PGPR might have simultaneously contributed to the enhancement of growth, yield, and nutrient uptake of peanut (*Arachis hypogaea*). Similarly, Shaharoona et al. (2006a, b) reported plant growth promotion by PGPR employing multiple mechanisms, such as ACC deaminase activity, chitinase activity, and root colonization. Furthermore, Hafeez et al. (2006) attributed increase in plant growth to multiple traits (such as production of IAA, and siderophores and P-solubilization) of PGPR. We have also found that PGPR expressing dual traits, such as ACC deaminase and nitrogenase activity or ACC deaminase with phosphatase activity performed better at enhancing growth and yield of wheat (*Triticum aestivum*) and maize (*Zea mays*) than PGPR possessing a single trait (Arshad et al., unpublished data).

## 7.3 Application of PGPR in Agriculture

PGPR are commonly used as inoculants for improving the growth and yield of agricultural crops. They represent an essential and large component of biofertilizer technology to improve the productivity of agricultural systems in the long run

(Zahir et al. 2004; Khalid et al. 2006; Naiman et al. 2009). Many PGPR have shown great promise as potential inoculants for agriculture uses and environmental protection, and can play very critical roles in maintaining the sustainability of agroecosystems. However, the current use of PGPR in agriculture is poor despite numerous reports on their fair performance under laboratory conditions.

PGPR possess the ability to colonize and establish an ongoing relationship with plants, resulting in better root growth, more biomass, and a substantial increase in crop yields. In this context, significant effects of PGPR have been observed on various agricultural crops, including legumes, cereals, and noncereals, and other environmentally important plant species. Furthermore, the impact of PGPR on root development with the consequent advantage on increasing water and nutrient use efficiencies has also been observed (Zahir et al. 2005; Ahmad et al. 2008; Arshad et al. 2008). The potential uses and benefits of PGPR in the improvement of overall performance of plants are discussed in the following sections.

### *7.3.1 Effect of PGPR on Plant Growth*

The potential of PGPR for improving growth and yields of various crops has been extensively documented (Table 7.2). However, most of the studies have been conducted under controlled environments rather than under natural field conditions. Results of these studies have demonstrated clearly that PGPR carry abundant potentials to enhance growth and yield of target crops. However, the selection of a functionally effective PGPR strain is very critical, and the plant responses are often variable depending upon the bacterial strain, plant genotypes, and experimental sites. It has also been claimed that the PGPR isolated from a particular crop or ecological zone are more effective in producing consistent results if reapplied to the same crop and reused in the same ecological zone (Chanway and Holl 1992; Nowak 1998). This might be due to a greater adaptability of the introduced PGPR in the given rhizosphere, while inconsistency in the responses of same crop to same PGPR could be attributed to (1) the poor quality of inocula, (2) short shelf life of PGPR, (3) lack of standard delivery systems, and/or (4) failure in maintaining a required density of PGPR onto seeds or roots. Moreover, the nature and composition of the material used as a carrier for a PGPR also plays a significant role in producing its impact on the inoculated plants.

Another important aspect of these trials is that the effects of PGPR have been investigated under different fertilizer doses, which has been shown to affect the efficiency of PGPR, leading to inconsistent performance under different agroecosystems (Shaharoona et al. 2008). Considering the acute demand for food supply, it is wise to make efforts to improve crop production by using PGPR, over and above what is achievable with optimum chemical fertilizers. It is also pertinent that most of the investigations have been focused on diazotrophs, which were tested under different N application rates. It could be very useful if selected PGPR were tested under different rates of all the three major nutrients (N, P, and K)

t2.1 **Table 7.2** Plant responses to inoculation with PGPR

| t2.2 PGPR | Plants | Comments | References |
|---|---|---|---|
| t2.3 *Azospirillum brasilense* Az1 and Az2, *P. fluorescens* Pf | *Oryza sativa* L | The inoculation increased aerial and root biomass and grain yield by 12, 40 and 16%, respectively, over uninoculated control | Naiman et al. (2009) |
| t2.4 *A. brasilense*, *Pantoea* dispersa | *Capsicum annuum* | Inoculation increased the concentration of citric, ascorbic and succinic acids in green fruit of sweet pepper compared to noninoculated control | Amor et al. (2008) |
| t2.5 *Bacillus* sp. | *Solanum tuberosum* | Bacterial inoculation caused increment in the growth of the plants compared to the noninoculated treatments | Ahmed and Hasnain (2008) |
| t2.6 *P. fluorescens*, *B. subtilis*, *Sinorhizobium meliloti*, *Bradyrhizobium* sp. | *Origanum majorana* L. | Only *P. fluorescens* and *Bradyrhizobium* sp. showed significant increases in shoot length, shoot weight, number of leaves and node, and root dry weight, in comparison to control plants or plants treated with other PGPR. Essential oil yield was also significantly increased relative to noninoculated plants, without alteration of oil composition | Banchio et al. (2008) |
| t2.7 *Phyllobacterium brassicacearum* STM196, *P. putida* UW4, *R. leguminosarum* bv. *viciae* 128C53K, *Mesorhizobium loti* | *Arabidopsis thaliana* | Root hairs of seedlings inoculated with the ACC deaminase strains were significantly longer | Contesto et al. (2008) |
| t2.8 *Rhizobiumtropici* (CIAT899), *Paenibacillus polymyxa* (DSM 36), *Rhizobium*, *P. polymyxa* strain Loutit (L), *Paenibacillus*, *Bacillus* sp. | *Phaseolus vulgaris* L. | Beans coinoculated with *R. tropici* (CIAT899) and *P. polymyxa* (DSM 36) had higher leghemoglobin concentrations, nitrogenase activity and $N_2$ fixation efficiency and thereby formed associations of greater symbiotic efficiency. Inoculation with *Rhizobium* and *P. polymyxa* strain Loutit (L) stimulated nodulation. PGPR also stimulated specific-nodulation (number of nodules per gram of root dry weight) and increased accumulated N | Figueiredo et al. (2008) |

| | | | | |
|---|---|---|---|---|
| t2.9 | *Bacillus* strains | *Capsicum annuum* | Stem diameter, root elongation, root dry weight, shoot dry weight and yield were increased in response to inoculation in the field experiment by 7.0–20.5, 7.0–17.0, 4.5–23.5, 16.5–38.5, and 11.0–33.0%, respectively | Mirik et al. (2008) |
| t2.10 | *Pseudomonas* spp. | *Triticum aestivum* L. | Inoculation significantly increased growth, yield and nutrient use efficiency of wheat | Shaharoona et al. (2008) |
| t2.11 | *Serratia oderifera* (J118), *Pantoea dispersa* (J112), *Enterobactor gergoviae* (J107) | *Cicer arietinum* L. | The PGPR in the presence of P-enriched compost resulted in a highly significant increase in fresh biomass (84%), number of pods plant$^{-1}$ (97%), grain yield (79%) and number of nodules plant$^{-1}$ (87%) compared to uninoculated control | Shahzad et al. (2008) |
| t2.12 | PGPR strains OSU-142, OSU-7, BA-8 and M-3) | *Malus domestica* Borkh) | Inoculation with OSU-142, OSU-7, BA-8 and M-3 PGPR increased average shoot length by 59.2, 18.3, 7.0 and 14.3% relative to the control and fruit yield by 116.4, 88.2, 137.5 and 73.7%, respectively. Bacterial inoculation increased shoot diameter from 7.0 to 16.3% compared to control | Aslantas et al. (2007) |
| t2.13 | *P. fluorescens*, *P. fluorescens* subgroup G strain 2, *P. marginalis*, *P. putida* subgroup B strain 1 and *P. syringae* strain 1) | *Lycopersicon esculentum* | *Pseudomonas putida* was shown to improve fruit yields in rockwool and in organic medium. The production of IAA was shown as a possible mechanism for plant growth stimulation by the bacterium. In addition, roots of tomato seedlings grown in the presence of increasing concentrations of IAA were significantly longer when seeds were treated with *P. putida* | Gravel et al. (2007) |
| t2.14 | *B. megaterium*, *B. subtilis*, *Pseudomonas corrugate* | *Zea mays* L. | All three bacterial inoculants resulted in an increment in grain yield of maize up to 122, 135 and 194%, respectively, compared to respective control. The overall beneficial effects of bacterial inoculations contributed to the colonization and survival of the introduced bacteria, and to stimulation of the indigenous microflora in the rhizosphere | Kumar et al. (2007) |

(*continued*)

t2.15 **Table 7.2** (continued)

| t2.16 PGPR | Plants | Comments | References |
|---|---|---|---|
| t2.17 *B. subtilis* BEB-lSbs (BS13) | *Lycopersicon esculentum* | Yield per plant, fruit weight and length were increased significantly by the *Bacillus subtilis* BEB-lSbs (BS13) treatment when compared to the control | Mena-Violante and Olalde-Portugal (2007) |
| t2.18 *Pseudomonas* sp., *Burkholderia caryophylli* | *Triticum aestivum* L. | Both PGPR containing ACC deaminase positively influenced growth and yield of wheat | Shaharoona et al. (2007b) |
| t2.19 *B. pumilus* 8 N-4 | | Inoculation of the wheat variety Orkhon with PGPR *B. pumilus* 8 N-4 (originated from Mongolia) resulted in the maximum increase in plant biomass, root length, and total N and P contents in plants. The isolate was also capable of producing auxin and siderophore | Hafeez et al. (2006) |
| t2.20 Cyanobacterial strains | *Oryza sativa* L. | Significant increases in grain and straw yield were observed when rice seedlings were inoculated with four cyanobacterial strains either applied alone or in combination with chemical fertilizer. In addition, a saving of 25 kg N $ha^{-1}$ was attained through cyanobacterial fertilization | Jha and Prasad (2006) |
| t2.21 *Pseudomonas* sp. | *Zea mays* L., *Vigna radiata* | Significant increases in plant height, root weight and total biomass were observed in response to inoculation with PGPR containing ACC deaminase. Similarly, inoculation significantly improved grain yield of maize in the presence of nitrogenous fertilizers. Effect of PGPR was also positive on nodulation of mung bean (*Vigna radiata*) | Shaharoona et al. (2006a, b) |
| t2.22 *Pseudomonas*, *Azotobacter*, *Azospirillum* | *Triticum aestivum* L. | Significant positive effects of inoculation on germination and growth of wheat were observed | Shaukat et al. (2006) |
| t2.23 *A. chroococcum*, *B. megaterium*, *B. mucilaginous* | *Zea mays* L. | The application of PGPR significantly increased the plant growth and resulted in the highest biomass and seedling height. Inoculation not only increased the nutritional assimilation of plant (total N, P, and K), but also improved soil properties, such as organic matter content and total N in soil | Wu et al. (2005) |

| | | | | |
|---|---|---|---|---|
| t2.24 | *Pseudomonas* spp. | *Arachis hypogaea* L. | Seed inoculation with PGPR containing ACC deaminase resulted in a significantly higher pod yield than the control, in pots, during rainy and post-rainy seasons. PGPR also significantly enhanced pod yield (23–26, 24–28, and 18–24%, respectively), haulm yield and nodule dry weight over the control under field conditions | Dey et al. (2004) |
| t2.25 | *B. licheniformis* CECT 5106, *B. pumilus* CECT 5105 | *Quercus ilex* ssp. *ballota* | Only *B. licheniformis* promoted the growth of *Q. ilex* seedlings. Furthermore, *B. licheniformis* inhibited fungal growth as revealed by ergosterol/chitin analysis | Domenech et al. (2004) |
| t2.26 | PGPR isolates | *Triticum aestivum* L. | Peat-based seed inoculation with selected PGPR strains capable of producing auxins exhibited stimulatory effects on grain yields of tested wheat cv. in pot (up to 14.7% increase over control) and field experiments (up to 27.5% increase over control); however, the response varied with cv. and PGPR strains. It was concluded that the strain which produced the highest amount of auxins in nonsterilized rhizosphere soil also caused maximum increase in growth and yield of both the wheat cultivars | Khalid et al. (2004a) |
| t2.27 | PGPR | *Quercus ilex* ssp. *ballota*, *Pinus pinea* | All strains significantly increased stem length, neck diameter and shoot dry weight of the inoculated plants | Garci´a et al. (2003) |
| t2.28 | *Enterobacter cloacae, P. putida, P. fluorescens* | *Brassica rapa* | Inoculation significantly enhanced root elongation of canola under gnotobiotic conditions | Penrose and Glick (2003) |
| t2.29 | Rhizobacteria | *Brassica juncea* | A significant increase in growth was observed in the inoculated seedlings. The PGPR were also capable of producing IAA | Asghar et al. (2002) |
| t2.30 | *P. putida* Am2, *P. putida* Bm3, *Alcaligenes xylosoxidans* cm4, *Pseudomonas* sp. Dp2 | *Brassica juncea* L. | Significant increase in root elongation of phosphorus-sufficient seedlings of rapeseed in a growth-pouch culture experiment was observed in response to inoculation | Belimov et al. (2002) |

(*continued*)

t2.31 **Table 7.2** (continued)

| t2.32 PGPR | Plants | Comments | References |
|---|---|---|---|
| t2.33 *P. putida* GR12-2 and an IAA-deficient mutant | *Brassica rapa*, *Vigna radiata* | Primary roots of canola seeds treated with wild-type strain were 35–50% longer than the roots from seeds treated with the IAA-deficient mutant and the roots from uninoculated seeds. Exposing mung bean cuttings to high levels of IAA by soaking in a suspension of the wild-type strain stimulated formation of many adventitious roots | Patten and Glick (2002) |
| t2.34 *Arthrobacter mysorens 7*, *Flavobacterium sp. L30, Klebsiella mobilis CIAM880* | *Hordeum vulgare* | All the PGPR actively colonized barley root system and rhizosphere, and significantly stimulated root elongation up to 25% | Pishchik et al. (2002) |
| *B. licheniformis* CECT 5106, t2.35 *B. pumilus* CECT 5105 | *Pinus pinea* | Both *Bacillus* strains promoted the growth of *P. pinea* seedlings | Probanza et al. (2002) |
| t2.36 *Rhizobium, Azospirillum* | *Oryza sativa* L. | Inoculation with diazotrophs had significant growth-promoting effects on rice seedlings | Biswas et al. (2000) |
| t2.37 *R. leguminosarum* (strain E11) | *Oryza sativa* L. | Growth-promoting effects of inoculation on rice seedlings were observed under axenic conditions | Dazzo et al. (2000) |
| *Azotobacter* t2.38 | *Zea mays* L. | Inoculation with strains efficient in IAA production had significant growth-promoting effects on maize seedlings | Zahir et al. (2000) |
| t2.39 *P. putida* GR 12-2 (wild-type), GR12-2/acd36 (ACC deaminase minus mutant), GR 12-2/aux1 (IAA over producers) | *Vigna radiata* | Only the wild-type strain produced longer roots | Mayak et al. (1999) |

instead of N only. Moreover, the soil fertility status should also be considered while using PGPR along with specific doses of N, P, and K fertilizers. Therefore, the proper understanding of mechanism(s) of action of chosen PGPR can substantially help in obtaining consistent responses in terms of improved growth and yield of bioprimed plants/crops. If all these factors are considered properly, the agriculture industry can draw significant benefits from the PGPR-based biotechnological approaches. Another aspect which requires urgent attention is the quality improvement of the agricultural produce following PGPR inoculation. Since PGPR exert their influence through different mechanism(s), it is very likely that they can affect quality of the produce; unfortunately, most of the studies have reported the effects of PGPR on growth and yield of inoculated plants in quantitative terms, but very little is known about the quality parameters of produce modified by the PGPR inoculations. Future efforts should, hence, be directed to assess how the quality of food produce is influenced by PGPR, besides their role in growth and development of plants.

### *7.3.2 PGPR in Stress Agriculture*

Agricultural crops are exposed to many stresses that are induced by both biotic and abiotic factors. These stresses invariably affect plant growth and yield of crops depending on the type and intensity of stress. Under stress conditions, such as salinity, drought, waterlogging, heavy metals and pathogenicity, the production of ethylene in plants at substantially accelerated rates is a very common feature, which adversely affects the root growth and, consequently, the development of the plants. As described earlier (see Sect. 2.2), certain PGPR lower ethylene synthesis by metabolizing ACC (an immediate precursor of ethylene biosynthesis in higher plants) into α-ketobutyrate and ammonia, and, thus, mitigate the negative impact of both biotic and abiotic stresses on plants (Saleem et al. 2007). Recently, several authors have documented profound effects of inoculation with PGPR containing ACC deaminase on plant growth under stress conditions (Table 7.3).

In the present scenario of several biotic and abiotic stresses to which agriculture is confronted, the role of PGPR containing ACC deaminase could be crucial for sustainable crop production. However, some beneficial aspects of these PGPR under salinity, drought, waterlogging, biocontrol, temperature, and nutritional stresses, and in the cut-flower industry and in nodulation in legumes have not been thoroughly exploited. Glick (2006) reported that transgenic tomato (*Lycopersicon lycopersicum*), canola (*Brassica napus*), and tobacco (*Nicotiana tabacum*) plants that express ACC deaminase exclusively in their roots behaved physiologically similarly to nontransformed plants treated with ACC deaminase-containing PGPR, both in the presence and absence of various stresses. Consequently, there is no apparent advantage to the use of ACC deaminase transgenic plants compared to treating the roots of the plants with ACC deaminase-containing PGPR. However, genetic modification of all plant species is not possible due to many limitations, such as proprietary rights and international trade agreements on genetically

t3.1 **Table. 7.3** Effect of PGPR on plant growth under various abiotic and biotic stresses

| t3.2 | Plant species | PGPR | Plant responses under stress | References |
|---|---|---|---|---|
| t3.3 | *Lactuca sativa* L. cv. Tafalla | *Pseudomonas mendocina* Palleroni | The inoculated plants had significantly greater shoot biomass than the control plants at low and high salinity levels. At the highest salinity level, the water content was greater in leaves of plants treated with *P. mendocina*. The plants also showed higher concentrations of foliar K and lower concentrations of foliar Na under high salt conditions | Kohler et al. (2009) |
| t3.4 | *Sorghum bicolor*, *Zea mays* L. | *Pseudomonas* spp. | Inoculation with PGPR containing ACC deaminase significantly improved fresh biomass under water-deficient field conditions | Arshad and Khalid (2008) |
| t3.5 | *Pisum sativum* L | *Pseudomonas* spp. | The inoculation partially eliminated the effects of water stress on growth, yield and ripening of *P. sativum* L., both in pot and field trials | Arshad et al. (2008), Zahir et al. (2008) |
| t3.6 | *Brassica rapa* | *P. putida* UW4 | Induced salt tolerance of plants by lowering the synthesis of salt-induced stress ethylene and promoted the growth of canola in a saline environment | Cheng et al. (2007) |
| t3.7 | *Apios americana* | *P. fluorescens* TDK1 | The PGPR strain enhanced the saline resistance in the plants and increased yield as compared to strains lacking ACC deaminase activity | Saravanakumar and Samiyappan (2007) |
| t3.8 | *Zea mays* L. | Unidentified PGPR | Significantly increased plant growth under salinity stress conditions | Nadeem et al. (2006, 2007) |
| t3.9 | *Vitis vinifera* L. | *Burkholderia phytofirmans* PsJN | Inoculation enhanced plant growth and physiological activity at both ambient (26°C) and low (4°C) temperatures. Inoculation also increased root growth and plantlet biomass. Moreover, the bacterium significantly improved plantlet cold tolerance compared to that of the nonbacterized control, which was more sensitive to exposure to low temperatures | Barka et al. (2006) |

| | | | | |
|---|---|---|---|---|
| t3.10 | *Solanum tuberosum* | PGPR | PGPR were capable of antagonizing at least one of the two potato pathogens *Ralstonia solanacearum* and *Rhizoctonia solani* | Rasche et al. (2006) |
| t3.11 | *Pisum sativum* | *Pseudomonas* sp. | Inoculation with bacteria counteracted the Cd-induced inhibition of nutrient uptake by roots | Safronova et al (2006) |
| t3.12 | *Brassica napus* | PGPR | Increases (up to 31%) in root elongation of inoculated rape seedlings compared to the control plants were observed. Inoculation with the isolates was found to increase root dry weight (ranging from 8 to 20%) and shoot dry weight (ranging from 6 to 25%) of rape in cadmium-amended soil in pot experiments. The bacterial isolates were also able to colonize and develop in the rhizosphere soil of rape after root inoculation | Sheng and Xia (2006) |
| t3.13 | *Brassica juncea* L. | *Variovorax paradoxus*, *Rhodococcus* sp. | Plant growth was improved in $Cd^{2+}$-supplemented media in response to inoculation | Belimov et al. (2005) |
| t3.14 | *Pisum sativum* | *Variovorax paradoxus* 5C-2 | Inoculated plants gave more seed yield (25–41%), seed number and seed nitrogen accumulation than uninoculated plants under moisture stress and watering conditions | Dodd et al. (2005) |
| t3.15 | *Chamaecytisus proliferus* | *P. fluorescens* | The bacterium showed positive effect in antagonizing the growth of *Fusarium oxysporum* and *Fusarium proliferatum* in growth medium. | Donate-Correa et al. (2005) |
| t3.16 | *Mimosa pudica* | *Burkholderia* sp. | The bacterium exhibited antagonistic activity against *Rhizoctonia solani* and *Sclerotinia sclerotiorum* | Pandey et al. (2005) |
| t3.17 | *Phragmites australis* | *Pseudomonas asplenii* AC | Inoculation resulted in normal plant growth under high levels of $Cu^{2+}$ and creosote | Reed et al. (2005) |
| t3.18 | *Lycopersicon esculentum* | *Achromobacter piechaudii* | Inoculation significantly increased the fresh and dry weights of tomato seedlings grown in the presence of NaCl salt concentration up to 172 mM. The bacterium also significantly increased the fresh and dry weights of both tomato and pepper seedlings exposed to transient water stress | Mayak et al. (2004a, b) |

(*continued*)

t3.19 **Table. 7.3** (continued)

| t3.20 Plant species | PGPR | Plant responses under stress | References |
|---|---|---|---|
| t3.21 *Glycine max* | *B. subtilis* NEB4, NEB5, *B. thuringiensis* NEB17, *Bradyrhizobium japonicum* | Coinoculation exhibited consistent and significant increases in nodule number, nodule weight, shoot weight, root weight, total biomass, total nitrogen, and grain yield at low (25, 17, and 15°C) root zone temperatures | Bai et al. (2003) |
| t3.22 *Brassica juncea* L. | *Pseudomonas* sp., *Alcaligenes* sp., *Variovorax paradoxus*, *B. pumilus*, *Rhodococcus* sp. | The bacteria were tolerant to $Cd^{2+}$ toxicity and stimulated root elongation of rape seedlings in the presence of 300 μM $CdCl_2$ in the nutrient solution | Belimov et al. (2001) |
| t3.23 *Lycopersicon esculentum* | *P. putida* UW4, *Enterobacter cloacae* CAL2, *P. Putida* | Inoculated plants showed substantial tolerance to flooding stress | Grichko and Glick (2001) |
| t3.24 *Brassica juncea* L., *Lycopersicum esculentum* Mill | *Kluyvera ascorbata* | Toxic effects of heavy metals ($Ni^{2+}$, $Pb^{2+}$ and $Zn^{2+}$) were not pronounced in inoculated plants | Burd et al. (2000) |
| t3.25 *Cucumis sativus* | *P. putida* UW4 | The bacterial strains were effective in biocontrol of *Pythium ultimum* | Wang et al. (2000) |
| t3.26 *Solanum tuberosum* | *Burkholderia phytofirmans* PsJN | PGPR helped potato plants in maintaining normal growth under heat stress | Bensalim et al. (1998) |

modified crops and restrictions in the use of genetically modified microorganisms or plants. The use of PGPR containing ACC deaminase activity along with other traits, such as the ability to synthesize nitrogenases, phosphatases, and chitinases could prove to be a cost-effective and environment-friendly strategy to ensure sustainable agriculture.

### *7.3.3 PGPR for Bioremediation*

Recently, the application of PGPR in association with plants has been expanded to remediate contaminated soils (Khan et al. 2009). The addition of PGPR increases the removal of pollutant most likely by enhancing germination, and by stimulating plant growth including root biomass and survival of plants, in soils that are heavily contaminated (Huang et al. 2004; Reed et al. 2005; Safronova et al. 2006; Arshad et al. 2007). Some rhizobacteria can enhance phytoremdiation by promoting plant growth through the synthesis of siderophores, phytohormones, enzymes, and antibiotics (Pattern and Glick 1996; Burd et al. 2000; Khalid et al. 2006; Arshad et al. 2007; Wani et al. 2008a), and/or through stimulation of certain metabolic pathways, such as nitrogen fixation and the uptake of N, P, S, Mg, Ca, and other nutrients (Belimov and Dietz 2000). Similarly, PGPR can increase the tolerance of plants to contaminants; the PGPR–plant system cannot survive in comparatively extreme environments such as with high concentrations of heavy metals (Wani et al. 2008a). Although microbial communities in polluted soils have been studied, little is known about the composition of microbial community in the plant rhizosphere growing on highly polluted soils. Usually, rhizosphere soil is more conducive to remediation due to high concentrations of nutrients exuded from the roots and dense bacterial populations. Many workers have reported bioremediation of both organic (Narasimhan et al. 2003; Huang et al. 2004, 2005; Villacieros et al. 2005; Muratova et al. 2005) and inorganic contaminants in the environment by using PGPR (Hallberg and Johnson 2005; Kao et al. 2006; Umrania 2006; Burd et al. 2000; Kamnev et al. 2005; Abou-Shanab et al. 2006; Wu et al. 2006; Zaidi et al. 2006; Sheng and Xia 2006; Wani et al. 2008b).

## 7.4 Formulations of Effective Biofertilizers

A number of steps are involved in developing effective PGPR-based biofertilizers for achieving consistent results in terms of crop productivity under field conditions (Fig. 7.1). The most critical steps involved in the development of biofertilizers include isolation of bacterial strains from the same habitat and/or crop followed by screening of PGPR under axenic conditions by conducting repeated trials. The strains showing better results under controlled conditions should be tested further for their performance under natural conditions by conducting pot and field trials.

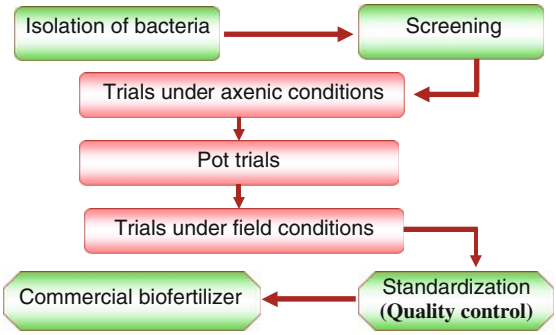



**Fig. 7.1** Steps involved in the development of an effective biofertilizer product

Finally, the PGPR strain selected for biofertilizer formulation should be investigated thoroughly to maintain its quality. Quality of biofertilizers is one of the most critical factors which determine their success or failure and acceptance or rejection by end users, the farmers. The functionality of selected PGPR must be defined well before using it as a candidate for biofertilizer formulation, which could be achieved by employing biochemical and molecular tests in the laboratory and then determining correlations between PGPR traits with the growth promotion of inoculated plants under axenic and natural conditions. However, there is no established basis for acceptance of a formulation as an effective biofertilizer. Since PGPR based biofertilizers contain living entities, which are very sensitive to environmental conditions, the consistency in effectiveness cannot be as good as observed in the case of chemical fertilizers. However, maintaining a particular population of a selected PGPR strain could help in enhancing the consistency of biofertilizer effects. A strict control over quality is the only answer to avoid failure of biofertilizers in different agronomic regions of the world.

The production of biofertilizer and its acceptance by farming communities are closely linked. For their use to expand globally at the farmers' end, quality management is essential and must be performed consistently in order to supply contaminant-free bioproducts to the users. For this, skilled personnel are required who know how to work with these materials and be able to respond to the modern conditions of agricultural production. In addition, they should be well aware of the sustainability and environmental protection measures. Furthermore, proper guidelines for the production and commercialization of biofertilizers should be framed in order to popularize the use of such bioagents for maintaining the sustainability of agro-ecosystems across the globe.

## 7.5 Conclusion

Enhancement in the use of PGPR is one of the newly emerging options for meeting agricultural challenges imposed by the still-growing aggregate demand for food. Moreover, this biotechnology is also likely to ensure conservation of our environments.

However, before PGPR can contribute to such benefits, scientists must learn more about them and explore ways and means for their better utilization in the farmers' fields.

Future research should focus on managing plant–microbe interactions, particularly with respect to their mode of actions and adaptability to conditions under extreme environments for the benefit of plants. Furthermore, scientists need to address certain issues, like how to improve the efficacy of biofertilizers, what should be an ideal and universal delivery system, how to stabilize these microbes in soil systems, and how nutritional and root exudation aspects could be controlled in order to get maximum benefits from PGPR application. Biotechnological and molecular approaches could possibly develop more understanding about PGPR mode of actions that could lead to more successful plant–microbe interaction. Efforts should also be directed towards the use of PGPR to reduce pesticide applications. In brief, PGPR biotechnology provides an excellent opportunity to develop environment-friendly biofertilizer to be used as supplements and/or alternatives to chemical fertilizers.

## References

Abou-Shanab RI, Angle JS, Chaney RL (2006) Bacterial inoculants affecting nickel uptake by *Alyssum murale* from low, moderate and high Ni soils. Soil Biol Biochem 38:2882–2889

Abou-Shanab RI, Angle JS, Delorme TA, Chaney RL, van Berkum P, Moawad H, Ghanem K, Ghozlan HA (2003) Rhizobacterial effects on nickel extraction from soil and uptake by *Alyssum murale*. New Phytol 158:219–224

Afzal A, Bano A (2008) *Rhizobium* and phosphate solubilizing bacteria improve the yield and phosphorus uptake in wheat (*Triticum aestivum*). Int J Agri Biol 10:85–88

Ahmad R, Arshad M, Khalid A, Zahir ZA (2008) Effectiveness of organic-/bio-fertilizer supplemented with chemical fertilizers for improving soil water retention, aggregate stability, growth and nutrients uptake of maize (*Zea mays* L.). J Sust Agri 31:57–77

Ahmed A, Hasnain S (2008) Auxin producing *Bacillus* sp.: auxin quantification and effect on the growth of *Solanum tuberosum*. J Biotechnol 136:766–767

Akhtar MJ, Arshad M, Khalid A, Mehmood MH (2005) Substrate-dependent biosynthesis of ethylene by rhizosphere soil fungi and its influence on etiolated pea seedlings. Pedobiologia 49:211–219

Almonacid S, Quintero N, Martinez M, Vela M (2000) Determination of quality parameters of bacterial inocula based on liquid formulation elaborated with strains producing indole acetic acid (IAA). Auburn university websitehttp://www.ag.auburn.edu/argentina/pdfmanuscript/almonacid.pdf

Amor FMD, Martínez AS, Fortea MI, Legua P, Delicado EN (2008) The effect of plant-associative bacteria (*Azospirillum* and *Pantoea*) on the fruit quality of sweet pepper under limited nitrogen supply. Sci Hortic 117:191–196

Arshad M, Frankenberger WT Jr (1998) Plant growth regulating substances in the rhizosphere: microbial production and functions. Adv Agron 62:146–151

Arshad M, Frankenberger WT Jr (2002) Ethylene: agricultural sources and applications. Kluwer Academic, New York

Arshad M, Saleem M, Hussain S (2007) Perspectives of bacterial ACC deaminase in phytoremediation. Trends Biotechnol 25:356–362

Arshad M, Khalid A (2008) Annual report. University of Agriculture, Faisalabad, Pakistan
Arshad M, Shaharoona B, Mahmood T (2008) Inoculation with plant growth promoting rhizobacteria containing ACC-deaminase partially eliminates the effects of water stress on growth, yield and ripening of *Pisum sativum* L. Pedosphere 18:611–620
Asghar HN, Zahir ZA, Arshad M, Khaliq A (2002) Relationship between in vitro production of auxins by rhizobacteria and their growth-promoting activities in *Brassica juncea* L. Biol Fertil Soils 35:231–237
Aslantas R, Cakmakci R, Sahin F (2007) Effect of plant growth promoting rhizobacteria on young apple tree growth and fruit yield under orchard conditions. Scientia Hort 111:371–377
Bai Y, Zhou X, Smith DL (2003) Enhanced soybean plant growth resulting from coinoculation of *Bacillus* strains with *Bradyrhizobium japonicum*. Crop Sci 43:1774–1781
Banchio E, Bogino PC, Zygadlo J, Giordano W (2008) Plant growth promoting rhizobacteria improve growth and essential oil yield in *Origanum majorana* L. Biochem Syst Ecol 36: 766–771
Barka EA, Nowak J, Clément C (2006) Enhancement of chilling resistance of inoculated grapevine plantlets with a plant growth-promoting rhizobacterium, *Burkholderia phytofirmans* Strain PsJN. Appl Environ Microbiol 72:7246–7252
Belimov AA, Dietz KJ (2000) Effect of associative bacteria on element composition of barley seedlings grown in solution culture at toxic cadmium concentrations. Microbiol Res 155: 113–21
Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick BR (2005) Cadmium-tolerant plant growth-promoting bacteria associated with the roots of Indian mustard (*Brassica juncea* L. Czern.). Soil Biol Biochem 37:241–250
Belimov AA, Safronova VI, Mimura T (2002) Response of spring rape (*Brassica napus* var. *oleifera* L.) to inoculation with plant growth promoting rhizobacteria containing 1-aminocyclopropane-1-carboxylate deaminase depends on nutrient status of the plant. Can J Microbiol 48:189–199
Belimov AA, Safronova VI, Sergeyeva TA, Egorova TN, Matveyeva VA, Tsyganov VE, Borisov AY, Tikhonovich IA, Kluge C, Preisfeld A, Dietz KJ, Stepanok VV (2001) Characterization of plant growth promoting rhizobacteria isolated from polluted soils and containing 1-aminocyclopropane-1-carboxylate deaminase. Can J Microbiol 47:242–252
Bensalim S, Nowak J, Asiedu SK (1998) A plant growth promoting rhizobacterium and temperature effects on performance of 18 clones of potato. J Potato Res 75:145–152
Biswas JC, Ladha JK, Dazzo FB, Yanni YG, Rolfe BG (2000) Rhizobial inoculation influences seedling vigor and yield of rice. Agron J 92:880–886
Blaha D, Prigent-Combaret C, Mirza MS, Moenne-Loccoz Y (2006) Phylogeny of the 1-aminocyclopropane-1-carboxylic acid deaminaseencoding gene *acd*S in phytobeneficial and pathogenic *Proteobacteria* and relation with strain biogeography. FEMS Microbiol Ecol 56: 455–470
Burd GI, Dixon DG, Glick BR (2000) Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. Can J Microbiol 46:237–245
Çakmakçi R, Dönmez F, Aydm A, Şahin F (2006) Growth promotion of plants by plant growth-promoting rhizobacteria under greenhouse and two different field soil conditions. Soil Biol Biochem 38:1482–1487
Chanway CP, Holl FB (1992) Influence of soil biota on Douglas fir (*Pseudotsuga menziesii*) seedling growth: the role of rhizosphere bacteria. Can J Bot 70:1025–1031
Cheng Z, Park E, Glick BR (2007) 1-Aminocyclopropane-1-carboxylate (ACC) deaminase from *Pseudomonas putida* UW4 facilitates the growth of canola in the presence of salt. Can J Microbiol 53:912–918
Contesto C, Desbrosses G, Lefoulon C, Bena G, Borel F, Galland M, Gamet L, Varoquaux F, Touraine B (2008) Effects of rhizobacterial ACC deaminase activity on *Arabidopsis* indicate that ethylene mediates local root responses to plant growth-promoting rhizobacteria. Plant Sci 175:178–189

Dazzo FB, Yanni YG, Rizk R, DeBruijn FJ, Rademaker J, Squartini A, Corich V, Mateos P, Martinez-Molina E (2000) Progress in multinational colaboraive studies on the beneficial association between *Rhizobium leguminoserum* bv. Trifolii and rice. In: Ladha JK, Reddy PM (eds) The quest for nitrogen fixation in rice. IRRI, Los Banos, pp 167–189

Dell'Amico E, Cavalca L, Andreoni V (2008) Improvement of *Brassica napus* growth under cadmium stress by cadmium-resistant rhizobacteria. Soil Biol Biochem 40:74–84

Dey R, Pal KK, Bhatt DM, Chauhan SM (2004) Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. Microbiol Res 159:371–394

Dodd IC, Belimov AA, Sobeih WY, Safronova VI, Grierson D, Davies WJ (2005) Will modifying plant ethylene status improve plant productivity in water-limited environments? Fourth international crop science congresshttp://www.cropscience.org.au/icsc2004/poster/1/3/4/510_doddicref.htm. Accessed 17 June 2007

Domenech J, Solano BR, Probanza A, Garca JAL, Colon JJ, Gutierrez-Manero FJ (2004) *Bacillus* spp. and *Pisolithus tinctorius* effects on *Quercus ilex* ssp. *ballota*: a study on tree growth, rhizosphere community structure and mycorrhizal infection. Forest Ecol Manage 194:293–303

Donate-Correa J, Leon-Barrios M, Perez-Galdona R (2005) Screening for plant growth-promoting rhizobacteria in *Chamaecytisus proliferus* (tagasaste), a forage tree-shrub legume endemic to the Canary Islands. Plant Soil 266:261–272

Egamberdiyeva D, Hflich G (2004) Effect of plant growth-promoting bacteria on growth and nutrient uptake of cotton and pea in a semiarid region of Uzbekistan. J Arid Environ 56: 293–301

Figueiredo MVB, Martinez CR, Burity HA, Chanway CP (2008) Plant growth-promoting rhizobacteria for improving nodulation and nitrogen fixation in the common bean (*Phaseolus vulgaris* L.). World J Microbiol Biotechnol 24:1187–1193

Frankenberger WT Jr, Arshad M (1995) Phytohormones in soils: microbial production and function. Marcel Dekker, New York

Garci'a JL, Probanza A, Ramos B, Manero FG (2003) Effects of three plant growth-promoting rhizobacteria on the growth of seedlings of tomato and pepper in two different sterilized and nonsterilized peats. Arch Agron Soil Sci 49:119–127

Glick BR (1995) The enhancement of plant growth by free living bacteria. Can J Microbiol 41:109–117

Glick BR (2003) Phytoremediation: synergistic use of plants and bacteria to clean up the environment. Biotechnol Adv 21:383–393

Glick BR (2004) Teamwork in phytoremediation. Nature Biotechnol 22:526–527

Glick BR (2005) Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase. FEMS Microbiol Lett 251:1–7

Glick BR (2006) Plant responses to ACC deaminase-containing PGPR. 7th international workshop on plant growth promoting rhizobacteria, 28 May–2 June 2006. Noordwijkerhout, The Netherlands, p 30

Glick BR, Penrose DM, Li J (1998) A model for lowering plant ethylene concentration by plant growth promoting rhizobacteria. J Theor Biol 190:63–68

Gravel V, Antoun H, Tweddell RJ (2007) Growth stimulation and fruit yield improvement of greenhouse tomato plants by inoculation with *Pseudomonas putida* or *Trichoderma atroviride*: possible role of indole acetic acid (IAA). Soil Biol Biochem 39:1968–1977

Gray EJ, Smith DL (2005) Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. Soil Biol Biochem 37:395–412

Grichko VP, Glick BR (2001) Flooding tolerance of transgenic tomato plants expressing the bacterial enzyme ACC deaminase controlled by the 35 S, rolD or PRB-1b promoter. Plant Physiol Biochem 39:19–25

Guo JH, Qi HY, Guo YH, Ge HL, Gong LY, Zhang LX, Sun PH (2004) Biocontrol of tomato wilt by plant growth-promoting rhizobacteria. Biol Control 29:66–72

Hafeez FY, Yasmin S, Ariani D, Mehboob-ur-Rahman ZY, Malik KA (2006) Plant growth-promoting bacteria as biofertilizer. Agron Sust Dev 26:143–150

Hallberg KB, Johnson DB (2005) Microbiology of a wetland ecosystem constructed to remediate mine drainage from a heavy metal mine. Sci Total Environ 338:53–66

Huang XD, El-Alawi Y, Penrose DM, Glick BR, Greenberg BM (2004) Responses of three grass species to creosote during phytoremediation. Environ Pollut 130:453–63

Huang XD, El-Alawi Y, Penrose DM, Glick BR, Greenberg BM (2005) Responses of hydrocarbons (TPHs) from soils. Microchem J 81:139–47

Jeun YC, Park KS, Kim CH, Fowler WD, Kloepper JW (2004) Cytological observations of cucumber plants during induced resistance elicited by rhizobacteria. Biol Control 29:34–42

Jha MN, Prasad AN (2006) Efficacy of new inexpensive cyanobacterial biofertilizer including its shelf-life. World J Microbiol Biotechnol 22:73–79

Kamnev AA, Tugarova AV, Antonyuk LP, Tarantilis PA, Polissiou MG, Gardiner PHE (2005) Effects of heavy metals on plant-associated rhizobacteria: comparison of endophytic and non-endophytic strains of *Azospirillum brasilense*. J Trace Elem Med Biol 19:91–5

Kao PH, Huang CC, Hseu ZY (2006) Response of microbial activities to heavy metals in a neutral loamy soil treated with biosolid. Chemosphere 64:63–70

Karadeniz A, Topcuoglu SF, Inan S (2006) Auxin, gibberellin, cytokinin and abscisic acid production in some bacteria. World J Microbiol Biotechnol 22:1061–1064

Kennedy IR, Choudhury ATMA, Kecskés ML (2004) Non-symbiotic bacterial diazotrophs in crop-farming systems: can their potential for plant growth promotion be better exploited? Soil Biol Biochem 36:1229–1244

Khalequzaman KM, Hossain I (2008) Effect of seed treatment with *Rhizobium* strains and biofertilizers on foot/root rot and yield of bushbean in *Fusarium oxysporum* infested soil. J Agric Res 46:55–64

Khalid A, Arshad M, Zahir ZA (2004a) Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. J Appl Microbiol 96:473–480

Khalid A, Tahir S, Arshad M, Zahir ZA (2004b) Relative efficiency of rhizobacteria for auxin biosynthesis in rhizosphere vs. non-rhizosphere soil. Aust J Soil Res 42:921–926

Khalid A, Arshad M, Zahir ZA (2006) Phytohormones: microbial production and applications. In: Uphoff N et al (eds) Biological approaches to sustainable soil systems. Taylor & Francis, Boca Raton, Florida, pp 207–220

Khan AG (2005) Role of soil microbes in the rhizospheres of plants growing on trace metal contaminated soils in phytoremediation. J Trace Elem Med Biol 18:355–364

Khan MS, Zaidi A, Wani PA (2007) Role of phosphate solubilizing microorganisms in sustainable agriculture: a review. Agron Sustain Dev 27:29–43

Khan MS, Zaidi A, Wani PA, Oves M (2009) Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. Environ Chem Lett 7:1–19

Kloepper JW, Scher FM, Tripping B (1986) Emergence promoting rhizobacteria: description and implication for agriculture. In: Swinburne TR (ed) Iron, siderophores and plant diseases. Plenum, New York, pp 155–164

Kohler J, Hernández JA, Caravaca F, Roldán A (2009) Induction of antioxidant enzymes is involved in the greater effectiveness of a PGPR versus AM fungi with respect to increasing the tolerance of lettuce to severe salt stress. Environ Exp Bot 65:245–252

Kumar B, Trivedi P, Pandey A (2007) Pseudomonas corrugata: a suitable bacterial inoculant for maize grown under rainfed conditions of Himalayan region. Soil Biol Biochem 39:3093–3100

Ladha JK, Reddy PM (2000) The quest for nitrogen fixation in rice. IRRI, Los Banos

Lebeau T, Braud A, Jézéquel K (2008) Performance of bioaugmentation-assisted phytoextraction applied to metal contaminated soils: a review. Environ Pollut 153:497–522

Lucy M, Reed E, Glick BR (2004) Applications of free living plant growth-promoting rhizobacteria. Antonie Van Leeuwenhoek 86:1–25

Madhaiyan M, Suresh Reddy BV, Anandham R, Senthilkumar M, Poonguzhali S, Sundaram SP, Sa TM (2006) Plant growth promoting *Methylobacterium* induces defense responses in groundnut (*Arachis hypogaea* L.) compared to rot pathogens. Curr Microbiol 53:270–276

Masoud A, Abbas ST (2009) Evaluation of fluorescent pseudomonads for plant growth promotion, antifungal activity against *Rhizoctonia solani* on common bean, and biocontrol potential. Biol Control 48:101–107

Mayak S, Tirosh T, Glick BR (2004a) Plant growth-promoting bacteria that confer resistance to water stress in tomato and pepper. Plant Sci 166:525–530

Mayak S, Tirosh T, Glick BR (2004b) Plant growth-promoting bacteria confer resistance in tomato plants to salt stress. Plant Physiol Biochem 42:565–572

Mayak S, Tivosh T, Glick BR (1999) Effect of wild type and mutant plant growth-promoting rhizobacteria on the rooting of mungbeen cuttings. J Plant Growth Regul 18:49–53

Mena-Violante HG, Olalde-Portugal V (2007) Alteration of tomato fruit quality by root inoculation with plant growth-promoting rhizobacteria (PGPR): *Bacillus subtilis* BEB-13bs. Sci Hortic 113:103–106

Mirik M, Aysan Y, Cinar O (2008) Biological control of bacterial spot disease of pepper with *Bacillus* strains. Turk J Agri For 32:381–390

Mubeen F, Aslam A, Radl V, Schloter M, Malik KA, Hafeez FY (2008) Role of nature's fertility partners with crop protectants for sustainable agriculture. In: Dakora FD et al (eds) Biological nitrogen fixation: towards poverty alleviation through sustainable agriculture. Springer, The Netherlands, pp 153–154

Muratova AY, Turkovskaya OV, Antonyuk LP, Makarov OE, Pozdnyakova LI, Ignatov VV (2005) Oil-oxidizing potential of associative rhizobacteria of the genus *Azospirillum*. Microbiol 74:210–215

Nadeem SM, Zahir ZA, Naveed M, Arshad M, Shahzad SM (2006) Variation in growth hizobacteand ion uptake of maize due to inoculation with plant growth promoting rria under salt stress. Soil Environ 25:78–84

Nadeem SM, Zahir ZA, Naveed M, Arshad M (2007) Preliminary investigations on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC-deaminase activity. Can J Microbiol 53:1141–1149

Naiman AD, Latronico A, Garca de Salamone IE (2009) Inoculation of wheat with *Azospirillum brasilense* and *Pseudomonas fluorescens*: impact on the production and culturable rhizosphere microflora. Eur J Soil Biol 45:44–51

Nakkeeran S, Fernando WGD, Siddiqui A (2005) Plant growth promoting rhizobacteria formulations and its scope in commercialization for the management of pests and diseases. In: Siddiqui ZA (ed) PGPR: biocontrol and biofertilization. Springer, The Netherlands, pp 257–296

Narasimhan K, Basheer C, Bajic VB, Swarup S (2003) Enhancement of plant-microbe interactions using a rhizosphere metabolomics-driven approach and its application in the removal of polychlorinated biphenyls. Plant Physiol 132:146–53

Narayanasamy P (2008) Molecular biology in plant pathogenesis and disease management. Springer, The Netherlands

Nowak J (1998) Benefits of in vitro 'biotization' of plant tissue cultures with microbial inoculants. In Vitro Cell Dev Biol Plant 34:122–130

Orhan E, Esitken A, Ercisli S, Turan M, Sahin F (2006) Effects of plant growth promoting rhizobacteria (PGPR) on yield, growth and nutrient contents in organically growing raspberry. Sci Hortic 111:38–43

Pandey P, Kang SC, Maheshwari DK (2005) Isolation of endophytic plant growth promoting *Burkholderia* sp. MSSP from root nodules of *Mimosa pudica*. Curr Sci 89:170–180

Patten CL, Glick BR (1996) Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol 42:207–220

Patten CL, Glick BR (2002) Role of *Pseudomonas putida* indoleacetic acid in development of the host plant root system. Appl Environ Microbiol 68:3795–3801

Penrose DM, Glick BR (2003) Methods for isolating and characterizing ACC deaminase-containing plant growth-promoting rhizobacteria. Physiol Plant 118:10–15

Pishchik VN, Vorobyev NI, Chernyaeva II, Timofeeva SV, Kozhemyakov AP, Alexeev YV, Lukin SM (2002) Experimental and mathematical simulation of plant growth promoting rhizobacteria and plant interaction under cadmium stress. Plant Soil 243:173–186

Poi SC, Kabi MC (1979) Effect of *Azotobacter* inoculation on growth and yield of jute and wheat. Indian J Agri Sci 49:478–480

Probanza A, Garca JAL, Palomino MR, Ramos B, Mañero FJG (2002) *Pinus pinea* L. seedling growth and bacterial rhizosphere structure after inoculation with PGPR *Bacillus* (*B. licheniformis* CECT 5,106 and *B. pumilus* CECT 5,105). Appl Soil Ecol 20:75–84

Raj SN, Deepak SA, Basavaraju P, Shetty HS, Reddy MS, Kloepper JW (2003) Comparative performance of formulations of plant growth promoting rhizobacteria in growth promotion and suppression of downy mildew in pearl millet. Crop Prot 22:579–88

Rasche F, Velvis H, Zachow C, Berg G, Van Elsas JD, Sessitsch A (2006) Impact of transgenic potatoes expressing anti-bacterial agents on bacterial endophytes is comparable with the effects of plant genotype, soil type and pathogen infection. J Appl Ecol 43:555–566

Reed MLE, Warner BG, Glick BR (2005) Plant growth-promoting bacteria facilitate the growth of the common reed *Phragmites australis* in the presence of copper or polycyclic aromatic hydrocarbons. Curr Microbiol 51:425–429

Römkens P, Bouwman L, Japenga J, Draaisma C (2002) Potentials and drawbacks of chelate-enhanced phytoremediation of soils. Environ Pollut 116:109–121

Safronova VI, Stepanok VV, Engqvist GL, Alekseyev YV, Belimov AA (2006) Root-associated bacteria containing 1-aminocyclopropane-1-carboxylate deaminase improve growth and nutrient uptake by pea genotypes cultivated in cadmium supplemented soil. Biol Fertil Soils 42:267–272

Saleem M, Arshad M, Hussain S, Bhatti AS (2007) Perspectives of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. J Indust Microbiol Biotechnol 34:635–648

Saravanakumar D, Samiyappan R (2007) ACC deaminase from *Pseudomonas fluorescens* mediated saline resistance in groundnut (*Arachis hypogea*) plants. J Appl Microbiol 102:1283–1292

Senthilkumar M, Madhaiyan M, Sundaram SP, Kannaiyan S (2009) Intercellular colonization and growth promoting effects of *Methylobacterium* sp. with plant-growth regulators on rice (*Oryza sativa* L. Cv CO-43). Microbiol Res 164:92–104

Serdyuk OP, Smolygina LD, Muzafarov EN, Adanin VM, Arinbasarov MU (1995) 4-Hydroxyphenethyl alcohol – a new cytokinin-like substance from the phototrophic purple bacterium *Rhodospirillum rubrum* 1R. FEBS Lett 365:10–12

Sessitsch A, Coenye T, Sturz AV, Vandamme P, Barka E, Wang-Pruski G, Faure D, Reiter B, Glick BR, Nowak J (2005) *Burkholderia phytofirmins* sp. Nov., a novel plant-associated bacterium with plant beneficial properties. Int J Syst Evol Microbiol 55:1187–1192

Shaharoona B, Arshad M, Zahir ZA, Khalid A (2006a) Performance of *Pseudomonas* spp. containing ACC-deaminase for improving growth and yield of maize (*Zea mays* L.) in the presence of nitrogenous fertilizer. Soil Biol Biochem 38:2971–2975

Shaharoona B, Arshad M, Zahir ZA (2006b) Effect of plant growth promoting rhizobacteria containing ACC-deaminase on maize (*Zea mays* L.) growth under axenic conditions and on nodulation of mungbean (*Vigna radiata*). Lett Appl Microbiol 42:155–159

Shaharoona B, Arshad M, Khalid A (2007a) Differential response of etiolated pea seedling to 1-aminocyclopropane-1-carboxylate and/or L-methionine utilizing rhizobacteria. J Microbiol 45:15–20

Shaharoona B, Jamro GM, Zahir ZA, Arshad M, Memon KS (2007b) Effectiveness of various *Pseudomonas spp*. and *Burkholderia caryophylli* containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum L.)*. J Microbiol Biotechnol 17:1300–1307

Shaharoona B, Naveed M, Arshad M, Zahir ZA (2008) Fertilizer dependent efficiency of Pseudomonads containing ACC-deaminase for improving growth, yield and nutrient use efficiency of wheat (*Triticum aestivum* L.). Appl Microbiol Biotechnol 79:147–155

Shahzad MS, Khalid A, Arshad M, Khalid M, Mehboob I (2008) Integrated use of plant growth promoting bacteria and P-enriched compost for improving growth, yield and nodulation of chickpea. Pak J Bot 40:1735–1744

Shaukat K, Affrasayab S, Hasnain S (2006) Growth responses of *Triticum aestivum* to plant growth promoting rhizobacteria used as a biofertilizer. Res J Microbiol 1:330–338

Sheng XF, Xia JJ (2006) Improvement of rape (*Brassica napus*) plant growth and cadmium uptake by cadmium-resistant bacteria. Chemosphere 64:1036–1042

Sindhu SS, Gupta SK, Dadarwal KR (1999) Antagonistic effect of *Pseudomonas* spp. on pathogenic fungi and enhancement of plant growth in green gram (*Vigna radiata*). Biol Fertil Soils 29:62–68

Sindhu SS, Suneja S, Goel AK, Parmar N, Dadarwal KR (2002) Plant growth promoting effects of *Pseudomonas* sp. on coinoculation with *Mesorhizobium* sp. *Cicer* strain under sterile and "wilt sick" soil conditions. Appl Soil Ecol 19:57–64

Singh B, Kahlon RS, Sahoo SK et al (2005) Residues of lindane and endosulfan in soil and their effect on soil microbial population and dehydrogenase activity. Pesticide Res J 17:88–90

Stiens M, Schneiker S, Keller M, Kuhn S, Pühler A, Schlüter A (2006) Sequence analysis of the 144-kilobase accessory plasmid psmesm11a, isolated from a dominant *Sinorhizobium meliloti* strain identified during a long-term field release experiment. Appl Environ Microbiol 72:3662–3672

Timmusk S, Nicander B, Granhall U, Tillberg E (1999) Cytokinin production by *Paenibacillus polymyxa*. Soil Biol Biochem 31:1847–1852

Tsavkelova EA, Cherdyntseva TA, Botina SG, Netrusov AL (2007) Bacteria associated with orchid roots and microbial production of auxin. Microbiol Res 162:69–76

Tuomi T, Rosenquist H (1995) Detection of abscisic, gibberellic and indole-3-acetic acid from plant and microbes. Plant Physiol Biochem 33:725–734

Umrania VV (2006) Bioremediation of toxic heavy metals using acidothermophilic autotrophes. Bioresour Technol 97:1237–1242

Van Loon LC (2007) Plant response to plant growth-promoting rhizobacteria. Eur J Plant Pathol 119:243–254

Van Loon LC, Glick BR (2004) Increased plant fitness by rhizobacteria. In: Sandermann H (ed) Molecular ecotoxicology of plants. Springer, Berlin, pp 178–205

Vig K, Singh DK, Sharma PK (2006) Endosulfan and quinalphos residues and toxicity to soil microarthropods after repeated applications in a field investigation. J Environ Sci Health Part B 41:681–692

Villacieros M, Whelan C, Mackova M, Molgaard J, Sanchez-Contreras M, Lloret J (2005) Polychlorinated biphenyl rhizoremediation by *Pseudomonas fluorescens* F113 derivatives, using a *Sinorhizobium meliloti* nod system to drive bph gene expression. Appl Environ Microbiol 71:2687–2694

Wang CKE, Glick BR, Defago G (20 00) Effect of transferring 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase genes into pseudomonas fluorescens strain CHA0 and its gacA derivative CHA96 on their growth-promoting and disease-suppressive capacities. Can J Microbiol 46:898–907

Wani PA, Khan MS, Zaidi A (2007) Co-inoculation of nitrogen fixing and phosphate solubilizing bacteria to promote growth yield and nutrient uptake in chickpea. Acta Agron Hung 55: 315–323

Wani PA, Khan MS, Zaidi A (2008a) Effect of metal tolerant plant growth promoting *Rhizobium* on the performance of pea grown in metal amended soil. Arch Environ Contam Toxicol 55: 33–42

Wani PA, Khan MS, Zaidi A (2008b) Chromium reducing and plant growth promoting *Mesorhizobium* improves chickpea growth in chromium amended soil. Biotechnol Lett 30:159–163

Wei G, Kloepper JW, Tuzun S (1996) Induced systemic resistance to cucumber diseases and increased plant growth by plant growth promoting rhizobacteria under field conditions. Phytopathol 86:221–224

Weingart H, Volksch B, Ullrich MS (1999) Comparison of ethylene production by *Pseudomonas syringae* and *Ralstonia aolanacearum*. Phyopathology 89:360–365

Wu SC, Caob ZH, Lib ZG, Cheunga KC, Wonga MH (2005) Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. Geoderma 125:155–166

Wu SC, Luo YM, Cheung KC, Wong MH (2006) Influence of bacteria on Pb and Zn speciation, mobility and bioavailability in soil: a laboratory study. Environ Pollut 144:765–773

Zahir ZA, Abbas SA, Khalid A, Arshad M (2000) Substrate-dependent microbially derived plant hormones for improving growth of maize seedlings. Pak J Biol Sci 3:289–291

Zahir ZA, Arshad M, Frankenberger WT (2004) Plant growth promoting rhizobacteria: applications and perspectives in agriculture. Adv Agron 81:96–168

Zahir ZA, Asghar HN, Akhtar MJ, Arshad M (2005) Precursor (L-tryptophan)-inoculum interaction for improving yields and nitrogen uptake of maize. J Plant Nutr 28:805–817

Zahir ZA, Munir A, Asghar HN, Arshad M, Shaharoona B (2008) Effectiveness of rhizobacteria containing ACC-deaminase for growth promotion of peas (*Pisum sativum*) under drought conditions. J Microbiol Biotechnol 18:982–987

Zaidi S, Usmani S, Singh BR, Musarrat J (2006) Significance of *Bacillus subtilis* strain SJ-101 as a bioinoculant for concurrent plant growth promotion and nickel accumulation in *Brassica juncea*. Chemosphere 64:991–997

Zhuang X, Chen J, Shim H, Bai Z (2007) New advances in plant growth promoting rhizobacteria for bioremediation. Environ Int 33:406–413

# Chapter 8
# Soil Health – A Precondition for Crop Production

**Niharendu Saha and Biswapati Mandal**

**Abstract** Soil health is an assessment of ability of a soil to meet its range of ecosystem functions appropriate to its environment. Soil health is generally used to measure the competence of soil to sustain plant and animal productivity, determine structural and functional diversity of microbes, maintain or enhance water and air quality, and support human health and habitation. Direct assessment of such parameters is a herculean task. As biological attributes are indicators of soil health, they are used for the diagnosis of soil heath. A comprehensive methodology integrating those indicators for soil health diagnosis is discussed. Further, the impact of soil health on crop production, quality improvement, production sustenance, and human, animal and environmental health is reviewed and discussed. In addition, the influence of soil management practices, like tillage, application of pesticides, fertilization, organic amendments, etc., on soil health is discussed. Inclusion of farmers and their experiences in the whole exercise of soil research are advocated for development of handy and farmer-friendly soil diagnosis protocols.

## 8.1 Introduction

Soil is the vital nonrenewable natural resource base. It is under competitive demand for increased crop production to feed the ever-growing population. This has led to intensification of agriculture with extensive use of chemicals, exploitation of surface and ground water for irrigation, and adoption of mechanization. The rapid pace of development in agriculture during recent years has resulted in an overall deterioration in soil-based ecosystems. The most important of such effects are the depletion of soil organic matter (SOM) and loss of biodiversity in the soil.

N. Saha (✉) and B. Mandal
Bidhan Chandra Krishi Viswavidyalaya, Kalyani, 741235, Nadia, West Bengal, India
e-mail: nihar_bckv@rediffmail.com

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_8, 

This leads to an overall deterioration of soil health resulting in damage to essential ecosystem functions, sustainability of agricultural production, and soil resilience capacity (Buresh et al. 1997; Doran and Zeiss 2000). The attendant problems are evident from the gradual decline in the rate of responses to external inputs and also deterioration in the quality of the product, as often observed even with the best possible management practices. The more the degree of intensification, the more is the severity of the problem (Abrol et al. 2000). Soil degradation and concomitant decline in soil health are often emphasized as constraints to crop productivity (Askegaard et al. 2004; Dick and Gregorich 2004; Wang et al. 2003). As a result, soil health has emerged as the key for assessing soil condition in the context of land management and sustainable crop production (Doran 2002; Karlen et al. 2004; Schjonning et al. 2004).

What does one mean by the term soil health? Does it include the soil with good bioavailability of plant nutrients? Does it embody the soil with good aggregate, proper aeration and high water-holding capacity? Or does it mean the empowerment of ecosystem processes leading to soil functioning for better crop production? The concept of Doran et al. (1996) and Gregory et al. (2002) includes all the attributes as mentioned and extends to ensure human, animal, plant and environment health. More than that, soil health is the specific condition of soil resulting from soil quality. As the term 'health' is normally associated with living entities, in this chapter, the soil's biotic component, particularly microbiological attributes, will be used for soil health diagnosis. The healthy condition of soil is vital both for production and for environmental sustainability (Karlen et al. 2003a, 2003b; Carter et al. 2004). Direct measurement of soil health is difficult, so indicators are used. The attributes which are most sensitive to management are considered to be the best indicators. Larson and Pierce (1991) proposed a minimum dataset (MDS) which was later on modified by Doran and Parkin (1994), who included biological properties in it, which is also supported by Schjonning et al. (2004).

Soil health assessment using physical and chemical indicators is sometimes misleading as it takes a longer time to exhibit a perceptible change in chemical and physical properties owing to the great buffering capacity of soil (Norfleet et al. 2003). So, the measurement of the biological component of soils in the first instance will be more suitable to assess soil health. Whenever there is any aberration in the soil system, soil organisms receive the stresses and respond immediately, and meaningfully, as they have a higher surface to volume ratio. In most cases, changes in populations of soil organisms or their activities can be detected much earlier than any change occurring in the physico-chemical properties of soil. This leads to being able to show improvement or degradation of soil health (Pankhurst et al. 1997), but there is inadequate available information on this aspect (Dick 1992; Heyer et al. 2003). Thus, many researchers have emphasized that biological indicators should be used to assess soil health (Elliot et al. 1996). Some of the biological indicators include microbial biomass C and N, dehydrogenase activity, acid and alkaline phosphatase activity, urease activity, arylsulphatase activity, soil respiration and ergosterol concentration, microbial diversity, and functional groups of soil fauna, etc. (Karlen et al. 1992; Hseu et al. 1999). Indicators influencing soil biological

health, however, vary according to the locations and the production systems involved (Karlen et al. 1994). Wylie (1994) concluded that it is not possible to develop a single list of biological indicators that could be suitable for all purposes and management practices. However, a few good biological indicators for assessing soil health for different production systems have already been constructed and are ready to use in soil health diagnosis (Anonymous 2005).

Microbe-mediated processes like decomposition, fixation, solubilization, filtration, remediation- and suppression of pathogens make the soil healthy and fertile. Soils with rich microbial diversity also protect crop from losses due to unpredictable climatic conditions of inundation, flood, and drought, etc. Experimental evidence showed microbial indicators like diversity, biomass and enzyme activities supporting higher biological yield and quality of crude protein and oil content (Anonymous 2005). So, identifying and quantifying the biotic component of soil for assessing aggradation or degradation of soil health for different production systems and subsequent mitigation options are a precondition for successful crop production.

## 8.2 Soil Health Concept

Soil is a wonderful gift of nature to humankind whose good health is essential for societal existence. But the high demographic pressure including nonagricultural operations and intensive cultivation is imposing tremendous stresses on soils. Some of the common stresses affecting soils are presented in Table 8.1. These are ultimately manifested in declined productivity of crops even under best possible management practices, and make soil "sick", so that it cannot respond efficiently to fertilization and other inputs. To arrest this deterioration, the general recommendation is to measure the nutrient status of soil and subsequently correct the nutrient deficiency, as well as control pest and disease incidents and involve other conservative steps. Routine measurement of such attributes of soils and recommendations for soil testing is not currently a problem, but on long-term basis it could be misleading and result in decreased productivity. Mere tests of soil for a few

**Table 8.1** Common soil stress and related degradation processes

| Stress | Degradation process |
|---|---|
| 1. Heavy load due to vehicular traffic | 1. Crusting, compacting, structural decline |
| 2. Poor internal drainage, slow surface drainage | 2. Soil wetness and anaerobiosis |
| 3. Intensive cropping | 3. Chemical degradation, nutrient imbalance, soil organic matter depletion, and habitat destruction |
| 4. Intensive use of agrochemicals and monoculture | 4. Biological degradation, acidification, reduction in soil biodiversity |
| 5. Monoculture | 5. Reduction in belowground diversity, infestation of soil-borne crop associated diseases, layer specific nutrient depletion, structural deterioration, etc. |

parameters inadequately address the problems of farmers, and, hence, the health of soil (fertility) under intensive agriculture cannot be properly judged. So, to address such problems associated with the deficiencies in agricultural practices, vital forces and processes should be identified and quantified. The vital forces and the associative processes conferring good living conditions to soil leading to healthy crop production may be termed as soil health.

Various researchers have expressed their views differently regarding soil health (Kinyangi 2007). Keeping pace with social priorities and increasing understanding of soil science, the concept of soil health has consistently changed (Warkentin 1995). Generally, the modern concept about soil health is mainly based on various functions that soil performs in any ecosystem. Based on the functional approach, Anderson and Gregorich (1984) defined soil health as "the sustained capability of a soil to accept, store and recycle water, nutrient and energy". Larson and Pierce (1991) further defined soil health as "the capacity of a soil to function within its ecosystem boundaries and interact positively with the environment external to that ecosystem". Later on, Acton and Gregorich (1995) stressed environmental concerns by defining soil health as "the soil's capacity or fitness to support crop growth without resulting in soil degradation or otherwise, harming the environment". A more detailed definition was developed by Soil Science Society of America (1995) as "the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain environmental quality and promote plant, animal and human health". This definition is similar to that of Doran et al. (1996), who defined soil quality as the "capacity of a soil to function, within ecosystem and land use boundaries, to sustain biological productivity, maintain environmental quality, and promote plant, animal and human health".

## 8.3 Soil Health Indicators and Criteria for Selection

Direct measurement of vital soil forces is next to impossible, so indicators are used to attain the goals. These indicators may be defined as measurable soil properties that influence the capacity of the soil to perform a specific function (Acton and Padbury 1993; Andrews and Moorman 2002). Attributes that are most sensitive to management and having low ecological redundancy are most desirable as indicators. In general, appropriate indicators include ecosystem process, the soil's physical, chemical and biological properties and processes, and a component of existing soil databases. Such indicators should also be sensitive to variation in management practices and climate, be easily measurable and reproducible, accessible to many users and applicable to field conditions, able to measure changes in soil function both at plot and landscape, and should lead to management decisions (Bouma 2002; Menge 2003).

The productivity of agricultural soils is known to depend greatly upon the activities of diverse microbial communities (Giller et al. 1997). But recently, soil

biodiversity is being threatened by agricultural intensification, particularly by land use change which is projected to have the largest global impact on soil biodiversity by the year 2100. This is more acute in tropical agriculture. Functional diversity, the key to ecological strategy to bring sustainability to production, is largely affected by long-term cultivation. Such cultivation leads to a decline in diversity and, hence, to poor soil health. Similarly, genetic diversity, particularly the diversity among microbes like mycorrhizal fungi (Boggs et al. 2003; Boddington and Dodd 2000), symbiotic nitrogen-fixing rhizobia (McGarth et al. 1998; Coutinho et al. 1999), and denitrifiers and nitrifiers (Cavigelli and Robartson 2000; Bruns et al. 1999) are also affected by high input agricultural activities. As functional diversity provides sustainability to agro-ecosystems and the genetic diversity imparts stability to speces (Hooper et al. 2000), they create the environment around themselves in such a way that other organisms can live only in the conditions thus created. These parameters can be included as indicators in soil health studies (Pompili et al. 2006; Saito 2007). However, high diversity is not always essential to run ecosystems because sometimes microorganisms fail to perform a specific function. Moreover, under unpredictable ecosystems (e.g., flood, drought, inundation, etc.), high microbial diversity is deemed necessary for imparting resistance against those stresses and resilience. In fact, some European countries are now including those parameters in routine soil monitoring programs (Nielsen and Winding 2002). For smallholder farmers, these tools need to be simple measures of soil health, such as consistency, color and workability. For extension and policy personnel, they provide basic information needed to arrive at management decisions. For researchers, there is a need to conduct detailed tests while controlling variations in order to develop meaningful assessments of soil fertility (Kang et al. 2005).

## 8.4 Significance of Soil Health Indicators

Soil biological parameters are early indicators of soil degradation and contamination (Visser and Parkinson 1992). A biological indicator is defined as “an organism, part of an organism, product of organism (e.g., enzyme), collection of organisms or biological processes which can be used to obtain information on the status of all or part of the environment” (Pankhurst et al. 1997).

Agricultural practices such as tillage, crop rotation, fertilization, and irrigation are generally known to have a significant effect on physico-chemical properties of soil; less is known about the associated changes in soil organisms and soil biochemical properties, and how such changes influence plant production and sustainability (Heyer et al. 2003). The activity of soil biota brings out nutrient transformation in soils and underpins a number of fundamental soil properties such as fertility and structure (Filip 2002; Osinski et al. 2003). Therefore, changes in soil organism activity are indicative of, and extremely sensitive to, changes in soil health (Nielsen and Winding 2002; Anderson 2003). Despite their small volume in the soil, microorganisms play a role in the cycling of elements by

decomposing organic residues. The organic residues are converted to biomass or mineralized to $CO_2$, $H_2O$, mineral N, P, and other nutrients by these soil residents. Mineral nutrients immobilized in microbial biomass are subsequently released by mineralization. In addition to the effect on nutrient cycling, microorganisms also affect the physical properties of soil. For example, production of extracellular polysaccharides and cellular debris by microorganisms helps in improving soil structure and stabilizing soil aggregates. Thereby, they also affect WHC, infiltration rate, crusting, erodibility, and susceptibility to compaction. Thus, populations of soil organisms and/or biochemical processes mediated by them are potentially sensitive bioindicators of changes in soil health (Arshad and Martin 2002). Many researchers have, therefore, stressed the importance of identifying biological indicators (Doran and Parkin 1994; Elliot et al. 1996). In some instances, changes in an organism's populations or soil biotic activities could precede detectable changes in the physico-chemical properties of soil, thereby providing an early sign of improvement or warning of soil degradation. So, monitoring of soil quality includes the assessment of biomass C and N, respiration measurement, mineralization rate, soil enzyme activities, microbial diversity, and functional groups of soil fauna. Indicators influencing soil health, however, vary according to the locations, production system, and management practices adopted (Table 8.2). Therefore, it is not possible to develop a single list of biological indicators which could be suitable for all purposes and management practices (Wylie 1994). However, microbial biomass carbon (MBC) is almost universally accepted as a biological indicator irrespective of production system and management practice (Anonymous 2005). The microbial biomass C is used as a sensitive indicator of changes in soil processes due to changes in management practices and cropping systems, because it has a much faster turnover rate than the total SOM and, hence, is the most labile C pool in soils (Jenkinson 1981; Paul 1984). Moreover, nutrient availability and productivity of agro-ecosystems depend on the size and activity of microbial populations; a strong link between soil microbial biomass, soil fertility, and soil health is reported (Hart and Brookes 1996; Kandeler and Eder 1993).

Microbial quotient (% $C_{mic}/C_{org}$) can be used as an indicator of changing soil processes under different cropping sequences and management practices and is a

**Table 8.2** Microbial/biochemical indicators identified for different soil types and cropping systems (Adapted from Anonymous 2005)

| Soil type | Cropping system | Identified indicators |
|---|---|---|
| Clay loam | Rice–rice | Dehydrogenase enzyme activity, organic carbon |
| Sandy loam | Groundnut–redgram | Organic carbon, microbial biomass carbon (MBC) |
| Sandy loam | Rice–lentil | Dehydrogenase enzyme activity, organic carbon |
| Sandy | Sorghum–castor | MBC |
| Sandy loam | Jute–rice–wheat | Dehydrogenase enzyme activity |
| Sandy clay loam | Rice–rice | Dehydrogenase enzyme activity and microbial biomass nitrogen |
| Silty clay loam | Rice–field pea | MBC |
| Sandy loam | Rice–wheat | Organic carbon, alkaline phosphatse enzyme activity, and potentially mineralizable nitrogen |

more useful measure than either MBC or total carbon assessed individually (Dilly and Munch 1998), and it also helps in comparing soils with different organic matter contents. Entry et al. (1996) suggested that microbial biomass C and the $C_{mic}$:$C_{org}$ ratio are poor predictors of annual crop yield but may be an accurate indicator of soil health and a good predictor of long-term crop yield. Furthermore, Insam and Haselwandter (1989) reported that the respiratory quotient (μg $CO_2$–C $h^{-1} mg^{-1}$ MBC) could be used as an indicator of soil development, substrate quality, ecosystem development, and ecosystem stress.

Enzyme activities are an important index of the biological activity of a soil because they are involved in the dynamics of soil nutrient cycling and energy transfer (Table 8.4). Enzymatic processes are closely associated with soil fertility as they mediate the conversion of unavailable forms of nutrients to forms that are readily assimilable by plants and microbes (Sarkar et al. 1989). Moreover, enzyme activity of depends not only on nutrient status and availability but also on the turnover of N, P, and other nutrients in soils. As enzymes do not react readily to environmental changes as do the microbes, enzyme activity is considered a more stable indicator of biological processes (Bandick and Dick 1999).

## 8.5 Approaches Used to Diagnose Soil Health

### *8.5.1 Methodology for Selection of Master Indicators*

#### 8.5.1.1 Data screening, Representative Variables and Redundancy

Data are reduced to meaningful manageable size, i.e., to a MDS of soil quality indicators, through a series of uni- and multivariate statistical methods. Both parametric (randomized block design) as well as nonparametric statistics (Kruskal–Wallis $\chi^2$) are used to identify indicators with significant treatment differences. Only variables with significant differences between treatments are chosen for the next step in MDS formation. To select a subset from a large dataset, principal component analysis (PCA) for each statistically significant variable is performed, assuming that principal components receiving high Eigen values and variables with high factor loadings best represent system attributes, and examining only the principal components that explain at least 5% of the variation in the data up to 85% of the cumulative variation. Within each principal component, only highly weighted factors, i.e., those with absolute values within 10% of the highest weight, are retained for the MDS. To reduce redundancy and rule out spurious groupings among the highly weighted variables within each principal component, multivariate correlation coefficients are run to determine the strength of the relationships among variables. Well-correlated variables are considered redundant and candidates for elimination from the dataset. Conversely, any uncorrelated, highly weighted variables are considered important and, therefore, retained in the MDS (Andrews et al. 2004).

#### 8.5.1.2 MDS Validation and Indicator Transformation (Scoring)

Multiple regressions and hierarchical cluster analysis are run using the final MDS components as the independent variables and each management-goal attribute (e.g., yield, sustainable yield index, protein yield, etc.) as a dependent variable. These regressions and cluster analysis serve to check the MDS representation of management system goals. After determining the variables for the MDS, every observation of each MDS indicator is transformed for inclusion in the SHI methods (e.g., linear scoring technique). In this technique, indicators are ranked in ascending or descending order depending on whether a higher value is considered "good" or "bad" in terms of soil function. For 'more is better' indicators, each observation is divided by the highest observed value such that the highest observed value received a score of 1. For 'less is better' indicators, the lowest observed value (in the numerator) is divided by each observation (in the denominator) such that the lowest observed value receives a score of 1. (Anonymous 2005).

#### 8.5.1.3 Indicator Integration Into Indices

After transforming MDS into scoring, they are integrated into soil health indices following additive SHI (ADD SHI) and a weighted, additive SHI (WTD SHI). The additive index is a summation of the scores from the MDS indicators. From this summed score, the ADD SQI treatment means and standard deviations are calculated. In the weighted additive index after transformation, the MDS variables for each observation are weighted using the PCA results. The percentage, standardized to unity, provides the weight for variables chosen under a given PC. The weighted MDS variables scores for each observation are then summed and the treatment means and standard deviations are calculated. For all the indexing methods, SHI scores for the management treatment are compared using a two-way ANOVA. Higher index scores are assumed to mean better soil quality.

## 8.6 Soil Health and Its Importance

Soil contains soil organisms which perform different functions and facilitate crop production and value addition. In addition, they detoxify all the nuisance stuffs dumped into soil to make the earth clean and fit for living organisms. The multifaceted activities of soils are discussed in the following section.

### *8.6.1 Crop Production*

Healthy soil fosters fertility which in turn increases crop production (Table 8.3). The health of soil can affect not only the productivity but also the quality of produce

**Table 8.3** Yield of Kharif rice and sustainability yield index for 18 years of cultivation with organic and inorganic sources of nutrients

| Treatment | Soil health index (additive linear index) | Yield (kg $ha^{-1}$) | Sustainable yield index |
|---|---|---|---|
| Control | 2.783 | 1,493[b] | 0.234[c] |
| N[a] | 2.688 | 2,000[b] | 0.616[d] |
| NP | 3.188 | 2,560[e] | 0.684[f] |
| NPK | 3.628 | 3,240[f] | 0.638[b] |
| NPK + FYM | 3.726 | 3,320[b] | 0.669[e] |
| Mean | – | 2,523 | 0.568 |
| SE (m) | – | 169.34 | – |

[a]Recommended dose of fertilizer (Adapted from Anonymous 2005)
[b–f]Values indicate the mean of three replicates. Mean values followed by different letters are significantly different within rows or column at $P \leq 0.05$ according to Tukey test.

and the sustainability of land (Acton and Gregorich 1995; Doran 2002; Wander et al. 2002; Carter et al. 2004). Under healthy soil conditions, numerous nutrient cycles influenced by microbes operate simultaneously in soil. Soil thus contributes nutrients uninterruptedly to the nutrient pool of soil ensuring balanced plant nutrition. Moreover, healthy soil conserves nutrients and releases them more or less synchronously with the demand of growing crops. Thus, nutrient use efficiency and nutrient conversion ratio remain favorable for reasonable production. Well-managed soils harbor a wide spectrum of microorganisms performing a variety of activities including synthesis of phytohormones, antibiotics, growth regulators, siderophores, etc., which directly or indirectly affect crop productivity. Under good soil health, internal soil defensive mechanisms remain constantly strong and vital to combat soil-borne diseases. Thus, crop loss due to diseases can be minimized and consequently yield is increased.

### *8.6.2 Crop Quality*

Lack of balanced nutrition is the most important reason for poor quality produce in unhealthy soil. Under intensified agriculture practices where soil health is in a miserable condition, the fundamental ratio of N:P:K has shifted from 2:1:1 to different imbalance ratios in different regions. Thus, production may be increased for a while but crop quality does not increase with the productivity. Production, productivity and quality of agricultural produce largely depend on the state of soil, microbial status and their specific functions leading to consistent soil health. The influence on crop quality is triggered by the specific biochemical reaction of microbes. For example, polyunsaturated fatty acid, Omega 3 fatty acid content in oil, essential amino acid in proteins, inulin and fructan in carbohydrates (Saha et al. 2007), and antioxidant and antiaging agents like β-carotene, lycopene, etc., are the quality attributes of agricultural produce. Experimental evidence showed that microbial indicators like diversity, biomass and enzyme activities have supported higher biological yield and certain qualities like protein and oil content

of crops (Anonymous 2005), and the quality under organically managed soil has been found to be superior to conventionally managed soil.

### 8.6.3 Production Sustainability

The sustainability of any production system depends on the stability of that system. Stability, on the other hand, of an ecosystem encompasses functional resistance, i.e., the capacity of the system to endure stresses imposed and to carry out all the essential ecosystem services during the entire period of stress leading to good production and functional resilience (Seybold et al. 1997, 1999). The two basic ecosystem attributes measure soil health and impart sustainability in agricultural production system. Healthy soil with strong lifeline activities capable of resisting all unusual situations emerging from indiscriminate agricultural practices maintains a satisfactory level of production. The influence of soil health on sustainability of agricultural production is more appropriate in unpredictable agro-ecosystems like drought, flood, inundation, and contaminated soils. Narrow ecological associations, such as legume–rhizobia sysbiosis and sustainability of legume production are highly dependent on soil health (Table 8.3).

### 8.6.4 Human Health

The nutritional health and well being of humans are entirely dependent on plant foods either directly or indirectly (Leo et al. 2002). Plant foods provide almost all essential vitamins and minerals, carbohydrate, protein, fat, and a number of other health-promoting phytochemicals like antioxidants and antiaging substances. Healthy soil provides a conducive environment for plants to grow better so that plants can synthesize all the basic molecules which, through the food chain enter the human system and get metabolized for carrying out particular biochemical reactions. Generally, micronutrient concentrations are low in cereals and fortification of multi-micronutrients in food stuffs causes antagonistic effects on the micronutrients. Sustainable soil management leading to improving the bioavailability of micronutrients in order to improve crop nutritional quality is the best alternative. The clinical health of humans is also dependent on quality plant food to some extent. The antioxidant and antiaging molecules like β-carotene and lycopene, and cardiovascular disease-inducing agents like, triglycerides in oilseed crops, etc., are largely influenced by balanced nutrition. Organically produced food generally contains higher amounts of antioxidant and antiaging agents and lower magnitude triglycerides, indicating that soil health under an organic system is perfectly good.

### 8.6.5 Animal Health

The modern soil health concept encompasses sound animal health as one of its components. Healthy soil is essential for producing nutrient-rich fodder for healthy animals in any farming system. Under good soil conditions, a micronutrient like iodine content in fodder crops increases, which might result in better growth of animals, higher iodine content of animal products such as meat and milk, and indirectly affect human health. Soil management practices has led to exhaustion of micronutrients in a given area and, hence, water derived from this area may cause micronutrient deficiency which could be reflected in animal health (Lee et al. 1999; Ellison 2002; Vaarst et al. 2003).

### 8.6.6 Environmental Health

Soils via microbes perform myriads of ecosystem services including decomposition, transformation, detoxification, infiltration, bioremediation, fixation, emission, solubilization, disinfection (antibiosis), commensalisms, protocooperation, symbiosis, predation, and resistance and resilience against different stresses; these collectively represent the lifeline activities of healthy soil. If soil is healthy, lifeline activities remain in resonance. Farming methods and activities sometimes disrupt the rhythm and, thus, soil becomes sick. Unhealthy soil cannot sequester carbon but rather triggers $CO_2$, methane, and other greenhouse gas emissions which in turn affects ecosystems. For instance, high concentrations of greenhouse gasses have caused an increase in global temperature that has threatened both food security and shelter. Changes in land use contribute about 14% of the total human-generated emissions of greenhouse gases, and much of this land development is for agricultural purposes (Rosenzweig and Hillel 1998). But healthy soil can prevent global warming by sequestering carbon in the soil as a way to slow down the concentration of $CO_2$ in the air, thus slowing the rate of climate change. Indiscriminate use of fossil fuel-based inputs may lead to a gradual decline in nutrients and WHC of soils. Thus, a plant nutrient is either leached down to pollute groundwater or emitted out to pollute air. Due to poor WHC along with poor carbon sequestration, desertification is now a phenomenal development of unhealthy soils in different parts of the globe, particularly in the sub-Saharan region of Africa. Reports suggest that soil influences environmental quality and the overall functioning of the biosphere by functioning as a living filter, through which water is cycled and xenobiotics are altered (Karlen et al. 2001). Consequently, the manner in which soils are managed has a tremendous impact on the environment and its inhabitants. This fact is supported by archaeological evidence showing that soil degradation was responsible for the extinction and collapse of the Harappa civilization in India, Mesopotamia in Asia, and the Mayen culture in Central America (Olson 1981).

## 8.7 Problems in Agricultural Practices

### *8.7.1 Lack of Good Soil Husbandry*

The rise and fall of many civilizations in the world was associated with the soil husbandry followed by the citizens of the nations (Ponting 1992). Poor land care by the inhabitants was one of the serious causes of ruin of the great Harappan civilization of ancient Indian Subcontinent (Olson 1981). In contrast, the glorious Egyptian Civilization was due to good stewardship of soils of the Nile basin. In ancient times, the soil was treated as 'Mother' and any agricultural activities in the soil were started with the worship of the land. Soil is now considered as the machinery for crop production. Soils are extracted in all possible ways without paying due care. Round the year cultivation resulting in soaring cropping intensity allows little rest to the land to recover its deficits in its health status. Thus, soil health further deteriorates. Previously, soil was routinely fed with organic matter from diverse sources, but the overnight magical effect turns the farmers towards proponents of chemical fertilizers in present day agriculture. The avoidance of organics, particularly in the post-green revolution era, has damaged soil health including structural aberrations leading to a hostile habitat for microbes, poor aeration, WHC, and nutrient retention capacity. Soils, thus, become sick. The health of soil, hence, requires greater attention as to how the fertility could be restored in degraded lands.

### *8.7.2 Agricultural Intensification and Inappropriate Cropping System*

The increasing demands for food to satisfy the need of the ever-expanding human population has led to intensification of agricultural practices with extensive use of chemicals, exploitation of surface and ground water for irrigation, and adoption of mechanization. These practices have resulted in severe adverse effects on the physical, chemical, and biological properties of soil. The most important of such effects is the depletion of SOM and loss of microbial diversity which together lead to damage to the essential ecosystem functions, the sustainability of agricultural production system and to soil resilience capacity (Buresh et al. 1997; Doran and Zeiss 2000).

A particular cropping system in any area has its own history. After being tested for a long time, it has been agronomically accepted not only for its good economic return but also for its role in maintaining soil health. In a traditional cropping system, legumes have been the farmers' choice. But recently, with the introduction of vegetables (particularly hybrid vegetables), the space for the legumes is shrinking, leading to poor soil health. Vegetable cultivation round the year degrades soil

aggregates into talc-like powder which facilitates fertile topsoil erosion, higher leaching of nutrients to ground water, as well as to eutrophication of water bodies. And, hence, fertilizer use efficiency declines, production decreases, and human, animal and environmental health are affected.

### 8.7.3 Blanket Application of Agrochemicals

From the very beginning of the green revolution, farmers have been desperately and indiscriminately using fertilizers and pesticides. Moreover, new molecules of biologically active metabolites, growth regulators, and hormones are being frequently used in high input agriculture. All those chemicals are foreign bodies to soil interacting differently with the soil's inherent entities and creating hostile environments leading to poor soil health. In this context, genetically modified inputs like seed and biocontrol agents are in the queue to join modern agricultural activities. Though confusing, they may have a long-term soil health hazard problem directly or indirectly (US National Research Council, 2000; Nordlee et al. 1996).

### 8.7.4 Reduction in Agricultural Biodiversity/Monoculture

Practicing a very few restricted cropping systems and/or monocultures over the years results in decreases in aboveground plant biodiversity as well as a sharp decline in belowground biodiversity including the organisms antagonist to plant pathogens. The production system thus becomes vulnerable to harmful soil organisms and the soil becomes sick. Emergence of nematode diseases as one of the important problems in recent years is a fallout of such soil sickness.

### 8.7.5 Heavy Vehicle Traffic and Indiscriminate Use of Sewage Sludge

Low energy-based conventional bullock-driven agricultural operations are almost absent in modern agriculture. They have been substituted by the introduction of energy-intensive heavy vehicles, like tractors, power tillers, combined harvesters, etc. The operation of such machines in agricultural practices results in soil compaction leading to destruction of microhabitats, reduction of soil biological entities, decline in nutrient transformation, poor aeration, and poor drainage. The long-term effects of such operations in agricultural soils causes soil health to deteriorate.

Due to the low capacity of sewage treatment plants, agricultural soils receive raw effluent emerging from different industrial or agrochemical sources. Raw sewage

water contains organic and inorganic pollutants, such as heavy metals, as well as human and plant pathogenic organisms. The discharge of heavy metals into the soil or the use of sewage sludges in agronomic practices as a source of irrigation leads to loss in soil fertility, disturbances in microbial equilibrium, and eventually makes the soil unsuitable for cultivation (Khan et al. 2009).

### 8.7.6 Lack of Appropriate Soil Testing and Diagnostic Protocol

Routine soil testing measures the status of soil nutrients and a few physical properties of the soils. Such analysis of soil is done to find out the nutrient deficiency in soils which further help to design the nutrient strategy for the deficient soils. The current concept of fertilizer application is directed to feed the plants but not the soil. Thus, underestimating the contribution of soil and its role in consistently supplying nutrients to plants is important. Moreover, even though soil microflora drives the key processes of soil, no serious attempt has yet been made to assess the functional diversity of microbes impacting on soil health. Furthermore, laboratory-based indicators for assessing soil are not easily available to farmers and ready to use soil health indicators at the field level are poorly developed.

### 8.7.7 Insufficient Awareness Among Farmers About Soil Health

Farmers are the central figures of any soil health management program. They are the stewards of soil health. But, presently there is a gap between skills and knowledge acquired by individual farmers, while at the community level there is inadequate awareness about modern scientific innovations. Effective participation of farmers in combined learning and experimental research for soil health assessment and management is still lacking. Approaches adopted to motivate farmers, such as state of the art information and how to make maximum use of modern agronomic tools are not sufficiently available to agrarian communities.

### 8.7.8 Lack of Proper Legislation and Contractual Farming

Unlike air, water and sound pollution, no systematic and proper guidelines for assessing soil health is currently in action in many countries experiencing soil deterioration. Although the European Union has initiated legislation to protect soil health, no governmental initiative regarding soil health protection is in action

in developing countries. Furthermore, since no legal institutions are available in almost all countries to police over the misuse and abuse of soil, no ethics are followed in the use of soil for specific purposes. Licensees of contractual farms try to extract all possible benefits from the leased land within the stipulated contract period without considering soil as a nonrenewable resource. So, a piece of healthy land turns sick by the time it is returned to the licensor. This is a common picture in contractual farming systems. Moreover**,** farmers in some countries, like India, sell the top soil to brick factories or to contractors for land fill and other nonagricultural purposes after the crop harvest. Thus healthy soil, generated over a decade or more, is destroyed with the stroke of a spade.

### *8.7.9 Government Policy*

Government subsidies often help perpetuate unsustainable practices. Subsidies often stimulate greater use of chemicals, despite their environmental and public health risks. Rice farmers in Japan, Taiwan, and Korea use just over one-half of all insecticides applied to rice worldwide yet produce only 2% of the world's crops. The reason is that large government price support makes it profitable to increase insecticide use even when the resulting production gains are small (Vorley and Keeney 1998). This is equally true for India which indiscriminately uses highly subsidized nitrogenous fertilizer. Besides encouraging harmful practices, farm subsidy programs often fail to reward good stewardship. They tend to emphasize a handful of major crops and put resource-conserving crop rotations at a financial disadvantage (Faeth and Westra 1993). Farmers receive no government incentives for sustainable practices such as growing alfalfa or *dhaincha* (*Sesbania rostata*).

## 8.8 Soil Health Management

### *8.8.1 Crop Rotation and Organic Amendments*

Crop rotation is a viable strategy for increasing SOM for the establishment of healthy, fertile, and productive soil. Crop rotation fulfils nutrient demand from different depth of soil, thus reducing nutrient mining and hardpan by root penetration (Corselius et al. 2001). For example, rotations that include cereals leave a significant amount of leftover stubbles after harvest. By including these crops in vegetable rotations, a grower can reduce the incidence of potentially devastating soil-borne diseases and outbreaks of phytopathogenic nematodes (FAO 2008). Rotations can also check weeds and insect pests that use the weeds as alternative hosts for perpetuation. The practice of crop rotation with legumes can thus improve

**Table 8.4** Soil health index (SHI) value for different management at various experimental sites

| Treatment/site | AAU | ANGRAU | BHU | CRIDA | CRIJAF | CRRI | OUAT | BCKV |
|---|---|---|---|---|---|---|---|---|
| Control | 2.27 | 0.92 | 1.63 | 0.95 | 1.04 | 2.77 | 0.31 | 2.78 |
| N[a] | 2.60 | – | 1.48 | – | 1.38 | 2.91 | 0.35 | – |
| NP | 2.59 | – | – | 1.02 | 1.66 | 3.21 | 0.78 | – |
| NPK | 2.79 | 0.97 | 1.52 | – | 1.87 | 3.10 | 0.81 | 2.69 |
| NPK + FYM | 2.84 | 2.00 | 1.87 | 1.27 | 2.10 | 4.00 | 1.13 | 3.63 |

[a]Recommended dose of fertilizer (Adapted from Anonymous 2005)

soil quality, protect top fertile soil erosion, and improve SOM status, which in turn improves productivity and consistency in production system,. Furthermore, the appropriate land management, such as fertilization combined with crop rotation and reducing 1-year-old fallows, would be useful ways to improve or maintain soil fertility (Jia et al. 2005).

Deliberate and routine carbon sources are essential to achieving good soil health in agricultural production systems (Majumder et al. 2008; Mitchell et al. 2000). Special care is needed to select carbon sources that will ensure short-term productivity while building long-term soil health. Farm yard manure (FYM) is considered as an organic amendment to improve soil health in different production systems. In a case study, the soil health index was computed from a minimum dataset in an integrated nutrient management system with FYM as sole organics: the soil health index was superior to conventional farming with chemical fertilizers (Table 8.4). However, as there is no definite protocol for FYM preparation, quality varies tremendously. Thus, compost, phosphocompost, and vermicompost with relatively long-lasting and strong persistence of carbon prepared under defined protocols have fair scope to be used as organics for better soil health. Furthermore, concentrated organics, like edible and nonedible oil cakes, rice bran, pulse bran, etc., can be used for improving soil health. In situ stubble incorporation in combination with cellulolytic microorganisms and green manuring are of great practical importance for maintaining soil health under rice-based cropping systems (Bhattacharya 2004). Such organic amendments add significant amounts of carbon to soil and are generally associated with rich microbial diversity in protected and conserved ecosystems for carrying out lifeline activities of soil.

## 8.9 Cover crops and Tillage Conservation

Cover cropping (also called green manuring) is a wise prescription for a soil health management program (Leo et al. 2002). Cover crops can provide a practical and economical means for supplying organic matter, enhancing soil fertility, suppressing weed growth, attracting beneficial insects, spiders and predatory mites, and reducing nitrate leaching looses to the groundwater (FAO 2008). Soils differing in fertility as well as health properties routinely receive these inputs compared to conventionally managed soil (Mitchell et al. 2000).

The beneficial effect of the tillage systems (no till and low till farming) are based on the premise that minimizes the disturbance to the soil leading to an increase in the retention of water, nutrients, and the topsoil itself (Pretty 1995; Leo et al. 2002). In tillage conservation, crops are grown with minimal cultivation of the soil. When the amount of tillage is reduced, the stubble or plant residues are not completely incorporated, and most or all remain on top of the soil rather than being ploughed or disked into the soil. The new crop is planted into this stubble or into small strips of tilled soil. Weeds are controlled with cover crops or herbicides rather than by cultivation. Fertilizer and lime are either incorporated earlier in the production cycle or placed on top of the soil at planting (FAO 2008). The tillage encourages soil flora and fauna under the protected habitat to bring out the biogeochemical processes leading to optimization of carbon turnover and bioavailability of nutrient elements in the soil (Mitchell et al. 2000). Natural processes involved in conservation farming include insect tunnels made by the movement of earthworms (drillosphere) and arthropods, and root tunnels created after the decomposition of leftover root debris make the soil porous and a loose fit for root ramification of the next crop with a good stand. Successful cultivation of relay cropping of legumes and/or mustard with winter rice is an example of no till farming in different parts of India.

### *8.9.1 Agricultural Diversification*

There is a relationship between aboveground and belowground diversity. After the green revolution, there has been a dramatic reduction in crop diversity which in turn has had a negative impact on belowground microbial diversity. The soil foodweb and its internal regulatory system have been badly disrupted. Furthermore, soil becomes vulnerable to diseases and pests and, consequently, production declines. This can be repaired by agricultural diversification. Growing a variety of crops will ensure rich belowground diversity which in turn revitalizes soil health and also provides a buffer against both ecological and economical problems. This can be achieved by the following.

#### 8.9.1.1 Selection of Right Cropping System

Cropping system as a whole has a great influence on soil health (Johnson et al. 2003). A production system causing deterioration in an agroecosystem is recommended to be omitted to minimize further deterioration. A system which leaves more residues and produce more exudates (rhizodeposition) can sequester higher carbon in the soil. For example, a cereal-based cropping system which retains stubble and huge root biomass and debris is preferred in degraded and fragile ecosystems for restoration of soil health. In addition, double-cropping rice practice also helps to maintain soil carbon and, thus, soil health (Mandal et al. 2008). In a

rice–rice system, kharif rice may be coupled with lentil as a relay crop, and winter rice followed by a green manuring crop to revitalize the soil internal regulatory system with a huge supply of easily accumulative carbon. Moreover, farmers can harvest a third crop with minimal investment and disturbance to the soil.

#### 8.9.1.2 Tailoring of Existing Cropping System

The cropping system may affect soil health by modifying soil microbial community structure leading thereby to a negative impact on soil fertility and, hence, crop yields (Kowalchuk et al. 2002; Mandal et al. 2008). Immediately after identifying the crops severely damaging soil biological health, it must be eliminated and a new cropping system be developed by incorporating suitable means for repairing the damage. For this, the following options may be explored. In rice–potato–sesame, sesame (*Sesamum indicum* L.) may be replaced by cowpea, whereas in rice–vegetable–vegetable, late cauliflower may be replaced by bean. The basis of replacement of sesame by cowpea is (1) sesame is susceptible to *Macrophomina* spp. causing low yield, so (b) to arrest this, a new crop such as cowpea may be introduced, as legumes have a special role in N-economy. However, in rice–vegetable–vegetable, late cauliflower may be replaced by bean because (1) early cauliflower followed by late cauliflower may impair the soil health by increasing inocula density of cauliflower associated soilborne pathogens, and (2) a cauliflower–cauliflower system causes seasonal glut in the market that hardly helps farmers to earn a remunerative price.

#### 8.9.1.3 Farming System Diversification

Crop diversification alone cannot bring stability to soil health. Rather, farming system diversification is necessary for overall improvement of soil health. Integrating animal and plant production has been considered as a wise policy to maintain a higher biotic diversity in sustainable farming. Agriculture and its allied activities, i.e., horticulture, agroforestry, organic farming, animal husbandry, fishery, poultry, etc., can benefit each other. Fish cum rice cultivation can be readopted in some specific rice growing areas for sustainable rice production and farm income. Resources can be properly recycled under diverse activities in mixed farming. Thus, resource utilization becomes optimum under mixed farming systems which in turn impart sustainability to any production system (Leo et al. 2002).

### *8.9.2 Organic Husbandry*

This is a holistic production management system that promotes and enhances agro-ecosystem health, including biodiversity, biological cycles, and soil biological

activities. Organic production systems are based on specific and precise standards of production which aim to achieve optimal agro-ecosystems which are socially, ecologically, and economically sustainable. The primary goal of organic agriculture is to optimize the health and productivity of interdependent communities of soil life, plants, animals, and people. Organic agriculture manages locally available resources to optimize competition for food and space between different plant and animal species. The manipulation of the temporal and spatial distribution of biodiversity is the main productive input of organic farmers. Organic practices such as crop rotation, cover crops, organic fertilizers, and minimum tillage increase the diversity and richness of indigenous soil life which in turn impact essential ecosystem services.

### *8.9.3 Soil-Based Fertilizer and Development of Farmer's Friendly Indicator*

There is a conspicuous gap between nutrient demand and addition. This is mostly due to nonconsideration of crop nutrition with crop demand. Thus, a net nutrient deficit increases day by day. The nutrient imbalance in soil has, thus, caused a poor growth in agricultural production necessitating soil testing and fertilizer recommendation on the basis of the test values. Such nutritional analysis of soils will help to design balance fertilization for various crops and will also help to take measures against environmental pollution. Farmers have a vested interest in soil health, and, hence, its maintenance has been the top priority for them. Farmer interest in soil health may have been encouraged by their desire to examine and validate the management practices they use in their own farm. Thus, they need familiar soil characteristics easy to interpret by themselves as soil indicators. The working knowledge of farmers obtained through experience will help researchers to articulate their experience with research findings to design farmer-friendly indicators. The soil taxonomies and intricate methods for fertility management should be taken into consideration while developing handy soil health indicators.

### *8.9.4 Legislation for Soil Health Protection*

Attempts should be made to enact legislation for protecting soil against its abuse and misuse. Selling of top soil for other nonagricultural activities should be treated as a punishable offence. In addition, government-endorsed drafts of agreement between licensor and license in regard to the status quo of soil health in contractual farming systems should be followed. And, at the time of return of the land to the licensor, if the soil health has deteriorated, the licensee should be liable to pay extra at certain rates to rejuvenate it.

## 8.10 Conclusion

Food demand for rapidly increasing the global human population is expected to expand substantially over the next decades, which in turn could make them food insecure. The excessive inputs and unethical activities in agriculture in developing countries are some of the reasons why fertile land is degrading rapidly and destroying ecosystems. Due to these problems, soil resource inventory and monitoring have gained momentum. And, as a consequence, the concept of soil health has generated awareness among agriculturists regarding the importance of soil in maintaining plant productivity and environment quality. There is a need to better understand relationships between the status of soil health indicators and fluctuating soil functions. In this context, state of the art research is needed in order to find and develop proper indicators, applicable at the farmer scale. The technology thus developed would be likely to help in setting up effective and ready to use protocols which may guarantee to generate the necessary baseline soil data in order to develop appropriate MDS encompassing all aspects of agro-ecosystem performance. In this regard, innovative use of geographical information systems and remote sensing will significantly improve the acceptance of soil health information by stakeholders. Laboratory-based indicators so far developed definitely have a substantive role in the scientific and more precise monitoring of terrestrial ecology. But sometimes such parameters lose their relevancy to the farmers and, hence, there is an urgent need to develop handy, meaningful and farmer-friendly sets of indicators.

## References

Abrol IP, Bronson KF, Duxbury JM, Gupta RK (2000) Long-term fertility experiments in rice-wheat cropping systems. Rice-wheat consortium paper series 6. Rice-wheat consortium for the Indo-Gangetic Plains, New Delhi, India, p 171

Acton DF, Gregorich IJ (1995) Understanding soil health. In: DF Acton, IJ Gregorich (eds) The health of our soils: towards sustainable agriculture in Canada. Centre for Land and Biological Resource Research, Agricultural and Agri-Food, Ottawa, Ontario, Canada, pp 5–10

Acton DF, Padbury GA (1993) A conceptual framework for soil quality assessment and monitoring. In: Acton DF (ed) A program to assess and monitor soil quality in Canada: soil quality evaluation program summary (Interim). Centre for land and biological resources research. Contribution No. 93–94. Research Branch, Agriculture Canada, Ottawa, pp 2–7

Anderson TH (2003) Microbial eco-physiological indicators to assess soil quality. Agric Ecosyst Environ 98:285–293

Anderson DW, Gregorich FG (1984) Effect of soil erosion on soil quality and productivity. In: Soil erosion and degradation. Proceedings of 2nd Annual Western Prov. Conference on National Water Soil Research management, Saskatoon, Canada, pp 105–113

Andrews SS, Moorman TB (2002) Soil quality as an indicator of sustainable land management: demonstrated successes and continued needs. Agron J 94:1–2

Andrews SS, Karlen DL, Cambardella CA (2004) The soil management assessment framework: a quantitative soil quality evaluation method. Soil Sci Soc Am J 68:1945–1962

Anonymous (2005) Assessment and improvement of soil quality and resilience for rainfed production system completion report code no RRPS-20. National Agricultural Technology Project (NATP), CRIDA, Hyderabad, pp 10–12
Arshad MA, Martin S (2002) Identifying critical limits for soil quality indicators in agro-ecosystems. Agric Ecosys Environ 88:153–160
Askegaard M, Eriksen J, Johnson AE, Schjonning P, Elmholt S, Christensen BT (2004) Sustainable management of potassium. Managing soil quality: challenges in modern agriculture. CAB International, Wallingford, UK, pp 85–101
Bandick AK, Dick RP (1999) Field management effects on soil enzyme activities. Soil Biol Biochem 31:1471–1479
Bhattacharya B (2004) *In situ* utilization of rice stubble in relation to soil fertility vis-à-vis performance of wheat crop. PhD Dissertation, BCKV, Kalyani, India
Boddington CL, Dodd JC (2000) The effect of agricultural practices on the development of indigenous arbuscular mycorrhizal fungi. I. Field studies in an Indonesian Ultisol. Plant Soil 218:137–144
Boggs LC, Stahl PD, Lindstrom MJ, Schumacher TE (2003) Soil microbial properties under permanent grass, conventional tillage, and no-till management in South Dakota. Soil Till Res 71:15–23
Bouma J (2002) Land quality indicators of sustainable land management across scales. Agric Ecosyst Environ 88:129–136
Bruns MA, Stephen JR, Kowalchuk GA, Prosser JI, Paul EA (1999) Comparative diversity of ammonia-oxidizer 16 S rRNA gene sequences in native, tilled, and successional soils. Appl Environ Microbiol 65:2994–3000
Buresh RJ, Sanchez PA, Calhour F (1997) Replenishing Soil Fertility in Africa, vol 51. Soil Science Society of America, Madison, USA
Carter MR, Andrews SS, Drinkwater LE (2004) Systems approaches for improving soil quality. In: Schjonning P, Elmholt S, Christensen BT (eds) Managing soil quality: challenges in modern agriculture. CAB International, Wallingford, UK, pp 261–281
Cavigelli MA, Robertson GP (2000) The functional significance of denitrifier community composition in the terrestrial ecosystem. Ecology 81:1402–1414
Corselius K, Wisniewski S, Ritchie M (2001) Sustainable agriculture: making money, making sense. The Institute for Agriculture and Trade Policy, Washington, DC
Dick RP (1992) A review: long-term effects of agricultural systems on soil biochemical and microbial parameters. Agric Ecosyst Environ 40:25–36
Dick WA, Gregorich EG (2004) Developing and maintaining soil organic matter levels. In: Schjonning P, Elmholt S, Christensen BT (eds) Managing soil quality: challenges in modern agriculture. CAB International, Wallingford, UK, pp 103–120
Dilly O, Munch JC (1998) Ratios between estimates of microbial biomass content and microbial activity in soils. Biol Fertil Soils 27:374–379
Doran JW (2002) Soil health and global sustainability: translating science into practice. Agric Ecosyst Environ 88:119–127
Doran JW, Alice JJ (1996) Methods for assessing soil quality. Soil Sci Soc Am J 49:410
Doran JW, Parkin TB (1994) Defining and assessing soil quality. In: Doran JW, Coleman DC, Bezdicek DF, Stewart BA (eds) Defining soil quality for sustainable environment, vol 35. SSSA, Madison, Wisconsin, pp 3–21
Doran JW, Zeiss MR (2000) Soil health and sustainability: managing the biotic component of soil quality. Appl Soil Ecol 15:3–11
Doran JW, Coleman DC, Bezdicek D, Stewart BA (1994) Defining soil quality for a sustainable environment, vol 35. SSSA, Madison, Wisconsin, pp 3–21
Doran JW, Sarrantonio M, Liebig MA (1996) Soil health and sustainability. Adv Agron 56:1–54
Doran JW, Stamatiadis SI, Haberern J (2002) Soil health as indicator of sustainable management. Agric Ecosyst Environ 88:107–110
Elliot LF, Lynch JM, Papendick RI (1996) The microbial component of soil quality. In: Stozky G (ed) Soil biochemistry, vol 9. Marcel Dekker, New York, pp 1–21

Ellison RS (2002) Major trace elements limiting livestock performance in New Zealand. N Z Vet J 50:35–40

Entry IA, Mitchell CC, Backman CB (1996) Influence of management practices on soil organic matter, microbial biomass and cotton yield in Alabama's "Old Rotation". Biol Fertil soils 23:353–358

Faeth P, Westra J (1993) Alternatives to corn and soybean production in two regions of the United States. Agricultural policy and sustainability: case studies from India, Chile, the Philippines and the United States. World Resources Institute, Washington, DC

FAO (2008) Conservation agriculture. http://www.fao.org/ag/ca

Filip Z (2002) International approaches to assess soil quality by ecologically-related biological parameters. Agric Ecosyst Environ 88:169–174

Giller KE, Beare MH, Lavelle P, Izac AMN, Swift MJ (1997) Agricultural intensification, soil biodiversity and agro-ecosystem function. Appl Soil Ecol 6:3–16

Gregory PJ, Ingram JSI, Anderson R, Betts RA, Brovkin V, Chase TN, Grace PR, Gray AJ, Hamilton N, Hardy TB, Howden SM, Jenkins A, Meybeck M, Olssol M, Schulze RE, Thiem M, Valentin C, Wilkinson MJ (2002) Environment consequences of alternative practices for intensifying crop production. Agric Ecosyst Environ 88:279–290

Hart MR, Brookes PC (1996) Soil microbial biomass and mineralisation of soil organic matter after 19 years of cumulative field applications of pesticides. Soil Biol Biochem 28:1641–1649

Heyer W, Hulsbergen KJ, Wittman CH, Papaja S, Christen O (2003) Field related organisms as possible indicators for evaluation of land use intensity. Agric Ecosys Environ 98:453–461

Hooper DU, Bignell DE, Brown BK, Brussard L, Dangerfield JM, Wall DH, Wardle DA, Coleman DC, Giller KE, Lavelle P, van der Putten WH, Ruiter PCD, Rusek J, Silver WI, Tiedje JM, Wolters V (2000) Interactions between aboveground and belowground biodiversity in terrestrial ecosystems: patterns, mechanisms, and feedbacks. Bioscience 50:1049–1061

Hseu ZY, Chen ZS, Tasi CC (1999) Selected indicators and conceptual framework for assessment methods of soil quality in arable soils of Taiwan. Soil Enviorn 2:135–139

Insam H, Haselwandter K (1989) Metabolic quotient of the soil microflora in relation to plant succession. Oecologia 79:174–178

Jenkinson DS (1981) Microbial biomass in soil: measurement and turnover. In: Paul EA, Ladd JN (eds) Soil biochemistry. Marcel Dekker, New York, pp 415–471

Jia GM, Cao J, Wang G (2005) Influence of land management on soil nutrients and microbial biomass in the central loess plateau, northwest China. Land Degrad Dev 16:455–462

Johnson MJ, Lee KY, Scow KM (2003) DNA fingerprinting reveals link among agricultural crops, soil properties, and the composition of soil microbial communities. Geoderma 114:279–303

Kandeler E, Eder G (1993) Effect of cattle slurry in grassland on microbial biomass and on activities of various enzymes. Biol Fetil Soils 16:249–254

Kang GS, Beri V, Sidhu BS, Rupela OP (2005) A new index to assess soil quality and sustainability of wheat-based cropping systems. Biol Fertil Soils 41:389–398

Karlen DL, Eash NS, Unger PW (1992) Soil and crop management effects soil quality indicators. Am J Alter Agric 7:48–55

Karlen DL, Wollenhaupt NC, Erbach DC, Berry EC, Swan JB, Eash NS, Jordhal NS (1994) Crop residue effects on soil quality following 10 years of no-till corn. Soil Till Res 31:149–167

Karlen DL, Andrews SS, Doran JW (2001) Soil quality: current concepts and applications. Adv Agron 74:1–40

Karlen DL, Andrews SS, Weinhold BJ, Doran JW (2003a) Soil quality: humankind's foundation for survival. J Soil Water Conserv 58:171–179

Karlen DL, Ditzler CA, Andrews SS (2003b) Soil quality: why and how? Geoderma 114:145–156

Karlen DL, Andrews SS, Weinhold BJ (2004) Soil quality, fertility and health historical context, status and perspectives. In: Schjonning P, Elmholt S, Christensen BT (eds) Managing soil quality: challenges in modern agriculture. CAB International, Wallingford, UK, pp 17–33

Khan MS, Zaidi A, Wani PA, Oves M (2009) Role of plant growth promoting rhizobacteria in the remediation of metal contaminated soils. Environ Chem Lett 7:1–19

Kinyangi J (2007) Soil health and soil quality: a review. Available on http://www.cornell.edu.org
Kowalchuk GA, Buma DS, de Boer W, Klinkhamer PGL, Van Veen JA (2002) Effects of above-ground plant species composition and diversity on the diversity of soil-borne microorganisms. Antonie van Leeuwenhoek 81:509–520
Larson WE, Pierce FJ (1991) Conservation and enhancement of soil quality. In: Dumanski J, Pushparajah E, Latham M, Myers R (eds) Evaluation for sustainable land management in the developing world, vol 2. Technical Papers, IBSRAM Proceedings No 12(2), Bangkok, pp 175–203
Lee J, Masters DG, White CL, Grace ND, Judson GJ (1999) Current issues in trace element nutrition of grazing livestock in Australia and New Zealand. Aust J Agric Res 50:1341–1364
Leo H, Lawrence RS, Walker P (2002) How sustainable agriculture can address the environmental and human health harms of industrial agriculture. Environ Health Perspect 110:445–456
Majumder B, Mandal B, Gangopadhyay A, Mani PK, Kundu AL, Mazumdar D (2008) Organic amendments influence soil organic carbon pools and rice-wheat productivity. Soil Sci Soc Am J 72:775–785
Mandal B, Majumder B, Adhya TK, Bandyopadhyay PK, Gangopadhyay A, Sarkar D, Kundu MC, Gupta Choudhury S, Hazra GC, Kundu S, Samantaray RN, Mishra AK (2008) Potential of double-cropped rice ecology to conserve organic carbon under subtropical climate. Glob Chang Biol 14:1–13
Menge M (2003) Experiences with application, recordation and validation of agri-environmental indicators in agricultural practices. Agric Ecosyst Environ 98:443–451
Mitchell J, Gaskell M, Smith R, Fouche C, Koike ST (2000) Soil management and soil quality for organic crops. Organic vegetable production in California series, vol 7248. University of California, USA
Nielsen MN, Winding A (2002) Microorganisms as indicators of soil health. NERI technical report No. 388. Ministry of the Environment, National Environmental Research Institute, Denmark, p 82
Nordlee JA, Taylor SL, Townsend JA, Thomas LA, Bush RK (1996) Identification of a Brazil-nut allergen in transgenic soybeans. N Engl J Med 334:688–692
Norfleet ML, Ditzler CA, Puckett WE, Grossman RB, Shaw JN (2003) Soil quality and its relationship to pedology. Soil Sci 168:149–155
Nortcliff S (2002) Standardization of soil quality attributes. Agric Ecosyst Environ 88:161–168
Olson GE (1981) Archaeology: lessons on future soil use. J Soil Water Conserv 36:261–264
Osinski E, Meier U, Buchs W, Weickel J, Matzdrof J (2003) Application of biotic indicators for evaluation of sustainable land use–current procedure and future development. Agric Ecosyst Environ 98:407–421
Pankhurst CE, Doube BM, Gupta VVSR (1997) Biological Indicators of soil health. CAB International, Wallingford, UK, p 451
Paul EA (1984) Dynamick of organic matter in soils. Plant Soil 76:275–285
Pompili L, Mellina AS, Benedtti A (2006) Microbial indicators for evaluating soil quality in differently managed soils. Geophysic Res Abst.8.06991
Ponting C (1992) A green history of the world. St. Martin, New York
Pretty JN (1995) Regenerating agriculture: policies and practice for sustainability and self-reliance. Joseph Henry, Washington, DC
Saha N, Ulrich A, Becker R, Wirth S (2007) Assessing the changes in bacterial diversity in rhizosphere and phyllosphere of transgenic and non-transgenic potato plants. Plant Tissue Cult Biotechnol 17:87–95
Saito M (2007) Can soil biodiversity be used for an indicator of soil health? Case studies in Japan. www.webdomino1.oecd.org
Sarkar JM, Lenowiez P, Bollag JM (1989) Immobilization of enzymes on clay and soils. Soil Biol Biochem 21:223–230
Schjonning P, Elmholt S, Christensen BT (2004) Soil quality management-synthesis. In: Schjonning P, Elmholt S, Christensen BT (eds) Managing soil quality: challenges in modern agriculture. CAB International, Wallingford, UK, pp 315–333

Seybold CA, Mausbach MJ, Karlen DL, Rogers HH (1997) Quantification of soil quality. In: Lal R, Kimble JM, Follett RF, Stewart BA (eds) Soil processes and the carbon cycle. CRC, Boca Raton, New York, pp 387–404

Seybold CA, Herrick JE, Brejda JJ (1999) Soil resilience: a fundamental component of soil quality. Soil Sci 164:224–234

US National Research Council (2000) Genetically modified pest-protected plants: science and regulation. National Academy, Washington, DC

Vaarst M, Hovi M, Sundrum A, Younie D, Susanne P (2003) Sustaining animal health and food safety in European organic livestock farming. Presentation at 54th annual EAAP Meeting, Rome, 31 August 2003

Visser S, Parkinson D (1992) Soil biological criteria as indicator of soil quality: soil microorganisms. Am J Alter Agric 7:33–37

Vorley W, Keeney D (1998) Bugs in the system: redesigning the pesticide industry for sustainable agriculture. Earthscan, London

Wander MM, Walter GL, Nissen TM, Bollero GA, Andrews SS, Cavanaugh-Grant DA (2002) Soil quality: science and process. Agron J 94:23–32

Wang Z, Chang AC, Wu L, Crowley D (2003) Assessing the soil quality of long-term reclaimed wastewater-irrigated cropland. Geoderma 114:261–278

Warkentin BP (1995) The changing concept of soil quality. J Soil Water Conserv 5:226–228

Wylie P (1994) Indicators of sustainable cropping systems. In: Pankhurst CE, Doube BM, Gupta VVSR, Grace PR (eds) Soil biota: management in sustainable farming systems. CSIRO, East Melbourne, Australia, pp 224–229

# Chapter 9
# Recent Advances in Biopesticides

**Parvez Qamar Rizvi, Rummana A. Choudhury, and Arshad Ali**

**Abstract** Consistent and injudicious applications of pesticides leads to the development of resistance in insects, destruction of beneficial organisms, and increases in residual problems, thereby posing a threat to human health and its ecological partners in the living biome. The need of the hour is to develop an eco-friendly approach to combat insect pests that should be able to regulate pest populations by exploring naturally occurring products, including extracts of plants and animals, microbes, parasitic nematodes and insects, and certain minerals. This call for viable alternatives has led the scientific community to engage in unveiling the potential of biopesticides. Currently, some strains of *Bacillus thuringiensis*, nuclear polyhedrosis virus, fungi, and nematode parasites are commercially available. Exploiting the benefits of biopesticides as biocontrol agents appears to be a more promising approach, assuming that issues of phytopathogens and environmental problems caused by synthetic pesticides can be resolved. This chapter emphasizes the experiences and progress made in the potential and promise of biopesticides in the global scenario.

## 9.1 Introduction

For many years, the use of synthetics and chemical pesticides has dominated the scene of pest control. Their intensive use, misuse, and abuse have caused various ecological and environmental problems, the resurgence of various insect and mites pests, contaminated food commodities, have adversely affected non-target organisms, and have also progressively increased occupational poisoning cases in developing countries. This tirade against their use has led agriculture to envision a new

P.Q. Rizvi (✉), R.A. Choudhury, and A. Ali
Faculty of Agricultural Sciences, Department of Plant Protection, Aligarh Muslim University, Aligarh, Uttar Pradesh, India
e-mail: pqrizvi@rediffmail.com

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_9, 

stride forward in the wake of biotechnological and other innovative developments so as to open up new vistas of scientific advancement, which requires sustained efforts and will certainly reap long-term benefits.

As a viable substitute to these conventional environmentally-unfriendly chemical pesticides, eco-friendly alternatives of crop-pest control by either biological agents or specifically designed synthetic anti-insect compounds are receiving greater attention. Though the initial acceptance of biopesticides was slow, owing to their non-availability in sufficient quantities, poor shelf life, lack of standard products, and collection/multiplication/application constraints, due to the organic food movement, the development and standardization of biopesticides have accelerated dramatically. Currently, the total world production of biopesticides is over 3,000 tons annually, which is increasing at a rapid rate. India has a vast potential for biopesticides as it utilizes more than 100,000 tons of pesticides each year. Most (80%) of the pesticides are used on cotton (45%), rice (30%), and vegetables (5%), the remaining crops receiving only 20% as their share (http://www.molecular-plant-biotechnology.info/industrial-microbiology/advantages-and-limitations-of-biopesticides-and-chemical-pesticides.htm).

Classical biological control involving *Bacillus thuringiensis (Bt),* nuclear polyhedral virus (NPV), *Trichoderma harzianum, Saccharopolyspora spinzosa* and *Pseudomonas* species have proved effective in checking pest infestations, along with pathogens like *Metarhizium anisopliae, Beauveria bassiana* and entomopathogenic nematodes such as *Steinernema carpcapse*, which has received much recognition. Great success in the reduction of populations rather than their elimination is also advocated in the cases of plant-feeding insects and pathogens as weed biocontrol agents (e.g., plant rusts) in rangelands, forests, and other natural habitats. Furthermore, a very critical approach is necessary towards the use of genetic engineering in agriculture in order to improve the properties of the biological control agents, and towards engineering crop plants to be resistant to pests using the recombinant DNA technology, which produces several transgenic crops with built-in resistance to insect pest and weeds. There is also a need to develop strategies based on the bio-chemical and ecological behavior of the pests. Thus, to complement and eventually substitute synthetic pesticides with biopesticides would represent an economically beneficial and ecologically sound alternative.

## 9.2 Shade of Biopesticide

Biopesticides are certain types of pesticides derived from natural materials such as animals, plants, bacteria, or some minerals. For example, garlic (*Allium sativum* L.), mint (*Mentha arvensis*), canola (*Brassica napus*) oil, and baking soda all have pesticidal activity and are considered biopesticides. However, as defined by Sudakin (2003), the term "biopesticide" encompasses a broad array of microbial pesticides, bio-chemicals derived from microorganisms and other natural sources, and processes involving the genetic incorporation of DNA into agricultural commodities that confer protection against pest damage (plant-incorporated protectants). According to Zechendorf (1995), the scope of biopesticides includes substances

such as plant extracts, hormones, pheromones, and toxins of organic origin. Copping and Menn (2000) defined the term biopesticides to encompass many aspects of pest control such as microbial, entomophagous nematodes, plant-derived pesticides, secondary metabolites from microorganisms, pheromones, and genes used to transform the crops to express resistance to pests. More recently, the encouragement of natural enemies (parasitoids, predators, microbes, etc.) and the use of transgenic crop varieties, pheromones, growth regulators, and plant-derived materials in pest management have been considered to constitute the biopesticide umbrella. At the end of 1998, there were approximately 175 registered biopesticide active ingredients and 700 products, which increased to 195 active ingredients and 780 products in 2001. Data on microbial biopesticide agents from Agriculture and Agri-food Canada (Kabulak and Gazdik 2005) and the US Environmental Protection Agency (EPA) indicate that more than 200 products are being sold in the United States, compared to only 60 comparable products in the European Union. In the UK, only 5 microbial products are currently being sold, compared to 10 in Germany, and 15 each in France and the Netherlands.

## 9.3 Types of Biopesticides

### *9.3.1 Microbial Pesticides*

Of the nearly 1 million known species of insects, about 15,000 are considered pests and about 300 require some form of control. Fortunately, most insect pests have pathogenic microorganisms associated with them. Entomopathogens have been highlighted as controlling agents of insect pests for over a century, and include species of bacteria, fungi, viruses, algae, and protozoa as the active ingredient. These pathogenic organisms are isolated from diseased insects during naturally occurring epidemics. Over 400 species of fungi and more than 90 species of bacteria which infect insects have been reported. These microbial control agents have a range of properties that make them desirable for integrated crop management (Hajek 2004). To achieve a double pay-off of better systems of environmental risk evaluation, and an effective and more sustainable microbial control, attention is required for the better understanding of phylogeny of microbial natural enemies (Rehner and Buckley 2005), their biogeography and factors determining bio-diversification of gene flow (Bidochka and Small 2005), and assessment of their background levels in agricultural and natural ecosystems (Mensink and Sheepmaker 2007).

#### 9.3.1.1 Bacteria

Among bioagents, bacteria are the most potent. On the basis of their pathogenecity, entomopathogenic bacteria are divided into four groups: (1) obligate pathogens (e.g., *Bacillus popilliae*), (2) crystalliferous spore-formers (e.g., *B. thuringiensis*), (3) facultative pathogens (e.g., *B. sphaericus*), and (4) potential pathogens (e.g., *Seerratia marscens*). Of these, *B. thuringiensis* is the most effective, with about 6,000 isolates

**Table 9.1** Molecular organization of the genes coding $\delta$ – endotoxin with target host insect

| Delta endotoxin gene | M.W. (kDa) | Insect host target |
|---|---|---|
| CrylA (a) | | Lepidoptera |
| CrylA (b) | 130 a 133 | Lepidoptera |
| CrylA (c) | 138 | Lepidoptera |
| CrylB | 135 | Lepidoptera |
| CrylC | 130 a 134 | Lepidoptera |
| CrylD,E,F | | |
| CryllA | 71 | Lepidoptera/diptera |
| CryllB | 71 | Lepidoptera |
| CryllC | 70 | Lepidoptera |
| CrylllA | 73 | Coleoptera |
| CrylllB | 74 | Coleoptera |
| CrylllC | 129 | Coleoptera |
| CrylllD | 73 | Coleoptera |
| CrylVA, CrylVB | 134 a 128 | Diptera |
| CrylVC | 78 | Diptera |
| CrylVD | 72 | Diptera |
| CryVA | 81 | Lepidopera/coleoptera |

Adapted from Nasrine Moazami. Biopesticide Production, Biotechnology (EOLSS) manuscript (http://www.eolss.net)

stored in various repositories throughout the world. This bacterium was first recorded in 1901 as the cause of the damaging "Sotto" disease in silkworms in Japan (Ishikawa 1936), and was isolated again in 1927 by Maltes in Germany and given the name *B. thuringiensis*. It occurs naturally in insect-rich locations, including soil, plant surfaces, and grain stores. So far, more than 40,000 strains of *Bacillus thuringiensis* have been isolated and identified as belonging to 39 serotypes. The *Bt* strains are classified according to their H antigens into 27 groups and 7 subgroups, and according to structure and molecular organization of the genes coding for the parasporal delta-endotoxins. Only a few strains are used commercially as bio-control agents. Chief among them includes *Bacillus thuringiensis, B. sphaericus* and *B. popilliae*. They are active against insect pests belonging mainly to the orders lepidoptera, diptera, or coleoptera (Table 9.1). *B. thuringiensis* produces three types of entomocidal toxins: alpha exotoxin, beta exotoxin, and delta endotoxin (insecticidal crystal protein, ICP). When ingested, the crystals paralyze the digestive tracts of insects, often killing them within 24–48 h. The stability of the *Bt* formulations, however, depends on the biological properties of insects, such as age and maturity, presence of the degenerative enzymes, pH, residual nutrient availability, ionic strength and osmotic pressure, type of preservatives used, type of surfactants used, and the temperature.

## Mode of Action

The toxic crystal *Bt* proteins in commercial formulations are effective only when eaten by insects with a specific (usually alkaline) gut pH and the specific gut

membrane structures required to bind the toxin. So, when (1) the insect has the correct physiology, (2) been at a susceptible stage of development, and (3) the bacterium has been eaten in sufficient quantity, only then does the toxic protein damage the gut lining, leading eventually to gut paralysis of the infected insect. As a consequence, the insect stops feeding and dies from the combined effects of starvation and tissue damage. However, *B. thuringiensis* spores do not usually spread to other insects or cause disease outbreaks on their own as occurs with many other pathogens (Whalon and McGaughey 1998). Formulation plays a significant role in determining the final efficacy of a *Bacillus*-based product. As a dry powder, *B. thuringiensis* is quite stable, whereas flowable formulations are difficult to prepare and stabilize, and are quite sensitive to heat and, hence, must be protected during storage. Formulations of *Bt* var. *tenebrionis* and *sandiego* have been registered for use against Colorado potato beetle larvae and elm leaf beetle adults and larvae. *B. thuringiensis* subsp. *israelensis* is marketed for use against black flies and mosquitoes, while *Bt* var. *aizawai* is used to control fungus gnats, wax moth larvae in bee hives and various caterpillars (Duan et al. 2004; Zehnder and Gelernter 1989).

#### 9.3.1.2 Virus

The efficacy of the insect-specific viruses can be seen among 1,600 viruses that have been recorded from more than 1,000 species of insects, particularly caterpillar pests. Different strains of naturally occurring nuclear polyhedrosis virus (NPV) and granulosis virus are present at low levels in many insect populations. No threat to humans or wildlife is posed by these insect viruses. A family of viruses called baculoviruses is the most popular choice for microbial control, which have been used regularly for pest control since the 1950s, particularly in forestry where they have been highly effective at controlling sawflies. They are usually small (less than 0.001 mm across), and are composed primarily of double-stranded DNA that codes for genes needed for virus establishment and reproduction. Because this genetic material is easily destroyed by exposure to sunlight or by conditions in the host gut, an infective baculovirus particle (virion) is protected by a protein coat called a polyhedron. Most insect baculoviruses must be eaten by the host to produce an infection, which is typically fatal to the insect. The majority of baculoviruses used as biological control agents belong to genus *Nucleopolyhedrovirus*. These viruses are considered suitable candidates for species-specific, narrow spectrum insecticidal applications, have no negative impacts on plants, mammals, birds, fish, or even on non-target insects, and are especially desirable when beneficial insects are being conserved to aid in an overall EPM program (Ramkrishnan 1992). They have a characteristic shiny-oily appearance, and are seen hanging limply from vegetation. They are extremely fragile to the touch, rupturing to release fluid filled with infective virus particles. This tendency to remain attached to foliage and then rupture is an important feature of the virus life-cycle. As discussed above, infection of other insects will occur only if they eat foliage that has been contaminated by

virus-killed larvae. Baculoviruses infect their hosts through ingestion. Virus particles invade the cells of the gut before colonizing the rest of the body. Infection reduces mobility and feeding, and insects are killed in 5–8 days.

Mass production of baculoviruses can be done only in insects, but this is economically viable for larger hosts such as caterpillars, and formulation and application are straightforward. At present, there are approximately 16 products available for use or under development, mostly for control of caterpillar pests. Commercial products are available in Switzerland, Germany, and Spain for the control of codling moth and the summer fruit tortrix. Based on their safety and potential to replace chemical pesticides, five baculoviruses have been registered as pesticides: *Helicoverpa zea* nuclear polyhedrosis virus (HzSNPV) in 1975, *Orgyia pseudotsugata* (Ot) MNPV in 1976, and *Lymantria dispar* (Ld) MNPV in 1993. However, the only privately produced and commercially available viral pesticide comes from the USA and is the SeMNPV (Spod-X$^{TM}$).

Mode of Action

Viruses invade an insect's body via the gut. They replicate in tissues and can disrupt components of an insect's physiology, interfering with feeding, egg laying, and movement. Different viruses cause different symptoms. NPV-infected larvae may initially turn white and granular or very dark. Some may climb to the top of the crop canopy, stop feeding, become limp, and hang from the upper leaves or stems, hence the common name "caterpillar wilt" or "tree top" disease. Victims of a granulosis virus may turn milky white and stop feeding, or contents of the dead larvae are liquefied and the cuticle ruptures easily to release infectious viral particles. Death from a virus infection usually occurs within 3–8 days. Their killing efficiency may be augmented by genetic modification of its genome with genes of other natural pathogens (Szewczyk et al. 2006).

#### 9.3.1.3 Entomopathogenic Fungi

Over 750 species of fungi kill insects. Entomopathogenic fungi are widely distributed throughout the fungal kingdom, although the majority occur in the *Deuteromycotina* and *Zygomycotina* families. Some insect-pathogenic fungi have restricted host ranges, for example, *Aschersonia aleyrodis* infects only scale insects and whiteflies, while other fungal species have a wide host range, with individual isolates being more specific, for example, *Metarhizium anisopliae* and *Beauveria bassiana*. The potential of fungal pathogens in the control of insect pests has been recognized since the latter part of the nineteenth century, when *M. anisopliae* was tested against the wheat cockchafer, *Anisoplia austriaca* and sugar beet curculionid, *Cleonus punctuventris*. Over the past century, there have been many attempts to exploit *Verticillium lecanii, A. aleyrodis, B. bassiana, Nomuraea rileyi, M. anisopliae*, and some species of *Entomophthorales* for pest control. At present, fungi are being used for pest control

on a moderate scale in China (Nasrine Moazami, Biopesticide Production, Biotechnology (EOLSS) manuscript http://www.eolss.net).

Entomopathogenic fungi invade their hosts using spores that grow through the cuticle, and hence they are particularly suited for control of pests with piercing mouthparts, such as aphids and whiteflies, which are unlikely to acquire pathogens through feeding. Infection requires high humidity at the insect surface, but this can be circumvented using oil-based formulations. *Beauveria bassiana* are becoming available for the control of a range of glasshouse pests. Many entomopathogenic fungi, especially those in the entomophthorales, are responsible for epizootics that often successfully regulate pest insect populations. Although inoculation of insect populations with entomopathogenic fungi has provided classical biological control of some pests, most notably against the gypsy moth, the most common method of employing fungi for insect control is through inundatory means. Development of effective methods for production of resting spores and competent mycelia of entomophthoralean species will ultimately increase the utility of these fungi. Fungi often become attenuated (lose virulence or antagonistic characteristics) when maintained on artificial media. Cultural conditions must be identified which retain virulence without increasing production costs. At present, little progress has been made in this area partly because the underlying mechanisms for attenuation have not been elucidated. While 750 species of entomopathogenic fungi are known, less than 20 have received serious attention as control agents of insect pests (Hawksworth et al. 1995; Copping 2004). Among fungal pesticides, five have been introduced since 1979, and three in 1981. Many countries with centrally planned economies have been using fungal pesticides successfully for many years. Nowadays, about 20 products are available worldwide for managing sap-feeding insects, beetles, caterpillars, flies, and locusts.

## Mode of Action

Entomopathogenic fungi invade their hosts by direct penetration of the host exoskeleton or cuticle. As occurs in many plant-pathogenic fungi, conidia germinate on the host surface and often differentiate to form an appressorium. An infected hypha penetrates down through the host cuticle and eventually emerges into the haemocoel of the insect. As with plant pathogens, entry into the host involves both enzymic degradation and mechanical pressure. A range of extracellular enzymes that can degrade the major components of insect cuticle is produced when *M. anisopliae* is grown in vitro with the cuticle as the sole carbon and nitrogen source. Growth of fungi in the haemolymph of insects may be as yeast-like blastospores, hyphal bodies, or protoplasts, rather than in the form of a mycelium (from which toxic compounds can be extracted). Death is caused by tissue destruction and, occasionally, by toxins produced by the fungus. The fungus frequently emerges from the insect's body to produce spores that, when spread by wind, rain, or contact with other insects, can spread infection. Destruxins (DTXs), a group of cyclic depsipeptides (peptides containing ester linkages) produced by *M. anisopliae*,

have received most attention, and are responsible for causing insect mortality by the fungus (Zimmermann 2007). DTXs are insecticidal by injection and, in some cases, when ingested by mouth; toxicity is most acute among lepidopteran larvae and adult Diptera.

#### 9.3.1.4 Nematodes

Entomopathogenic nematode worms are just visible to the naked eye, being about 0.5 mm in length. The entomopathogenic nematodes are a nematode–bacterium complex forming a strong mutualistic relationship. The nematode may appear as little more than a biological syringe for its bacterial partner. The bacteria break down the insect body, which provides food for the nematodes. After the insect dies, the juvenile nematodes develop to adults and reproduce. A new generation of infective juveniles emerges 8–14 days after infection. Entomopathogenic nematodes are extraordinarily lethal to many important soil insect pests, yet are safe for plants and animals. This high degree of safety means that, unlike chemicals, or even *Bacillus thuringiensis*, nematode applications do not require other safety equipment; and re-entry time, residues, groundwater contamination, chemical trespass, and pollinators are not major issues. Like other biological control agents, nematodes are constrained by being living organisms that require specific conditions to be effective. Thus, desiccation or ultraviolet light rapidly inactivates insecticidal nematodes; chemical insecticides are less constrained. Similarly, nematodes are effective within a narrower temperature range than chemicals, and are more impacted by suboptimal soil type, depth, and irrigation frequency. In addition, unlike other entomopathogens, nematodes are exempted from registration and so have been popular choices for commercialization. Over 60 products prepared from nematodes are available in Europe. Nematodes require moist conditions to operate and have been marketed predominantly against soil pests, such as vine weevil and sciarid fly larvae. However, they may also control foliar pests, for example Nemasys (Becker Underwood) which can be used to control western flower thrips.

##### Mode of Action

Juvenile nematodes parasitize their hosts directly by penetrating the cuticle through natural openings and introducing symbiotic bacteria, which multiply rapidly and cause death by septicaemia, often within 48 h. Nematode growth and reproduction depend upon conditions established in the host cadaver by the bacterium. The bacterium further contributes anti-immune proteins to assist the nematode in overcoming host defences, and anti-microbials that suppress colonization of the cadaver by competing secondary invaders. Conversely, the bacterium lacks invasive powers and is dependent upon the nematode to locate and penetrate suitable hosts.

#### 9.3.1.5 Entomopathogenic Protozoa and Microsporida

Protozoan diseases of insects are ubiquitous and perform an important regulatory role in insect populations. They are generally host specific and slow acting, most often producing chronic infections. They develop only in living hosts and many species require an intermediate host. Species in the Microsporida are among the most commonly observed. Their main advantages are persistence and recycling in host populations and their debilitating effect on reproduction and overall fitness of target insects. As inundatively-applied microbial control agents, only a few species have been moderately successful. The main disadvantages of the protozoa as inundatively-applied microbial control agents are the requirement for in vivo production and low levels on immediate mortality. *Nosema locustae* is the only commercially available species of microsporidium, marketed under several labels for the control of grasshoppers and crickets. It is applied with insect-attractant bait. Because of its slow mode of action, this product is better suited to long-term management of rangeland pests than to the more intensive demands of commercial crop or even home garden production.

### *9.3.2 Plant Incorporated Protectants*

#### 9.3.2.1 Botanicals

Every native plant is evolved with a specific recipe of natural controls. The rich plant bio-diversity of India which continues to be a major untapped source of a diverse range of bio-active molecules needs to be explored for safe crop protection. A range of secondary metabolites, like alkaloids, flavonoids, phenols, glycosides, sitosterols, and tannins, have a record of protecting plants against their herbivorous pests (Ahmad 2007). However, greater emphasis needs to be laid on developing innovative formulations with good storage ability and persistence. There is also a need to improve the knock-down characters in natural pesticides which are endowed with slow acting behavioral and physiological modes of action. Historically, plant materials have been in use longer than any other chemicals (with the exception of sulphur) but after the Second World War, they lost their importance with the introduction of synthetic organic chemicals. Tobacco (*Nicotiana tabacum*), pyrethrum (*Chrysanthemum cinerariifolium*), derris (*Derris elliptica*), hellebore (*Helleborus niger*), quassia (*Picrasma excels*), camphor (*Cinnamonum camphora*), and turpentine (*Syncarpia glomulifera*) were some of the important plant products in use before the organized and systematic search began in the 1940s. In the US, the use of botanical pesticides like pyrethrum, nicotine, and rotenone peaked during 1966, but due to some limitations like photosensitivity, degradation, and availability, their use declined thereafter. Chief among the adverse effects are structural modifications and structure–activity relationships which led to the discovery of synthetic pyrethrin-like materials called synthetic pyrethroids which are

stable in light and act even at very low concentrations. Similarly, nicotinoids (previously referred to as nitro-quanidines, neonicotinyls, neonicotinoids, and chloronicotines, and more recently referred as chloronicotinyls) are considered the siblings of structurally modified pesticides. Therefore, there is an urgent need to explore these virgin plants for their bioactivities. However, the major bottlenecks in the commercialisation of biopesticides are: (1) resource availability, (2) problems in chemical standardization and delivery systems, (3) less effective field reports than laboratory results, (4) lack of novel, effective, stable and economical formulations of botanical products, and (5) lack of general awareness among the end users which discourage product realization. Despite all these constraints, the Energy and Resources Institute (TERI) under the patronage of the Department of Biotechnology, Ministry of Science and Technology, Government of India, has developed a plant extract-based biopesticide formulation "*Bollcure*", that could be used to control cotton bollworm (*Helicoverpa armigera*), the most challenging larvae capable of inflicting severe losses to the yield of cotton (*Gossypium hirsutum)* crops.

## *9.3.3 Biochemical Pesticides*

### 9.3.3.1 Allellochemicals

Plant biodiversity provides a vast repository of biologically active compounds that find application in traditional medicines and crop protection systems. Among the estimated over a half a million species of the existing plant kingdom, nearly 2,500 species belonging to 235 plant families have exhibited measurable anti-pest properties (Saxena 1998). Phytochemicals have a wide range of activity including hormonal, neurological, nutritional, and enzymatic, many of which still need to be explored. Recent research in plant resistance to insect pests of *Nicotiana* spp. and *Solanum* spp. have demonstrated that leaf exudates produced by them play a significant role in determining resistance or susceptibility to infestation to insects in such plants (Severson et al. 1985). These exudates produce a protective waxy layer comprising numerous secondary metabolites such as glycolipids and glycerolipids as well as free fatty acids/esters and terpenes. The bioactive glycerolipids consisting of 1,2-diacylglycerol, 1,3-diacylglycerol and 1,2,3- triglycerol provide in-built plant resistance to invading pests and are active against certain phytophagous insects and pathogens. High levels of α-amaryl alkanoate and cycloartenyl alkanoate in epicular waxes from aphid-resistant raspberry (*Rubus* spp.) plants is responsible for its resistance. Moreover, some light-activated phytotoxins, like substituted acetylenes, thiophenes, acetylenic thiophenes, quinines, furanocoumarins and related compounds, have also shown significant pest control properties. Allelochemicals from microbes include spinosyns derived from aerobic fermentation of the soil-inhabiting actinomycetes *Saccharopolyspora spinosa* (Thompson

et al. 2000), while avermectin and milbemycin are derived from a family of macrocyclic lactones produced from *Streptomycin avermitilis* (Campbell 1989).

#### 9.3.3.2 Sex Pheromones

Pheromones specifically disrupt the reproductive cycle of harmful insects. In this way, farmers can reduce the amount of insecticide they need; spraying only when insects are in a vulnerable stage or when their numbers exceed certain levels. There is no alteration to the natural biological and ecological cycle, hence ensuring that there is no environmental or health hazard. They are portable, less expensive, and a more natural form of crop-protecting agent. Pheromones lure and trap is an insect-trapping apparatus which essentially works by using the sex pheromones generated by female insects to attract their male counterparts. Pheromones as monitoring tools provide cost-effective and simple techniques to time application of biological control agents and bio-pesticides in integrated pest management (IPM). In 1987, Pest Control of India (PCI) became the first company in India to commercially introduce pheromone technology for agricultural use by launching sex pheromone lures and traps for monitoring *Helicoverpa armigera* and *Spodoptera litura*. Since then, PCI has introduced commercial pheromone lures for monitoring a range of pests, including cotton bollworms, tobacco caterpillar, rice yellow stem borer, sugarcane borers, diamond back moth, brinjal shoot and fruit borer, and fruit flies. Furthermore, PCI has also been regularly introducing suitable traps for use with these pheromone lures, and today has in its trap range funnel traps, delta traps, McPhail traps, cross-vane traps, water traps, and bucket traps.

## 9.4 Potentials and Constraints of Biopesticides

All these biopesticide strategies employed to control insect pests have distinct advantages and disadvantages under specific conditions. Although there has been an extensive programme for mass rearing and release of beneficial insects such as *Trichogramma* species, perhaps the greatest potential for successful biological control through augmentation of natural enemies exists with pathogenic microorganisms. The utility of these agents has, however, been limited by difficulties in the production and formulations of products, particularly with viruses and fungi, and has limited spectra of activity against pest complexes in comparison with chemical insecticides. However, recent research has indicated that the efficacy of bacteria and viruses have improved through recombinant DNA technology. The advantages of microbial insecticides over chemical insecticides in terms of reduced threats to nontarget species and limited potential for environmental degradation are compelling reasons for increasing efforts to improve these agents and increase their utility for IPM programmes (Cuperus et al. 2004).

Although many compounds have been isolated, characterized, and evaluated as anti-insect compounds from plants, not much headway has been achieved in the commercialization of such products. The standardization of plant based anti-insect preparations has been the biggest constraint and has subsequently hindered their potential marketability compared with conventional pesticides. Generally, natural plant products tend to be rather slow acting, of modest toxicity, and rapidly degrade in the environment. The variation in efficacy of the compounds between test species is probably the greatest barrier to their commercialization. However, the concept of phytosynergistic strategy and assessing the toxicity of co-occurring toxins seems to have potential and could lead to further advancement in the development of biopesticides (Koul and Dhaliwal 2001).

### *9.4.1 Advantages of Using Biopesticides*

The potential advantages of bioinsecticides include that they: (1) are usually inherently less harmful than conventional pesticides, (2) do not leave harmful residues, (3) are designed to affect just the specific target organism in contrast to broad spectrum of conventional ones, (d) are effective in small quantities and often disperse quickly (Ware 1994), thereby resulting in lower exposures and largely avoiding the pollution problems caused by conventional pesticides, (e) as a component of IPM, greatly reduce the use of conventional synthetics while crop yields remain high, and (f) are often cheaper than chemical pesticides. The introduction of transgenic technology has opened up new vistas in crop protection management. Although investment in agricultural biotechnology is concentrated in industrialized countries, many developing countries like India, China, and Brazil have committed significant levels of public funding to biotechnology. Recent studies show that small-scale farmers in developing countries stand to benefit more from transgenic technology than commercial farmers in industrial countries, due to limited access to agrochemicals and greater losses associated with insect pests in tropical and subtropical climates. The best example of such progress in developing countries is the use of *Bt* cotton containing Cry 1ac gene from *Baccillus thuringiensis* which is commercially grown in India and China. Multi-level endocrine systems controlling a wide range of physiological processes (e.g., moulting, metamorphosis, diapause, reproduction, etc.) advocating multiple functional capacities of insect neuropeptides, also provide opportunities for new insect control strategies. As a biopesticide, they induce adult sterility, physical body change, water loss, and premature death in insects (Muraleedharan and Devi 1992). Their chief disadvantages are: (1) very high specificity, which will require an accurate identification of the pest/pathogen and may require multiple pesticides to be used, and (2) often variable efficacy due to the influences of various biotic and abiotic factors (since biopesticides are usually living organisms, which bring about pest/pathogen control by multiplying within the target insect pest/pathogen).

### *9.4.2 Constraints in the Promotion of Biopesticides*

#### 9.4.2.1 Technological

These includes (1) lack of technological advancement relating to the transfer of technology from pilot point scale to mega-scale, (2) lack of mass rearing facilities, (3) inconsistent field results, (4) limited or short shelf life (say 6 months), and (e) existence of very crude formulations.

#### 9.4.2.2 Financial

This includes (1) huge rates of tax, (2) benefit of economy of scale rather limited, and (3) lack of subsidy from Government bodies.

#### 9.4.2.3 Extension Related

- Existing mindset of farmers to compare biological vis-à-vis chemical pesticides
- Lack of field demonstration and training programmes

#### 9.4.2.4 Regulatory/Statutory

(1) Need for a separate body for registering biological compounds, (2) need to liberalize expensive and time-consuming registration processes, (3) require strict quality control and standardised formulations, and (d) lack of large number of nodal biopesticide testing laboratories.

## 9.5 Biopesticides Global Scenario

During the 1970s, research institutions around the globe had started opting for biopesticides as a new and sustainable alternatives to conventional pesticides. The tactics of using biopesticides seems ideal for IPM if they could spare the pests' natural enemies, as they are narrowly selective and pose few problems to the nontarget ones. Except for *Bacillus thuringiensis*, the predicted demand for commercial biopesticides has not been realised. Estimated world sales of all biopesticides amount to 0.5% of the total world pesticide market accounting for more than 90% of the total sales of *Bt* products (Rodgers 1993). Among the best examples of its demand in developing countries, Brazilian farmers have obtained exceptional control of the velvetbean caterpillar, *Anticarsia gemmatalis*, by spraying NPV onto soyabean, *Glycine max* (Moscardi and Sosa-Gomez 1996). Chinese farmers applied

*Beauveria bassiana* to the soil surface to infect the larvae dropping from the plants to overwinter (Kogan and Turnipseed 1987) but, like the Brazilians, they used it as an IPM tool and did not rely merely on these biopesticides. Among the plant pathologists, at least 30 different biological control organisms are presently available as commercial formulations to manage plant diseases (Lumsden et al. 1995). For example, *Agrobacterium tumefaciens* (strain K84), which is able to synthesize antibiotic agrocin 84, has been used to prevent crown gall (Copping 2004; Kabulak and Gazdik 2005). The control of the coconut rhinocerous beetle, *Oryctes rhinoceros*, on coconut (*Cocos nucifera)* in Asia and the Pacific Islands is a good example of how entomopathogens could provide benefits to such plants (Waterhouse and Norris 1987).

## 9.6 Biopesticide Production

### *9.6.1 Use of Genetic-Engineering Technology*

Major breakthroughs in molecular biology and biotechnology since the early 1980s indicate that rapid improvements in the competitive ability of biological control methods are possible, and that biopesticides can play a major role in crop protection in the future. It has become possible to improve some of the critical properties that earlier hampered the usefulness of many biocontrol agents. Valuable genes from completely unrelated organisms can now be utilized for biological control purposes. Biological control using recombinant DNA (genetic engineering) technology can be achieved in several different ways: control agents may be improved; crop plants can be engineered to carry better resistance genes; or organisms associated with the plant may be modified to provide protection. All these approaches have successfully been used experimentally in several different ways. Product development has been very active in the area of incorporating resistant genes – mainly from *Bt* directly into plants. Successes include potato (*Solanum tuberosum*), tomato (*Lycopersicon lycopersicum*), tobacco, and cotton. General root-colonizing bacteria of plants have also been engineered to produce insecticidal toxins, which protect against pests such as the corn rootworm.

### *9.6.2 Engineering Biological Control Agents*

The genetic improvement of biological agents is a relatively new concept. For this, a great deal must be known about the biology, ecology, and behavior of the organism.

### 9.6.3 Engineering Crop Plants

The first gene encoding the *Bt* toxin was cloned by Schnepf and Whiteley (1981), and *Bt* gene regulation was known by 1986 (Whiteley et al. 1987). The crop plants were tobacco and tomato, producing the delta endotoxin of *Bacillus thuringiensis* to make them resistant against caterpillars. The uses of genetically modified crops that express insecticidal genes (e.g., Bt crops) also provide an important tool to control Lepidopterous pests (Shelton et al. 2002). Similar strategies have been employed to develop mosquitocidal species of algae. Through genetic engineering techniques, the *Autographa californica* multinucleocapsid nucleopolyhedrosis virus (AcMNPV) was engineered to kill insects more quickly by expressing either enzymes or toxins soon after host invasion. Of particular interest is the possibility of making viruses produce insect neurohormones, which can cause rapid physiological disruptions in minutely defined target hosts. Parasexual recombination not only facilitates genetic analysis in asexually reproducing fungi, but also provides an important tool in strain improvement of bioprotectant fungi.

## 9.7 Biopesticide Regulations

Integrated pest management is being redefined as BIPM (biointensive integrated pest management) by the advent of biologically-based alternatives, which are environment-friendly and ensure food safety and security, apart from maintaining the ecological balance of the living biota. In India, all pesticides (including biopesticides) are regulated under the Insecticidal Act 1968 and require mandatory registration from the Central Insecticidal Board and Registration Committee (CIB and RC). This body has streamlined the registration guidelines and brought the quantum of data generation to a lower scale, granting the registration of the biopesticide on a priority basis.

## 9.8 Biopesticide Commercialization

Biopesticides have not been adequately evaluated in terms of their costs relative to their benefits to farmers and others. Examples from both developed countries (Benbrook 1996) and developing countries (Oka 1996) show that farmers commonly reduce insecticide use by 50–100% without any loss in crop yield after switching to IPM. In addition, the synthetic pesticides market is expected to show a declining trend at the rate of 1.5% per annum. At the same time, the biopesticide market is growing and is expected to reach more than a billion dollars in the next 5 years (Table 9.2 and Fig. 9.1). Key developments expected in the coming years are more R&D in biopesticides, an increase in genetically modified crops, the application of

**Table 9.2** The global market is rapidly expanding with 150–300% increase over next 5 years ($ millions)

| | Europe | NAFTA | Latin America | Africa | ASIA | Oceania | Total |
|---|---|---|---|---|---|---|---|
| Macrobials | 70 | 100 | 15 | 8 | 30 | 20 | 243 |
| Microbials | | | | | | | |
| Bacteria | 15 | 80 | 10 | 5 | 20 | 30 | 160 |
| Virus | 10 | 15 | 10 | 2 | 5 | 10 | 42 |
| Fungi | 25 | 45 | 20 | 3 | 15 | 20 | 128 |
| Total | 50 | 140 | 40 | 10 | 40 | 60 | 330 |
| Biorationals | | | | | | | |
| Natural | 30 | 70 | 25 | 10 | 40 | 15 | 180 |
| Semisynthetic | 40 | 80 | 20 | 10 | 30 | 20 | 200 |
| Total | 70 | 150 | 45 | 20 | 70 | 35 | 390 |
| TOTAL | 190 | 390 | 100 | 38 | 140 | 115 | 973 |

*Source*: International Biocontrol Manufacturer's Association (2004)

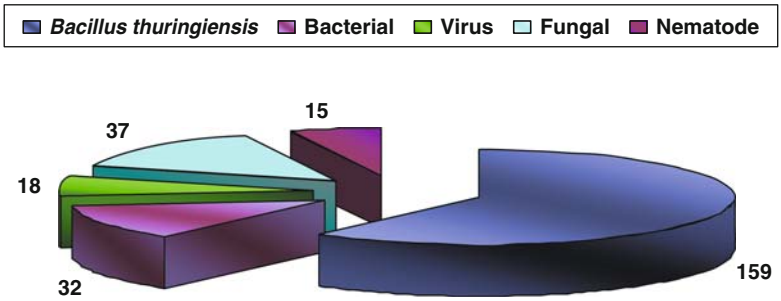



**Fig. 9.1** $260MM estimated 2005 sales projected to grow to $350–400MM by 2015 *Source*: Nasrine Moazami. Biopesticide Production, Biotechnology (EOLSS) manuscript (http://www.eolss.net)

integrated pest management concepts, and a widening of organic farming. Biopesticides today represent about 2.5% of the overall pesticides market, and are expected to grow to about 4.2% by 2010 (Fig. 9.2). Of all the crops, orchard crops hold the largest share of biopesticides use at 55%.

The high cost of labor has also slowed down the development of other biopesticides. The spiralling cost, coupled with regulatory constraints and problems with formulations and marketing, have led to serious setbacks to the application of biopesticides. To apprehend a slow acting biological control agent (the essence of a biopesticide) to challenge an impressive and rapidly acting synthetic chemical is quixotic. Ecosystems are highly variable and unpredictable and to assume that either their resident pests or biocontrol agents will respond constantly over space and time is unrealistic. The inconsistent performance may merely be alluding to the variable nature of their crop ecosystem and are not flaws in the biopesticide per se, and the state of affairs will continue if we treat them like conventional pesticides

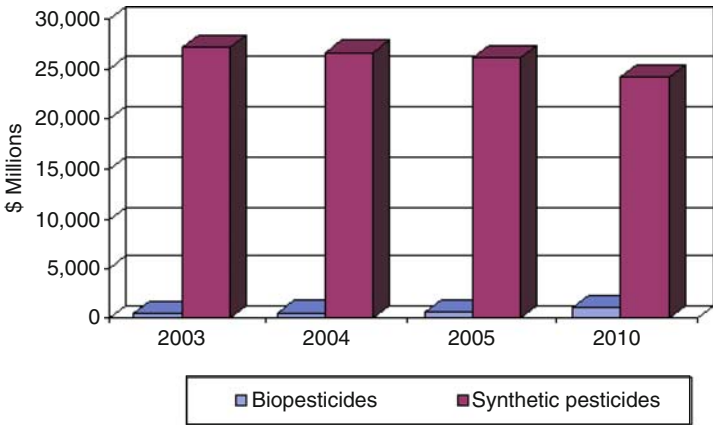



**Fig. 9.2** Global biopesticide market
Report ID: CHM029B, Published (adapted from Thakore 2006)

and expect them to perform evenly across a range of situations and apply them in an ecological abyss.

The successful commercialization of insect-pathogenic viruses has been limited. Thus far, NPV strains have only been mass produced in living insects, a costly procedure. Viral insecticide development is further hindered by the fact that the viruses are specific to one species or genus, ensuring a relatively small market. Genetic engineering offers a potential solution to these shortcomings. A foreign pesticidal gene can be inserted into the viral genome, and expression of the pesticidal gene product during replication will allow the virus to kill insects faster or cause rapid cessation of feeding. Foreign genes which have been inserted into baculoviruses for this purpose include the *Buthus eupeus* insect toxin-1, the Manduca sexta diuretic hormone, the *Bacillus thuringiensis* ssp. *kurstaki* HD-73 delta-endotoxin, the *Heliothis virescens* juvenile hormone esterase, the *Pyemotes tritici* TxP-I toxin, *Androctonus australis* neurotoxin, Dol m V gene and T-urf 13 genes [Nasrine Moazami.Biopesticide Production, Biotechnology (EOLSS) manuscript (http://www.eolss.net).]

## 9.9 Conclusion

Although chemicals characteristically have good storage life, relatively wide spectrum of activity, fast speed of kill, and relatively short persistence, frequent and injudicious applications leads to environmental hazards and poses a serious toxicological threat. In contrast, biological control agents tend to have relatively poor storage life, high target specificity, slow speed of kill, potentially long persistence through secondary cycling, and consequently lower frequency of application. They are environmentally friendly and have a low degree of hazard for humans and livestock. The need of the hour is for a biological agent that possesses the desirable

properties of a chemical pesticide, is highly toxic to the target organism, is able to be mass-produced on an industrial scale, has a long shelf life, can be safely transported, and is highly effective both in its capacity to kill and to reproduce on pests (compounding its killing action). It is important that microbial control of insect pests be further developed and that entomologists along with microbiologists should be able to quantify and make contributions to the regulation of insect populations by naturally occurring pathogens.

Biological control uses a different approach to pest management, focusing on natural enemies of plant pests and diseases to manage their populations. The strategies rely on detailed knowledge of the ecology, the life cycles, and the food chains in each system, thus developing highly target-specific control strategies that leave the nontarget plants, insects, or other animals unharmed. Researchers should employ a range of strategies, often looking for means to strengthen local, natural enemies or to produce them as biopesticides. Fungi, insect viruses, and competing but harmless strains of the same pest need to be tried so as to develop "safer pesticides".

## References

Ahmad M (2007) Insecticide resistance mechanisms and their management in *Helicoverpa armigera* (Hubner). Rev J Agric Res 45:319–335

Benbrook CM (1996) Pest management at the crossroads. Consumers Union, Yonkers, NY, p 272

Bidochka MJ, Small CL (2005) Phylogeography of *Metarhizium*, an insect pathogenic fungus. In: Vegaand FE, Blackwell M (eds) Insect fungal associations: ecology and evolution. Oxford University Press, Oxford, UK, pp 28–50

Campbell WC (1989) Ivermectin and abamectin. Springer, New York, p 363

Copping LG (2004) The manual of biocontrol agents. British Crop Protection Council, Farnham, UK, p 752

Copping LG, Menn JJ (2000) Biopesticides: a review of their action, applications and efficacy. Pest Manag Sci 56:651–676

Cuperus GW, Berberet RC, Noyes RT (2004) The essential role of IPM in promoting sustainability of agricultural production systems for future generations. In: Koul O, Dhaliwal GS, Cuperus GW (eds) Integrated pest management: potential, constraints and challenges. CAB International, Wallingford, UK, pp 265–280

Duan JJ, Head G, Jensen A, Reed G (2004) Effects of transgenic *Bacillus thuringiensis* potato and conventional insecticides for Colorado potato beetle (Coleoptera: Chrysomelidae) management on the abundance of ground-dwelling arthropods in Oregon potato ecosystems. Environ Entomol 33:275–281

Hajek A (2004) Natural enemies: an introduction to biological control. Cambridge University Press, Cambridge, UK

Hawksworth DL, Kirk PM, Sutton BC, Pegler DN (1995) Ainsworth and Bisby's dictionary of fungi, 8th edn. CAB International, Wallingford, UK, p 616

Ishikawa K (1936) Pathology of the silkworm. Meibundo, Tokyo, p 512

Kabulak T, Gazdik K (2005) Directory of microbial pesticides for agricultural crops in OECD countries. Agriculture and Agri-food Canada, http.//www.Agr.Gr.Ca/env/pdf/cat_e_pdf. Accessed 28.06.07

Kogan M, Turnipseed SG (1987) Ecology and management of soybean arthropods. Ann Rev Entomol 32:507–538

Koul O, Dhaliwal GS (2001) Phytochemical biopesticides. Harwood Academic, ISBN:90-5823-089-9.ISSN:1563-6712
Lumsden RD, Lewis JA, Fravel DR (1995) Formulation and delivery of biocontrol agents for use against soil-borne pathogens (unpubl. Report cited in National Research Council 1996). Ecologically based pest management: new solutions for a new century. Natl Acad Press, Washington, DC, p 144
Mensink BJWG, Sheepmaker JWA (2007) How to evaluate the environmental safety of microbialplant protection products: a proposal. Biocontrol Sci Technol 17:3–20
Moazami N (1996) Biopesticide production, biotechnology (EOLSS) manuscript (http://www.eolss.net)
Moscardi F, Sosa-Gomez DR (1996) Soybean in Brazil. In: Persley GJ (ed) Biotecnology and integrated pest management. CAB Int, Wellingford, UK, pp 98–112
Muraleedharan D, Devi DS (1992) Endocrine manipulations as an insect pest management strategy. Agric Zool Rev 5:253–272
Oka IN (1996) Integrated crop pest management: one way to empower farmers to develop efficient and environmentally sound agricultural practices. IARD J 18:1–12
Ramkrishnan N (1992) Nuclear polyhedrosis virus of *Spodoptera litura* : an approach for its efficient use. In: Ananthakrishnan TN (ed) Emerging trends in biological control of phytophagous insects. Oxford and IBH Pub. Co. Pvt. Ltd, New Delhi, pp 159–164
Rehner SA, Buckley E (2005) A Beauveria phylogeny inferred from nuclear ITS and EF1-a sequences: evidence for cryptic diversification and links to *Cordyceps telemorphs*. Mycologia 97:84–98
Rodgers PB (1993) Potential of biopesticides in agriculture. Pesticide Sci 39:117–129
Saxena RC (1998) Green revolutions without blues: botanicals for pest management. In: Dhaliwal GS, Randhawa NS, Arora R, Dhawan AK (eds) Ecological agriculture and sustainable development, vol 2. Indian Ecological Society and Centre for Research in Rural and Industrial Development, Chandigarh, pp 111–127
Schnepf HE, Whiteley HR (1981) Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. Proc Natl Acad Sci USA 78:2893–2897
Shelton AM, Zhao JH, Roush R (2002) Economic, ecological, food safety and social consequences of the deployment of *Bt* transgenic plants. Ann Rev Entomol 47:845–881
Sudakin DL (2003) Biopesticides. Toxicol Rev 22:83–90
Szewczyk B, Hoyos-Carvajal L, Paluszek M, Skrzecz I, Lobo de Souza M (2006) Baculoviruses emerging biopesticides. Biotechnol Adv 24:143–160
Thakore Y (2006) The biopesticide market for global agricultural use. Industrial Biotechnology, 2:194–208
Thompson GD, Dutton R, Sparks TC (2000) Spinosad – a case study: an example from a natural products discovery programme. Pest Manage Sci 56:696–702
Ware GW (1994) The pesticide book, 4th edn. Thomson Publications, Fresno, CA, pp 386–390
Waterhouse DF, Norris KR (1987) Biological control: pacific prospects. Australian Centre Int Agr Res. Inkata Press, Melbourne, p 454
Whalon ME, McGaughey WH (1998) *Bacillus thuringiensis*: use and resistance management. In: Ishaaya I, Deheele D (eds) Insecticides with novel modes of action, mechanism and application. Springer, New York, pp 106–137
Whiteley HR, Schnepf HE, Tomczak K, Lara JC (1987) Structure and regulation of the crystal protein gene of *Bacillus thuringiensis*. In: Maramorosch K (ed) Biotechnology in invertebrate pathology and cell culture. Academic Press, San Diego, pp 13–27
Zechendorf (1995) Novel approaches to integrated pest management. Lewis, Boca Raton, FL, pp 231–257
Zehnder GW, Gelernter WD (1989) Activity of the M-ONE formulation of a new strain of *Bacillus thuringiensis* against the Colorado potato beetle (Coleoptera: Chrysomelidae): relationship between susceptibility and insect life stage. J Econ Entomol 82:756–761
Zimmermann G (2007) Review on safety of the entomopathogenic fungus *Metarhizium anisopliae*. Biocontr Sci Technol 17:879–920

# Chapter 10
# Benefits of Arbuscular Mycorrhizal Fungi to Sustainable Crop Production

**M. Vosátka and J. Albrechtová**

**Abstract** The majority of agricultural crops form relationships with arbuscular mycorrhizal (AM) fungi, which affect physiology and, consequently, yields and food qualities of plants. Mycorrhiza occur naturally in agroecosystems, but their abundance decreases with soil degradation, pollution or excessive use of agrochemicals. Additionally, mycorrhiza may be negatively affected by soil management practices and disadvantageous crop rotation. Conversely, mycorrhizal fungi are usually more abundant in sustainable and medium-to-low-input production systems. In order to exploit the beneficial effects of AM fungi, appropriate management practices, such as the design of suitable crop rotations, appropriate tillage practices, the introduction of multi-microbial inoculants, and the regulated use of agrochemicals, have to be employed. An alternative strategy for improved use of AM in sustainable crop production is to target crop breeding programs involving AM-favoring traits. Understanding the multifarious activities of AM fungi is likely to provide a prospective tool for sustainable crop production in different agro-ecosystems.

## 10.1 Introduction

Mycorrhiza form a beneficial soil microbe–plant interaction that affects the physiological traits of many crop plants, including yield and food-quality. Recently, mycorrhiza have become a prospective tool for sustainable, low input crop production

J. Albrechtová (✉)
Faculty of Science, Department of Plant Physiology, Charles University in Prague, Vinicna 5, 128 44, Prague, Czech Republic
Institute of Botany, Academy of Sciences of the Czech Republic, 252 43 Pruhonice, Czech Republic
e-mail: albrecht@natur.cuni.cz

M. Vosátka
Institute of Botany, Academy of Sciences of the Czech Republic, 252 43 Pruhonice, Czech Republic

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_10, 

systems. Mycorrhizas inhabit the ecosystems of terrestrial plants as a natural way to acquire nutrients from soils (Pirozynski and Dalpe 1992). The majority of crop plants form relationships with arbuscular mycorrhizal (AM) fungi, and their responsiveness to mycorrhiza depends on numerous factors including genotype, soil-related and climate properties, and agrochemical inputs into the cultivation system. Extensive mycorrhizal research conducted during the last few decades has focused on the mechanisms of the symbiosis and its role in the management of a sustainable crop production. Systems high in sustainability include those which aim to make the best use of environmental factors and services without damaging these variables (Pretty 1995). The sustainability has, therefore, become one of the most prominent issues in agricultural practices. Sustainability incorporates the concepts of resilience (the capacity of systems to buffer shocks and stresses) and persistence (the capacity of systems to sustain over long periods), and in turn addresses many wider economic, social and environmental outcomes (Pretty 2008). Moreover, the soil quality is an integral indicator of sustainable ecosystems as well (Herrick 2000).

Soil microorganisms including AM fungi form the basis of healthy and productive soil environments. During the last century, soils have degraded considerably on a global level, and as a result microbial diversity is disturbed. When the world population was less than half what it is currently, its pressure on the environment did not have such far-reaching consequences, and concerns about the environment were not so vital (Lal 2008). The growing world population and human needs such as food, energy and living environments have, however, imposed increasing demands on agroecosystems. The twenty-first century faces many global challenges involving soil functioning where mycorrhiza could play an important role in combating these problems. Mycorrhiza's problem-solving potential applies to (1) increasing agronomic production, (2) enhancing carbon sequestration in terrestrial ecosystems to stabilize the atmospheric $CO_2$ concentration, (3) converting degraded, polluted or desertified soils to restored land or sustainable agroecosystems, and (4) developing sustainable farming/cropping systems aimed at improving water use efficiency and soil properties to combat increasing erosion and minimize risks of water pollution and eutrophication. Thus, AM fungi are regarded as essential components of sustainable soil–plant systems (Schreiner et al. 2003). As an ecological biofertilizer, a bioprotectant against environmental stresses, an agent controlling root pathogens and a soil improver acting as an anti-erosion agent, mycorrhiza possesses great potential in sustainable or organic agriculture. Moreover, it appears that its contribution to carbon sequestration is substantial; mycorrhizal fungi can be an important soil carbon sink and often constitute 20–30% of total soil microbial biomass (Leake et al. 2004). Under conditions of continuously increasing ambient $CO_2$ concentrations, AM fungi are expected to increase their role. Based on meta-analysis, global standing stocks of mycorrhizal fungi may increase substantially by up to 50% under elevated $CO_2$ (Treseder 2004).

Phosphorus (P) availability in soil is often a limiting factor in plant production, and the use of chemical fertilizers (e.g., manufactured water-soluble superphosphates)

has played a significant role in meeting the growing population demands for foods. However, the conventional agricultural practices based on the application of agrochemicals (chemical fertilizers and pesticides) cannot sustain the production base as a healthy plant–soil system for very long (Khan et al. 2007). Also, consistently increasing prices of chemical fertilizers warrants the implementation of sustainable approaches. The new approaches to farming, referred to as sustainable agriculture, require agricultural practices that maintain a long-term ecological balance in the soil ecosystem, including the sustainable management of soil microbes. Under low P or P-deficient conditions, interaction of plants with rhizospheric microorganisms either improves the uptake of the available P or renders unavailable P sources that would otherwise be accessible to the plant. Many lines of scientific evidence highlight the importance of a targeted and sustainable microbial management of soil resources. Management of sustainable agricultural systems needs to develop technologies and practices that do not have adverse effects on environmental variables. Additionally, these technologies and practices need to be accessible to farmers in an effective manner that could lead to enhanced food productivity (Pretty 2008). Mycorrhizal biotechnology meets the demands of sustainable agriculture, and in addition, it often improves food crop quality. However, efficient management of biological soil resources in sustainable cropping systems remains the largest challenge because of the complexity of its roles and interactions with other parts of cropping systems. Special attention is paid to highlight the effects of mycorrhiza on crop productivity and the principles underlying crop responsiveness to AM, including the occurrence and importance of AM in agro-ecosystems depending on environmental factors and management practices. Also, the specifics of AM fungi in horticultural production and the advantages, prospects, and drawbacks of mycorrhiza-friendly management in sustainable plant–soil systems is reviewed and discussed.

## 10.2 Principles of Crop Responsiveness to Mycorrhiza

Many important crops are mycotrophic, or responsive to mycorrhiza, except species from the non-mycotrophic families *Brassicaceae, Chenopodiaceae,* and *Amaranthaceae*, which are never or rarely mycorrhized (Gerdemann 1968). Mycotrophic agricultural and horticultural crops usually form symbioses with AM fungi, and there are reports that approximately 160 zygomycetous fungal taxa of the order *Glomales* (*Glomeromycota*) form mycorrhizal symbioses (Schüssler et al. 2001). A number of AM fungi, including the most common ones *Glomus intraradices*/*Glomus fasciculatum* group and *Glomus mosseae*, have a global distribution occurring in both natural and anthropogenic (disturbed) habitats (Opik et al. 2006). Morphological structures of mycorrhizal fungi and of entire symbiotic system with plant roots determine the symbiotic effects and formulations of mycorrhizal inocula.

In general, colonization with AM fungi result in improvements in plant fitness and nutrition (Smith and Read 1997). A network of extraradical mycorrhizal hyphae facilitate nutrient acquisition and transport many ions to roots, particularly less mobile ions such as P, N, K, S, Ca, Zn, etc. In addition, AM fungi may enhance the reabsorption of nutrients lost through root exudation (Hamel 2004). The contribution of AM fungi to soil fertility has been partly attributed to production of glomalin resulting in an accumulation of organic matter and protection against erosion via forming water stable soil aggregates (Rillig 2004a). The application of AM fungi facilitates the growth of plants by enhancing seedling growth and rooting of cuttings, reducing phosphate and nitrate requirements, increasing survival rate and development of plantlets, increasing resistance to abiotic stresses, increasing flowering and fruiting, and by increasing crop uniformity (Azcon-Aguilar and Barea 1997). In addition to the nutritional effects, AM fungi induce an array of biochemical and molecular responses in host plants. For example, AM fungi have shown an increased production of many allelochemicals (soluble and cell wall-bound phenolics, terpenes, alkaloids, essential oils, and other secondary compounds), and have also included up regulated expression of genes (e.g. phenylalanine ammonia-lyase, chalcone synthase, and chitinase coding genes) involved in plant defences (Bi et al. 2007; Yao et al. 2007). Furthermore, AM fungi often increase resistance or decrease susceptibility of plants to soil-borne pathogens (Dassi et al. 1998; Brimner and Boland 2003; Whipps 2004; Dalpe 2005; Franco et al. 2008). However, mycorrhizal-induced defenses and increased systemic resistance in host plants are still not fully understood (Toussaint 2007).

Generally, the mycorrhizal fungi can be regarded as efficient biofertilizers for sustainable agriculture (Piotrowski and Rillig 2008). In this regard, several aspects of their interaction with target crops and the environment should, however, be taken into account. Chief among them is the responsiveness of plants to mycorrhiza (i.e., the dependence of plant upon mycorrhiza), and the effectiveness of mycorrhizal fungi species and their mixtures. These are terms commonly used in mycorrhiza research with a variety of meanings, which have evolved during the last few decades. Responsiveness to mycorrhiza refers to the dependency of plants upon mycorrhiza, a measure of mycorrhizal fungus effectiveness (Janos 2007), and is represented by the difference in growth between plants with and without mycorrhiza at any designated level of P availability. The difference in performance between AM-colonized and non-colonized plants can be measured as a difference in biomass accumulation, improved morphological parameters, yield, etc. Mycorrhiza dependence denotes the inability of plants to grow or survive without mycorrhizas and without leading to any increase in soil fertility. It can be assessed by examining the effects of a range of soil nutrient contents (especially available phosphorus) on the performance of plants lacking mycorrhizas (Janos 2007). Mycorrhiza dependence can be calculated as the level of nutrient availability below which noncolonized plants either cease to grow or fall below a given performance threshold. By extension, variation in responsiveness can be partitioned into dependence and nondependence components (Sawers et al. 2008). Dependence variation relates to plant performance under a given set of abiotic conditions, and nondependence

variation describes differences in the interaction between plant and the fungus. As such, nondependence differences include variation in the ability of lines to establish colonization, variation in the efficiency of nutrient uptake, and variation in the regulation of nutrient exchange between the fungus and host (Sawers et al. 2008).

The degree of mycotrophy depends to a greater extent on the plant genotype and on environmental conditions (Hamel 1996). Many studies have investigated mycorrhizal responsiveness variation in existing crop varieties, and it is apparent that selection and breeding programs have often selected against AM associations, as the programs are mainly aimed at maximum plant performance in high-input production agro-ecosystems. For example, when comparing the performance of wheat (*Triticum aestivum*) varieties developed before and after 1900, varieties developed before 1900 were more responsive to AM colonization than those developed later (Hetrick et al. 1992). The principle of this phenomenon lies in selection for increased ability of modern lines to take up phosphate without AM fungi under high phosphorus availability in soils (Sawers et al. 2008).

More insight into the complexity of plant responsiveness to AM brought recent advances in the molecular basis of AM–plant interactions including P uptake by mycorrhized plants (Paszkowski 2006). Plants use two distinct P uptake pathways: one acquiring P directly from the soil via P membrane transporters located in the outer membrane of the rhizodermis and root hairs, and the second being active in colonized plants when P is uptaken by the extraradical hyphal network and delivered to arbuscules. In the arbuscules, it is absorbed by plant phosphate transporters in the periarbuscular membrane (Sawers et al. 2008). In mycorrhized plants, a switch from one pattern of gene expression to another corresponding to changes in P uptake occurs. This process is not a simple superimposition of additional gene expression on the noncolonized state (Sawers et al. 2008). Moreover, although cytological and physiological features of AM fungi seem to be conserved, the molecular components may differ significantly between distantly related plant species, as has been shown for P transporter genes in rice *(Oryza sativa)* (Paszkowski et al. 2002). Sawers et al. (2008), however, imply that the genetic determinants affecting the performance of AM-colonized and noncolonized plants vary independently.

Mycorrhizal development increases the availability of nutrients other than P. Nitrogen serves as a good example because of the ability of extraradical mycelium of AM fungi to access small soil pores in the event of low nutrient status or immobile nutrients such as $NH_4^+$ (Drew et al. 2004). Progress has been made in exploring metabolic pathways of N uptake and transport by AM fungi (Govindarajulu et al. 2005). For AM fungi, $NH^{4+}$ is the most likely form of N uptaken though the N uptake mechanisms remain largely unknown (Jackson et al. 2008). Nitrogen is synthesized and stored in the extraradical mycelium in the form of arginine and is transported to the intraradical mycelium in host cells following its generation from arginine breakdown (Jin et al. 2005). The presence of mycorrhiza can affect the uptake of nitrogen depending on the type of fertilizer supplied and the ratio of ammonium/nitrate in it. For example, corn (*Zea mays*) plants colonized by

*Glomus aggregatum* take up ten times more N from a $^{15}NH_4^+$ pathway than from a $^{15}NO_3^-$ pathway (Tanaka and Yano 2005).

## 10.3 Mycorrhiza in Agro-ecosystems

### *10.3.1 Occurrence*

Mycorrhizas occur naturally in agro-ecosystems but their abundance is usually lower compared to natural, nonmanaged ecosystems (Opik et al. 2006). The level of AM fungi in soils and their efficacy decreases with soil degradation, pollution or overfertilization. Also, high soil P fertility and/or fertilization, the use of systemic fungicides, and the use of nonmycotrophic or low mycotrophic crops in rotation have an adverse effect on mycorrhizal symbiosis (Plenchette et al. 2005). Generally, soil AM fungi are more abundant in low input cropping systems with low-to-medium soil P content usually having less than 50 mg $g^{-1}$ soil. In agro-ecosystems, the inoculation effects of mycorrhizal fungi increases with lower availability of essential nutrients and/or water, or where other environmental constraints are in force. The occurrence of mycorrhizal fungi in a particular agro-ecosystem is greatly dependent on the environmental conditions, management history, and current management practices. Agricultural practices can directly or indirectly influence mycorrhizal biodiversity. For example, the lowest AM species abundance has been found in arable fields (Helgason et al. 1998; Daniell et al. 2001) and metal-contaminated soils (Whitfield et al. 2004). Relatively low species richness (about five fungal taxa per plant species) has been reported in habitats that remain under anthropogenic influence (Opik et al. 2006). By contrast, taxon-rich fungal communities (over 20 taxa) were detected in different natural ecosystems, such as a boreal herb-rich coniferous forest (Opik et al. 2008). Recent evidence suggests that the richness of AM fungal taxa in soil is in a complex relationship with the management history of a particular site and its production scheme (Hijri et al. 2006). For example, conventional high-input agriculture with long-term maize monoculture demonstrated low AM fungal diversity, while substantially higher diversity of AM fungi was found in a field trial where a 7-year crop rotation was performed under lower levels of inorganic fertilizer input and chemical pest control (Hijri et al. 2006). Sustainable agricultural practices including crop rotations, reduced or no-tillage practices, and organic farming practices increase total microbial biomass and caused a shift in the soil microbial community structure towards a more fungal-dominated community (Six et al. 2006). It becomes apparent that low-input agriculture involving an AM-friendly crop rotation may provide better conditions to preserve AM fungi diversity by preventing the selection for the few AM fungi species tolerating high nutrient levels.

Changes in the sequence of crops grown on agricultural land – crop rotation – are known to enhance the yield of grain (e.g., wheat) crops by 20% or more (Kirkegaard

et al. 2008). Some of the principal mechanisms responsible for the benefits of crop rotation include (1) effects on disease control, and (2) improved nitrogen nutrition and water supply, besides affecting changes in rhizosphere biology and allelopathy effects or soil structure (Kirkegaard et al. 2008). To successfully integrate mycorrhiza in cropping systems, different factors of production systems and management practices have to be considered. However, the detailed knowledge of mycorrhizal conditions in sustainable agro-ecosystems is scarce (Rillig 2004b). To improve it further, efficient and fast quality indicators of AM fungi populations in the field, including relative field mycorrhizal dependency, soil mycorrhizal infectivity, and mycorrhizal fungi development on plants, need to be developed (Plenchette et al. 2005; Vosátka and Albrechtová 2008).

### *10.3.2 Mycorrhiza-Friendly Management in Sustainable Plant–Soil Production Systems*

In order to exploit beneficial effects of AM fungi in sustainable agricultural plant–soil production systems, the appropriate management practices have to be applied. These include the design of suitable crop rotation in cropping systems, appropriate tillage practices, the introduction of multi-microbial inoculants, and the reduced use of agrochemicals, including pesticides. The appropriate management of mycorrhizal symbiosis could enable a reduction in the use of chemical fertilizers and water supply (Verma and Arya 1998; Sharma and Adholeya 2004; Hart and Trevors 2005; Subramanian et al. 2006; Wu and Xia 2006). In this context, many reports suggest a substantial increase in soil fertility and, consequently, crop productivity following AM inoculation in low-to medium input cropping systems (Harrier and Watson 2003; Johansson et al. 2004; Hart and Trevors 2005; Gosling et al. 2006; Liebig et al. 2006; Perner et al. 2006). Practical management of AM fungal populations and AM symbiosis in agricultural soils and substrates can be of two types: (1) the manipulation of indigenous AM fungi through selected agronomical practices, or (2) artificial crop inoculation with AM inoculum (Sieverding 1991; Gianinazzi and Vosátka 2004; Vosátka and Albrechtová 2008). From an agronomic practice point of view, the most important factors in the management of indigenous AM fungi populations are fertilization, its intensity and method of application, pesticide management, tillage practices, and cropping system (Sieverding 1991). A better understanding of the impacts of agronomic practices on soil microorganisms and the dissemination of this information to end-users will ensure an opportunity for the utilization of the AM fungi that could lead to enhanced sustainable agricultural production.

One of the major priority management issues in sustainable systems is how to maintain nutrient levels (particularly P in the soluble form) compatible for AM development. A significant positive effect of AM inoculation is reported in low or P-deficient soils. This is particularly obvious when slow release biofertilizers are used. In contrast, highly available soil P often limits AM colonization.

For example, Hao et al. (2008) showed a negative effect of AM application on growth and P uptake by maize plants grown in soil rich in P compared to those having low P supply. When P availability is relatively high with simultaneous low N supply, AM fungi did not promote plant N acquisition suggesting that they can act as a carbon drain to plants (Reynolds et al. 2005; Nogueira and Cardoso 2006). Even when P availability is low and AM colonization levels are high, as may occur in organic and biodynamic agricultural systems, AM fungi may not always facilitate plant growth for reasons not yet fully explained (Ryan and Graham 2002).

Irrigation does not belong to the major factors affecting development and efficacy of AM fungi. Mycorrhiza can function under irrigated-to-dry conditions while frequent overlogging can be inhibitory to AM symbionts, since most AM fungi seem to be rather resistant to drought stress (Auge 2001). The application of agrochemicals to soils in order to attain optimum yields severely affects the mycorrhizal symbiosis (Kjoller and Rosendahl 2000; Schweiger et al. 2001). Such effects could be neutral, as reported for the impact of mefenoxam or fludioxonil on soybean (*Glycine max)* (Murillo-Williams and Pedersen 2008), or inhibitory, as observed with certain fungicides, herbicides, or insecticides (Thingstrup et al. 2000; Pimienta-Barrios et al. 2003). Another factor affecting the colonization of AM fungi are the tillage practices applied in agriculture systems. Tillage interferes with AM fungi abundance and lowers the AM fungal diversity (Jansa et al. 2002, 2003). Therefore, such practices need to be considered while designing sustainable farming systems (Gosling et al. 2006). To consolidate this hypothesis, different tillage treatments including moldboard, shred-bedding, subsoil-bedding (Alguacil et al. 2008), plowing, or chiseling (Jansa et al. 2003) have been investigated using maize as a test crop. Although no differences in the effects of tillage treatments on AM fungi diversity were observed, yet a moderate change in mycorrhizal diversity was reported. Such variations in mycorrhizal diversity could be due to (1) the differences in tolerance to the tillage-induced disruption of the hyphae among the different AM fungal species, (2) changes in nutrient content of the soil, (3) changes in microbial activity, and (4) changes in weed populations in response to soil tillage. These findings therefore suggest that the frequent disturbances caused by tillage practices should be avoided. Moreover, conservation tillage in combination with high mycorrhization seems to be a strategy to reduce root pathogens, as was shown for maize (Franco et al. 2008).

Crop rotation has proved to be an efficient tool for AM fungi management (Gosling et al. 2006), and adopting an appropriate crop sequence is an important part in developing sustainable production systems in many areas of the world (Kirkegaard et al. 2008). For example, the use of nonmycotrophic or low mycotrophic crops in crop sequences can lead to a substantial decrease or even the disappearance of indigenous mycorrhizal populations (Harrier and Watson 2003; Plenchette et al. 2005). The involvement of intercropping plants producing nonfavorable root exudates to AM fungi development can also have negative effects on the maintenance of indigenous AM fungal populations. This phenomenon was observed for some cruciferous plants that are inserted as cover crops between two cash crops to improve soil nitrate

fertility (Grodzinsky 1992). The consequences of practices using cruciferous nitrate trapping plants, such as mustard (*Brassica campestris*), on mycorrhizal fungi survival have not yet been fully considered (Plenchette et al. 2005). The allelopathic properties of cereal crops can be related to low responsiveness to AM fungi or even carbon drain effects in different crop rotation systems and have to be considered. The most important grain crops like wheat, rice, maize, and sorghum (*Sorghum bicolour)* may exhibit allelopathy and show variable response to mycorrhizal inoculation under different soil environments (Javaid 2008).

An appropriate design of crop rotation involving the use of AM fungi may result in improved mineral nutrition. The proper management of cover crops inducing enhanced AM fungi colonization can increase the availability of soil P pool (both organic and inorganic) by stimulating microbial activity and release of root exudates (Nelson and Janke 2007). For example, legumes fixing biologically atmospheric nitrogen are often supposed to be capable of bringing energy savings when used as a cover crop. For maize production, it has been shown that under P-deficient conditions, legumes supplied with phosphate rock increase P acquisition by a subsequent maize crop compared to direct application of phosphate rock to maize (Pypers et al. 2007). The results of this trial suggested that the legume in the rotation system had other positive effects, possibly soil-microbial effects enhancing maize growth and production. Nevertheless, caution should be exercised when simultaneous application of intercropping legumes and mycorrhizal inoculation is practised. Arbuscular mycorrhizas can alter competitive interactions between plants that markedly differ in their dependence upon mycorrhizas, and also during intra- and inter-specific competition between similarly dependent plant species (Schroeder-Moreno and Janos 2008). Yao et al. (2008), while using AM fungi in a citrus–leguminous *Stylosanthes gracilis* intercropping system, reported negative growth effects of citrus due to the increased nutrient-competing capacity of *S. gracilis* and, hence, suggested that AM fungi is not a potential biofertilizer candidate in a citrus–*S. gracilis* intercropping system. However, the study of Brehmer et al. (2008) concludes that legumes grown as bioenergy crops do not present energy savings, and thus do not contribute to sustainability. The authors state that the benefits of nitrogen fixation by legumes should be carefully assessed and best utilized within the emerging sector of nonfood applications. The influence of AM fungi on soil processes is complex and difficult to define, in part because it varies with plant and fungal genotypes as well as with environmental conditions (Hamel 2004). Biocontrol can be another benefit of suitable crop rotation. Integrating appropriate break crops into the farming system should contribute to disease control. Break crops impede the life cycle of crop-specific pathogens by growing a nonhost crop, and lead to better nutrient, water, and soil management (Kirkegaard et al. 2008). From a practical point of view, the adoption of sustainable agricultural practices may not yield desirable effects immediately, and hence numerous field trials in conventional agriculture have turned to organic systems. Due to various reasons, the natural populations of AM fungi in soils can be greatly reduced (Harrier and Watson 2003; Plenchette et al. 2005; Gosling et al. 2006).

### *10.3.3 Specifics of Mycorrhiza Applications in Horticultural Crop Production*

High responsiveness to mycorrhiza is common for the majority of horticultural crops (Plenchette et al. 1983; Azcon-Aguilar and Barea 1997). In this context, the mycorrhizal biotechnology, i.e., introduction and/or maintenance and management of mycorrhizal fungi populations in the cultivation system, should become an important component of horticultural production (Vosátka and Albrechtová 2008). In floriculture, mycorrhizal application has shown a substantial increase in the yield properties, such as aboveground biomass (Sramek et al. 2000), number of flowers per plant (up to 24%) for marigold (*Calendula officinalis*) plants (Flores et al. 2007), flower diameter, or shortening the period to flowering. The degree of crop mycorrhizal responsiveness plays an important role, particularly when cultivation conditions involve either soil-less or fumigated substrates where indigenous mycorrhizal fungal populations are either lacking or greatly reduced.

In high-value greenhouse crop production in horticulture, the introduction of mycorrhiza can be important in soil-less substrates lacking microorganisms and during the production of micropropagated plants (Vosátka and Albrechtová 2008). For example, the AM fungi are not usually present in peat, while some ericoid and ectomycorrhizal fungi are very common in peat substrates (Perner et al. 2006). Peat based soil-less substrate is usually enriched with nutrients, and when excessive P-enrichment of substrates is used, it can suppress the germination of endomycorrhizal spores. A mycorrhizal-friendly peat amendment can have a high N content and low P content promoting spore growth of *Gigaspora margarita* Becker and Hall (Ma et al. 2006). In horticultural crop systems, plants are usually transplanted after the first propagation stage, and the conditions found in the transplanting substrate are crucial for plant survival. Only a few reports are available on the use of mycorrhizal inoculation when plants are grown in soil-less hydroponic culture systems as described for the production of axenic arbuscular mycorrhizae (MacDonald 1981) or nutrient film technique (NFT) in hydroponic culture systems (Mathew and Johari 1988). George and Lee (2005) suggested that a modified NFT system might be a useful way to mass-produce mycorrhizal crops and inoculum for commercial horticultural purposes.

Mycorrhizal inoculation can be successfully used in horticulture for tissue cultured plants. Micropropagation is a well-established and widely used technology for propagation of many vegetables and spices (Vestberg and Estaun 1994). Plants propagated under in vitro conditions can suffer from an initial lack of beneficial soil microorganisms while they can greatly benefit from inoculation when transferred to post vitro unsterile conditions. To alleviate transplantation shock and to increase plant survival after transplantation, successful plant acclimatization has to be achieved. The most crucial phase of acclimatization is the development of a functional root system that mediates effective nutrient acquisition and water supply. There are three stages during which mycorrhizal inoculation could be included in the micropropagation procedures: (1) rooting phase, (2) at the beginning of acclimatization

(weaning) phase, and (3) after the acclimatization phase but before starting the post-acclimatization period under greenhouse conditions (Azcon-Aguilar and Barea 1997; Taylor and Harrier 2003).

Studies on the inoculation of micropropagated plants again underlined the necessity of selecting an efficient combination of mycorrhizal fungi and host plant genotype for a particular substrate and cultivation conditions (Vestberg et al. 2002). As was documented in the case of red raspberry (*Rubus idaeus*) inoculated with *Gigaspora* sp. (Taylor and Harrier 2000), the whole range of plant growth responses from enhancement to growth depression can occur. Current possibilities of using consortia of rhizotrophic microorganisms for the inoculation of micropropagated plants are under investigation, and could lead to the development of composite inoculants of mycorrhizal fungi and nitrogen-fixing and/or phosphate-solubilizing bacteria (Vosátka et al. 2000; Gryndler et al. 2002; Vestberg et al. 2004).

## 10.4 Drawbacks and Potentials of Mycorrhizal Applications in Sustainable Agriculture

During the last decade, mycorrhizal technology reached a more developed stage by becoming an industry supported by extensive research and commercial applications that emphasized ecological aspects of the use of mycorrhiza (Gianinazzi and Vosátka 2004; Vosátka et al. 2008). Since large-scale inoculations with AM fungi have become a reality, the drawbacks of these applications should be carefully considered with regard to their profitability.

An important question for using AM fungi in sustainable crop production systems is whether generic or tuned products should be used. One of the drawbacks of using mycorrhizal inoculants in field is that there are already indigenous populations of AM fungi in soils. Such indigenous fungi can be highly competitive in securing their colonization niche from the introduced fungi. Even though introduced strains can be less efficient than indigenous ones (Schwartz et al. 2006), in some cases the adaptation of the native fungi has shown a competitive advantage over the introduced strains (Batkhuugyin et al. 2000; Oliveira et al. 2005). This is often more pronounced in degraded ecosystems with extreme soil properties where native populations of AM fungi can perform better in the environment of their origin compared to other nonnative symbionts in degraded soils (Batkhuugyin et al. 2000). Furthermore, there can be significant differences in performance even among different geographical isolates that belong to the same fungal species. It is possible to isolate native mycorrhizal strains for a particular ecosystem, to produce inoculum, and to re-introduce native strains in the field on a large scale. Some commercial companies are applying inoculum tuning to target conditions where native mycorrhizal populations have been reduced (Gianinazzi and Vosátka 2004; Vosátka and Albrechtová 2008).

For the successful introduction of inocula into the field, numerous ecological factors have to be considered. These include soil properties, level of fertilizer input,

and the degree and potential of existing fungal population (Vosátka and Dodd 2002). To realize all the potentials of mycorrhizal management and applications, it is absolutely vital to conduct reference field trials prior to large-scale applications, which are of great importance for properly tuning the best combinations of mycorrhizal fungal inoculum for target conditions. And, hence, a careful selection of symbionts intended for use should be employed prior to large-scale production and application. By tuning the appropriate inoculants to the target cultivation system, it is possible to achieve economic feasibility of mycorrhizal technology (Vosátka and Dodd 2002).

Molecular identification of different isolates in a field to reliably distinguish native and introduced populations is needed to evaluate ecological and economic consequences of artificial inoculation into field conditions. Although, within species, genetic differences among AM fungi have been shown to differentially affect plant growth, very little is known about the degree of genetic diversity in AM fungal populations (Croll et al. 2008). It is apparent that differences in crop responsiveness to various mycorrhizal isolates can be based on genetic variability in AM fungal populations that affects host–plant fitness. The AM fungi are asexually reproducing organisms that show coexistence of a population of many genomes (Hijri and Sanders 2005). This implies a high genetic variation of within-AM fungi populations, though the effects of within-AM fungi species or within-population variation on plant growth have received less attention (Koch et al. 2006). Recently, success has been achieved in molecular identification of different isolates of *G. margarita* (Yokoyama et al. 2005) and *G. intraradices* using newly developed simple sequence repeat, nuclear gene intron, and mitochondrial ribosomal gene intron markers (Croll et al. 2008). Advances in molecular identification further support the importance of adjusting the tuning of introduced commercial inocula to a specific target combination of a crop production system.

Among different types of mycorrhiza, AM fungi exhibit relatively low specificity in host–symbiont formation, which is advantageous for natural spreading of arbuscular mycorrhiza in a plant community as well as for large-scale commercial use of mycorrhizal inoculants in agriculture in different agro-ecosystems. However, the last decade of AM fungi research has revealed far more selectivity of AM fungi–plant associations, and a greater dependence of resulting functions on particular host/fungus combinations than previously suggested (Piotrowski and Rillig 2008). It has been proposed that genetic diversity, either within or among AM fungal species, rather than the species richness of AM fungi, could play a pivotal role in the diversity, functioning and overall performance of plants (Sanders 2004). Host plants in a field could provide a heterogeneous environment favoring certain genotypes. Such preferences, in turn, may partly explain the patterns of genetic diversity within populations. The effects of specific host/AM fungi combinations range from beneficial to parasitic; hence, the next step in AM fungi application will entail an effort to employ advantageous combinations. The tuning of commercial inoculum is regarded not as management practices but as necessity to achieve optimum benefits from mycorrhizal association (Vosátka and Albrechtová 2008).

In crop breeding programs, progress has been made in the isolation and functional analyses of genes controlling yield and tolerance to abiotic stresses (Takeda

and Matsuoka 2008), which have to be considered with respect to the type of environment for which a crop cultivar is targeted. Crop breeding targeted to drought tolerance focuses on physiological yield drivers such as water uptake, water use efficiency, and harvest index (Reynolds and Tuberosa 2008). The AM fungi can directly mediate improved drought tolerance via changes of plant physiological traits, such as higher leaf water potential, transpiration rates, stomatal conductance, and relative water content, and alleviate drought stress conditions, as reported for citrus (Wu and Xia 2006), chillies (*Capsicum annuum* L.) (Mena-Violante et al. 2006), and tomato (*Lycopersicon lycopersicum)* plants (Subramanian et al. 2006). Indirect effects of mycorrhiza on crop drought resistance can be mediated by the introduction of glomalin into soil, quantified operationally in soils as glomalin-related soil protein, which is able to aggregate soil particles and increase water stable aggregates (Rillig 2004a) having a positive effect on soil moisture. In conventional agriculture, the breeding programs have not considered mycorrhiza-oriented breeding traits, and crops are selected to favor solely nutrient acquisition in high input systems without considering the role of mycorrhiza in nutrient management of soils (Sawers et al. 2008). This is a major drawback to mycorrhiza application, though it can be circumvented by the proper selection of crop genotype. Thus, there is an urgent need to manipulate plant genetic factors that impact the cost–benefit balance of mycorrhizal applications, or more specifically, the rate at which resources are diverted to the fungus in return for a given level of enhanced performance or reduction in production costs.

One of the main potentials of mycorrhizas for sustainable agriculture is that they induce plant physiological changes that affect the quality and safety of food crops, including a higher production of antioxidants or essential oils, or reduced uptake of pollutants such as heavy metals to plant tissues (Toussaint 2007; Toussaint et al. 2007; Vosátka and Albrechtová 2008). Although no clear mechanism other than an improvement in the nutritional status (mainly P) has been proposed (Toussaint 2007), yet the beneficial fungus–plant interactions has shown enhancement in productivity of crops by synthesizing an increased level of active compounds (Rai et al. 2001). For example, the suitable selection of host plant–fungus genotype led to an altered accumulation of essential oil levels in AM-colonized plants of *Mentha arvensis* (Freitas et al. 2004) and sweet basil *Ocimum basilicum* L. (Copetta et al. 2006, 2007; Toussaint et al. 2007). Thus, the use of mycorrhiza is being extended to herbal plants in order to get maximum benefits by the steadily increasing herbal medicine industry (Ernst 2000). However, to achieve good outcomes from mycorrhizal inoculations in crop production, it is necessary to incorporate inoculum in the production systems in the most effective manner. The mycorrhiza should be introduced in the plant propagation as soon as possible so that a reliable and effective mycorrhization of crop plants is achieved. Unfortunately, there is no universal method of inocula application, while some planting and plant production systems require specific ways of application. Mycorrhizal inocula, however, could be applied in several ways. For example, they can be applied in dry formulation form. For this, a layer of inoculum is placed below the seeds or can be mixed with the growing substrate for containerized plants. Either of these methods leads to its spreading into

the planting hole. When the mycorrhizal product is used as a gel formulation, it can be easily applied either to the planting hole or the bare roots during transplanting. Additionally, the root balls can be dipped into a gel product to cover their surface and ensure mycorrhizal colonization of newly emerging roots. For large-scale applications under field environments, mechanical devices are needed (mixing tanks for application in substrates or sowing machines for dispersing granulated inocula).

Currently, there is increasing demand for the incorporation of bioproducts from organic agriculture and new alternatives that enhance sustainability in agriculture. There are obviously certain problems that need to be seriously addressed. For example, it is necessary to educate farmers about the benefits of mycorrhizal inoculation and organic farming so that the reliance on agrochemicals is minimized. Also, the questionable capacity of organic agriculture to provide sufficient yield should be addressed (Connor 2008). Some analyses have estimated the capability of organic agriculture to feed three to four billion people, well below the present and projected world population (Smil 2001). Recently, a new concept of eco-agriculture was proposed for achieving productivity in perpetuity (Kesavan and Swaminathan 2008). That includes developing farming/cropping systems that improve water use efficiency and minimize risks of water pollution, contamination, and eutrophication thereby converting the degraded/desertified soils to restored land use for enhancing biodiversity and improving the environment (Lal 2008). The efficient management of mycorrhizas and soil biota, in general, forms an important component of this technology.

In developing countries, many farmers are using less agrochemicals and more biocontrol agents like species of *Trichoderma*, *Bacillus,* and also mycorrhizal products as an alternative to agrochemicals (Kesavan and Swaminathan 2008). Apparently such bioagents are more often used due to economic reasons, since agrochemicals are too expensive, rather than from mere interest in sustainable crop production. Official marketing of mycorrhiza as a biocontrol agent is currently unrealistic, because the registration of mycorrhizal products with biocontrol activity is complicated and too costly for inoculant producers (Whipps 2004). Mycorrhizal products are usually sold as growth promoters for two simple reasons: (1) a large-scale screening process would be needed to specify strains acting as biocontrol agents for specific plants and environmental conditions, and (2) biocontrol strains would have to be formally registered (Whipps 2004). Despite all these facts, the trend of using natural resources like AM fungi, seems to be increasing and could become prevalent due to increasing demands placed on safety and quality of foods across the globe. Another driving force for stronger focus on sustainable agriculture in developing countries is indeed continuous depletion of soil and water resources.

## 10.5 Conclusion

In properly managed agriculture systems, mycorrhiza can act as a biofertilizer, biocontrol agent and soil improver. When accurate inoculum tuning is accomplished and used for formulation of mycorrhizal products, the large-scale application of

such fungi is not a threat either to the biodiversity or to soil fertility. Genotype-related differences in host plant–AM fungus combinations can be discerned which could be attributed to the differences in the balance between P uptake by the fungal pathway and direct uptake via the roots of plants. Furthermore, maintaining sustainability in agriculture requires attention on how new breeding programs could help to attain maximum benefits following mycorrhizal symbiosis. Recent advances in molecular breeding approaches are helping to optimize the use of AM–fungal symbioses for improvement of crop productivity. However, there is also a need to produce efficient varieties which are able to use AM fungi in more effective ways for nutrient acquisition (Sawers et al. 2008).

Despite its potential and proven ability, microbe management currently plays only a secondary role in agricultural practices. One of the reasons is that there is a lack of legislative policy focusing explicitly on soil ecosystems and degradation processes. And, currently, there is no international policy framework to guide sustainable soil management, though there are some guidelines related to that issue in the United Nations Convention to Combat Desertification (Stringer 2008). Another area of concern is the lack of awareness about the benefits of AM fungi among farmers besides their inconsistent performance under different ecological niches. The application of soil microbial inoculations is further compounded by poor understanding of ecology, population dynamics, and functionality of AM fungi over a range of environments (Khan et al. 2007). And, hence, if the microbes are to be used in a predictable and useful way, focus has to be directed towards understanding the basic biology of these microbes and their interaction with host plants (Hart and Trevors 2005). Additionally, in order to expand the use of AM fungi, inoculum tuning to monitor and predict the functioning of introduced mycorrhiza in crop production systems able to improve soil fertility and pest management should be given priority. Despite such problems, the mycorrhiza industry has developed quickly. If farmers are provided with sufficient training as to how these organisms are properly applied, the use of AM fungi is likely to maximize not only the productivity of crops while reducing the use of agrochemicals but is also likely to reduce soil pollution.

**Acknowledgement** The authors acknowledge the funding of the Ministry of Education, Youth and Sports of the Czech Republic from the projects No. 1P04OE187, the EU project E! 3375 EUROAGRI +MYCOTAGRIF, the Centre for Bioindication and Revitalization 1M0571, and the Institutional project AV0Z60050516 of the Institute of Botany, Academy of Sciences of the Czech Republic.

## References

Alguacil MM, Lumini E, Roldan A, Salinas-Garcia JR, Bonfante P, Bianciotto V (2008) The impact of tillage practices on arbuscular mycorrhizal fungal diversity in subtropical crops. Ecol Appl 18:527–536

Auge RM (2001) Water relations, drought and vesicular-arbuscular mycorrhizal symbiosis. Mycorrhiza 11:3–42

Azcon-Aguilar C, Barea JM (1997) Applying mycorrhiza biotechnology to horticulture: significance and potential. Sci Hortic-Amsterdam 68:1–24

Batkhuugyin E, Rydlova J, Vosátka M (2000) Effectiveness of indigenous and non-indigenous isolates of arbuscular mycorrhizal fungi in soils from degraded ecosystems and man-made habitats. Appl Soil Ecol 14:201–211

Bi HH, Song YY, Zeng RS (2007) Biochemical and molecular responses of host plants to mycorrhizal infection and their roles in plant defence. Allelopathy J 20:15–27

Brehmer B, Struik PC, Sanders J (2008) Using an energetic and exergetic life cycle analysis to assess the best applications of legumes within a biobased economy. Biomass Bioenerg 32:1175–1186

Brimner TA, Boland GJ (2003) A review of the non-target effects of fungi used to biologically control plant diseases. Agr Ecosyst Environ 100:3–16

Connor DJ (2008) Organic agriculture cannot feed the world. Field Crop Res 106:187–190

Copetta A, Lingua G, Berta G (2006) Effects of three AM fungi on growth, distribution of glandular hairs, and essential oil production in *Ocimum basilicum* L. var. Genovese. Mycorrhiza 16:485–494

Copetta A, Lingua G, Bardi L, Masoero G, Berta G (2007) Influence of arbuscular mycorrhizal fungi on growth and essential oil composition in *Ocimum basilicum* var. Genovese. Caryologia 60:106–110

Croll D, Wille L, Gamper HA, Mathimaran N, Lammers PJ, Corradi N, Sanders IR (2008) Genetic diversity and host plant preferences revealed by simple sequence repeat and mitochondrial markers in a population of the arbuscular mycorrhizal fungus *Glomus intraradices*. New Phytol 178:672–687

Dalpe Y (2005) Mycorrhizae: a potential tool for plant protection but not a panacea. Phytoprotection 86:53–59

Daniell TJ, Husband R, Fitter AH, Young JPW (2001) Molecular diversity of arbuscular mycorrhizal fungi colonising arable crops. FEMS Microbiol Ecol 36:203–209

Dassi B, Dumas-Gaudot E, Gianinazzi S (1998) Do pathogenesis-related (PR) proteins play a role in bioprotection of mycorrhizal tomato roots towards *Phytophthora parasitica*? Physiol Mol Plant P 52:167–183

Drew EA, Murray RS, Smith SE, Jakobsen I (2004) Beyond the rhizosphere: growth and function of arbuscular mycorrhizal external hyphae in sands of varying pore sizes. Plant Soil 251:105–114

Ernst E (2000) Herbal medicines: where is the evidence? Growing evidence of effectiveness is counterbalanced by inadequate regulation. Brit Med J 321:395–396

Flores AC, Luna AAE, Portugal VO (2007) Yield and quality enhancement of marigold flowers by inoculation with *Bacillus subtilis* and *Glomus fasciculatum*. J Sustain Agr 31:21–31

Franco AD, Garcia JRS, Cano IG, Perez NM (2008) Impact of tillage and arbuscular mycorrhiza inoculation on charcoal rot and yield of maize under semiarid conditions. Revista Fitotecnia Mexicana 31:257–263

Freitas MSM, Martins MA, Vieira EIJC (2004) Yield and quality of essential oils of *Mentha arvensis* in response to inoculation with arbuscular mycorrhizal fungi. Pesqui Agropecu Bras 39:887–894

George E, Lee YJ (2005) Development of a nutrient film technique culture system for arbuscular mycorrhizal plants. HortScience 40:378–380

Gerdemann JW (1968) Vesicular-arbuscular mycorrhiza and plant growth. Annu Rev Phytopathol 6:397–418

Gianinazzi S, Vosátka M (2004) Inoculum of arbuscular mycorrhizal fungi for production systems: science meets business. Can J Bot 82:1264–1271

Gosling P, Hodge A, Goodlass G, Bending GD (2006) Arbuscular mycorrhizal fungi and organic farming. Agr Ecosyst Environ 113:17–35

Govindarajulu M, Pfeffer PE, Jin HR, Abubaker J, Douds DD, Allen JW, Bucking H, Lammers PJ, Shachar-Hill Y (2005) Nitrogen transfer in the arbuscular mycorrhizal symbiosis. Nature 435:819–823

Grodzinsky AM (1992) Allelopathic effects of cruciferous plant in crop rotation. In: Rizvi SJH, Rizvi V (eds) Allelopathy: basic and applied aspects. Chapman & Hall, London

Gryndler M, Vosátka M, Hrselova H, Catska V, Chvatalova I, Jansa J (2002) Effect of dual inoculation with arbuscular mycorrhizal fungi and bacteria on growth and mineral nutrition of strawberry. J Plant Nutr 25:1341–1358

Hamel C (1996) Prospects and problems pertaining to the management of arbuscular mycorrhizae in agriculture. Agr Ecosyst Environ 60:197–210

Hamel C (2004) Impact of arbuscular mycorrhizal fungi on N and P cycling in the root zone. Can J Soil Sci 84:383–395

Hao LF, Zhang JL, Chen FJ, Christie P, Li XL (2008) Response of two maize inbred lines with contrasting phosphorus efficiency and root morphology to mycorrhizal colonization at different soil phosphorus supply levels. J Plant Nutr 31:1059–1073

Harrier LA, Watson CA (2003) The role of arbuscular mycorrhizal fungi in sustainable cropping systems. Adv Agron 79:185–225

Hart MM, Trevors JT (2005) Microbe management: application of mycorrhyzal fungi in sustainable agriculture. Front Ecol Environ 3:533–539

Helgason T, Daniell TJ, Husband R, Fitter AH, Young JPW (1998) Ploughing up the wood-wide web? Nature 394:431

Herrick JE (2000) Soil quality an indicator of sustainable land management. Appl Soil Ecol 15: 75–83

Hetrick B, Wilson GWT, Cox TS (1992) Mycorrhizal dependence of modern wheat-varieties, landraces, and ancestors. Can J Bot 70:2032–2040

Hijri M, Sanders IR (2005) Low gene copy number shows that arbuscular mycorrhizal fungi inherit genetically different nuclei. Nature 433(7022):160–163

Hijri I, Sykorova Z, Oehl F, Ineichen K, Mäder P, Wiemken A, Redecker D (2006) Communities of arbuscular mycorrhizal fungi in arable soils are not necessarily low in diversity. Mol Ecol 15:2277–2289

Jackson LE, Burger M, Cavagnaro TR (2008) Roots nitrogen transformations, and ecosystem services. Annu Rev Plant Biol 59:341–363

Janos DP (2007) Plant responsiveness to mycorrhizas differs from dependence upon mycorrhizas. Mycorrhiza 17:75–91

Jansa J, Mozafar A, Anken T, Ruh R, Sanders IR, Frossard E (2002) Diversity and structure of AMF communities as affected by tillage in a temperate soil. Mycorrhiza 12:225–234

Jansa J, Mozafar A, Kuhn G, Anken T, Ruh R, Sanders IR, Frossard E (2003) Soil tillage affects the community structure of mycorrhizal fungi in maize roots. Ecol Appl 13:1164–1176

Javaid A (2008) Allelopathy in mycorrhizal symbiosis in the Poaceae family. Allelopathy J 21:207–217

Jin H, Pfeffer PE, Douds DD, Piotrowski E, Lammers PJ, Shachar-Hill Y (2005) The uptake, metabolism, transport and transfer of nitrogen in an arbuscular mycorrhizal symbiosis. New Phytol 168:687–696

Johansson JF, Paul LR, Finlay RD (2004) Microbial interactions in the mycorrhizosphere and their significance for sustainable agriculture. Fems Microbiol Ecol 48:1–13

Kesavan PC, Swaminathan MS (2008) Strategies and models for agricultural sustainability in developing Asian countries. Philos T R Soc B 363:877–891

Khan MS, Zaidi A, Wani PA (2007) Role of phosphate-solubilizing microorganisms in sustainable agriculture – A review. Agron Sustain Dev 27:29–43

Kirkegaard J, Christen O, Krupinsky J, Layzell D (2008) Break crop benefits in temperate wheat production. Field Crop Res 107:185–195

Kjoller R, Rosendahl S (2000) Effects of fungicides on arbuscular mycorrhizal fungi: differential responses in alkaline phosphatase activity of external and internal hyphae. Biol Fert Soils 31:361–365

Koch AM, Croll D, Sanders IR (2006) Genetic variability in a population of arbuscular mycorrhizal fungi causes variation in plant growth. Ecol Lett 9:103–110

Lal R (2008) Soils and sustainable agriculture. A review. Agron Sustain Dev 28:57–64

Leake JR, Johnson D, Donnelly DP, Muckle GE, Boddy L, Read DJ (2004) Networks of power and influence: the role of mycorrhizal mycelium in controlling plant communities and agroecosystem functioning. Can J Bot 82:1016–1045

Liebig M, Carpenter-Boggs L, Johnson JMF, Johnson JMF, Wright S, Barbour N (2006) Cropping system effects on soil biological characteristics in the Great Plains. Renew Agr Food Syst 21:36–48

Ma N, Yokoyama K, Marumoto T (2006) Stimulatory effect of peat on spore germination and hyphal growth of arbuscular mycorrhizal fungus *Gigaspora margarita*. Soil Sci Plant Nutr 52:168–176

MacDonald RM (1981) Routine production of axenic vesicular-arbuscular mycorrhizas. New Phytol 89:87–93

Mathew J, Johari BN (1988) Propagation of vesicular-arbuscular mycorrhizal fungi in moong (*Vigna radiata* L) through nutrient film technique (NFT). Curr Sci India 57:156–158

Mena-Violante HG, Ocampo-Jimenez O, Dendooven L, Martinez-Soto G, Gonzalez-Castaneda J, Davies FT, Olalde-Portugal V (2006) Arbuscular mycorrhizal fungi enhance fruit growth and quality of chile ancho (*Capsicum annuum* L. cv San Luis) plants exposed to drought. Mycorrhiza 16:261–267

Murillo-Williams A, Pedersen P (2008) Arbuscular mycorrhizal colonization response to three seed-applied fungicides. Agron J 100:795–800

Nelson NO, Janke RR (2007) Phosphorus sources and management in organic production systems. Horttechnology 17:442–454

Nogueira MA, Cardoso EJBN (2006) Plant growth and phosphorus uptake in mycorrhizal rangpur lime seedlings under different levels of phosphorus. Pesqui Agropecu Bras 41:93–99

Oliveira RS, Vosátka M, Dodd JC, Castro PML (2005) Studies on the diversity of arbuscular mycorrhizal fungi and the efficacy of two native isolates in a highly alkaline anthropogenic sediment. Mycorrhiza 16:23–31

Opik M, Moora M, Liira J, Zobel M (2006) Composition of root-colonizing arbuscular mycorrhizal fungal communities in different ecosystems around the globe. J Ecol 94:778–790

Opik M, Moora M, Zobel M, Saks U, Wheatley R, Wright F, Daniell T (2008) High diversity of arbuscular mycorrhizal fungi in a boreal herb-rich coniferous forest. New Phytol 179: 867–876

Paszkowski U (2006) A journey through signaling in arbuscular mycorrhizal symbioses. New Phytol 172:35–46

Paszkowski U, Kroken S, Roux C, Briggs SP (2002) Rice phosphate transporters include an evolutionarily divergent gene specifically activated in arbuscular mycorrhizal. P Natl Acad Sci USA 99:13324–13329

Perner H, Schwarz P, George E (2006) Effect of mycorrhizal inoculation and compost supply on growth and nutrient uptake of young leek plants grown on peat-based substrates. Hortscience 41:628–632

Pimienta-Barrios E, del Castillo-Aranda MEG, Muñoz-Urias A, Nobel PA (2003) Effects of benomyl and drought on the mycorrhizal development and daily net $CO_2$ uptake of a wild platyopuntia in a rocky semi arid environment. Ann Bot 92:239–245

Piotrowski JS, Rillig MC (2008) Succession of arbuscular mycorrhizal fungi: patterns, causes, and considerations for organic agriculture. Adv Agron 97:111–130

Pirozynski KA, Dalpe Y (1992) The geological history of the Glomaceae with particular reference to mycorrhizal symbiosis. Symbiosis 7:1–36

Plenchette C, Fortin JA, Furlan V (1983) Growth responses of several plant species to mycorrhizae in a soil of moderate P- fertility. I. Mycorrhizal dependency under field conditions. Plant Soil 70:199–209

Plenchette C, Clermont-Dauphin C, Meynard JM, Fortin JA (2005) Managing arbuscular mycorrhizal fungi in cropping systems. Can J Plant Sci 85:31–40
Pretty J (1995) Regenerating agriculture: policies and practice for sustainability and self-reliance. Earthscan, London, UK; National Academy Press, Washington, DC, p 320
Pretty J (2008) Agricultural sustainability: concepts, principles and evidence. Philos T R Soc B 363:447–465
Pypers P, Huybrighs M, Diels J, Abaidoo R, Smolders E, Merckx R (2007) Does the enhanced P acquisition by maize following legumes in a rotation result from improved soil P availability? Soil Biol Biochem 39:2555–2566
Rai M, Acharya D, Singh A, Varma A (2001) Positive growth responses of the medicinal plants *Spilanthes calva* and *Withania somnifera* to inoculation *by Piriformospora indica* in a field trial. Mycorrhiza 11:123–128
Reynolds M, Tuberosa R (2008) Translational research impacting on crop productivity in drought-prone environments. Curr Opin Plant Biol 11:171–179
Reynolds HL, Hartley AE, Vogelsang KM, Bever JD, Schultz PA (2005) Arbuscular mycorrhizal fungi do not enhance nitrogen acquisition and growth of old-field perennials under low nitrogen supply in glasshouse culture. New Phytol 167:869–880
Rillig MC (2004a) Arbuscular mycorrhizae, glomalin, and soil aggregation. Can J Soil Sci 84:355–363
Rillig MC (2004b) Arbuscular mycorrhizae and terrestrial ecosystem processes. Ecol Lett 7: 740–754
Ryan MH, Graham JH (2002) Is there a role for arbuscular mycorrhizal fungi in production agriculture? Plant Soil 244:263–271
Sanders IR (2004) Plant and arbuscular mycorrhizal fungal diversity – are we looking at the relevant levels of diversity and are we using the right techniques? New Phytol 164:415–418
Sawers RJH, Gutjahr C, Paszkowski U (2008) Cereal mycorrhiza: an ancient symbiosis in modern agriculture. Trends Plant Sci 13:93–97
Schreiner RP, Mishra RL, Mc Daniel KL, Benthlenfalvay GJ (2003) Mycorrhizal fungi influence plant and soil functions and interactions. Plant Soil 188:199–209
Schroeder-Moreno MS, Janos DP (2008) Intra- and inter-specific density affects plant growth responses to arbuscular mycorrhizas. Botany-Botanique 86:1180–1193
Schussler A, Schwarzott D, Walker C (2001) A new fungal phylum, the Glomeromycota: phylogeny and evolution. Mycol Res 105:1413–1421
Schwartz MW, Hoeksema JD, Gehring CA, Johnson NC, Klironomos JN, Abbott LK, Pringle A (2006) The promise and the potential consequences of the global transport of mycorrhizal fungal inoculum. Ecol Lett 9:501–515
Schweiger PF, Spliid NH, Jakobsen I (2001) Fungicide application and phosphorus uptake by hyphae of arbuscular mycorrhizal fungi into field-grown peas. Soil Biol Biochem 33: 1231–1237
Sharma MP, Adholeya A (2004) Effect of arbuscular mycorrhizal fungi and phosphorus fertilization on the post vitro growth and yield of micropropagated strawberry grown in a sandy loam soil. Can J Bot 82:322–328
Sieverding E (1991) Vesicular-arbuscular mycorrhiza management in tropical agrosystems. Deutsche Gesellschaft fur Technische Zasammenarbeit (GTZ) GmBH. Eschborn, Germany, p 371
Six J, Frey SD, Thiet RK, Batten KM (2006) Bacterial and fungal contributions to carbon sequestration in agroecosystems. Soil Sci Soc Am J 70:555–569
Smil V (2001) Feeding the world: a challenge for the twenty-first century. MIT Press, Cambridge, MA, p 360
Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic Press, San Diego, p 604
Sramek F, Dubsky M, Vosátka M (2000) Effect of arbuscular mycorrhizal fungi and *Trichoderma harzianum* on three species of balcony plants. Plant Prod Sci 46:127–131

Stringer L (2008) Can the UN convention to combat desertification guide sustainable use of the world's soils? Front Ecol Environ 6:138–144

Subramanian KS, Santhanakrishnan P, Balasubramanian P (2006) Responses of field grown tomato plants to arbuscular mycorrhizal fungal colonization under varying intensities of drought stress. Sci Hortic-Amsterdam 107:245–253

Takeda S, Matsuoka M (2008) Genetic approaches to crop improvement: responding to environmental and population changes. Nat Revs Gen 9:444–457

Tanaka Y, Yano K (2005) Nitrogen delivery to maize via mycorrhizal hyphae depends on the form of N supplied. Plant Cell Environ. 28:1247–1254

Taylor J, Harrier LA (2000) A comparison of nine species of arbuscular mycorrhizal fungi on the development and nutrition of micropropagated *Rubus idaeus* L. cv. Glen Prosen (red raspberry). Plant Soil 225:53–61

Taylor J, Harrier LA (2003) Beneficial infuences of arbuscular mycorrhizal (AM) fungi on the micropropagation of woody and fruit trees. In: Jain SM, Ishii K (eds) Micropropagation of woody trees and fruits. Kluwer, Dordrecht, the Netherlands, pp 129–150

Thingstrup I, Kahiluoto H, Jakobsen I (2000) Phosphate transport by hyphae of field communities of arbuscular mycorrhizal fungi at two levels of P fertilization. Plant Soil 221:181–187

Toussaint JP (2007) Investigating physiological changes in the aerial parts of AM plants: what do we know and where should we be heading? Mycorrhiza 17:349–353

Toussaint JP, Smith FA, Smith SE (2007) Arbuscular mycorrhizal fungi can induce the production of phytochemicals in sweet basil irrespective of phosphorus nutrition. Mycorrhiza 17:291–297

Treseder KK (2004) A meta-analysis of mycorrhizal responses to nitrogen, phosphorus, and atmospheric $CO_2$ in field studies. New Phytol 164:347–355

Verma RK, Arya ID (1998) Effect of arbuscular mycorrhizal fungal isolates and organic manure on growth and mycorrhization of micropropagated D*endrocalamus asper* plantlets and on spore production in their rhizosphere. Mycorrhiza 8:113–116

Vestberg M, Estaun V (1994) Micropropagated plants, on opportunity to positively manage mycorrhizal activities. In: Giananazzi S, Schuepp H (eds) Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Birkhauser Verlag, Basel, Switzerland, pp 217–225

Vestberg M, Cassells AC, Schubert A, Cordier C, Gianinazzi S (2002) Arbuscular mycorrhizal fungi and micropropagation of high value crops. In: Giananazzi S, Schuepp H, Barea JM, Hasselwandter K (eds) Mycorrhizal technology in agriculture. Birkhauser Verlag, Basel, Switzerland, pp 223–233

Vestberg A, Kukkonen S, Saari K, Parikka P, Huttunen J (2004) Microbial inoculation for improving the growth and health of micropropagated strawberry. Appl Soil Ecol 27: 243–258

Vosátka M, Albrechtová J (2008) Theoretical aspects and practical uses of mycorrhizal technology in floriculture and horticulture. In: Teixeira da Silva JA (ed) Floriculture, ornamental and plant biotechnology: advances and topical issues, vol 5, 1st edn. Global Science Books, Isleworth, UK, pp 466–479

Vosátka M, Dodd JC (2002) Ecological considerations for successful application of arbuscular mycorrhizal fungi inoculum. In: Gianinazzi S, Schuepp H, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture. Birkhauser Verlag, Basel, pp 235–248

Vosátka M, Jansa J, Vohnik M, Gryndler M (2000) Post-vitro mycorrhization and bacterization of micropropagated strawberry, potato and azalea. Acta Hortic 530:313–324

Vosátka M, Albrechtová J, Patten R (2008) The international market development for mycorrhizal technology In: Varma A (ed), Chapter 21. Springer, Berlin, pp 419–438

Whipps JM (2004) Prospects and limitations for mycorrhizas in biocontrol of root pathogens. Can J Bot 82:1198–1227

Whitfield L, Richards AJ, Rimmer DL (2004) Relationships between soil heavy metal concentration and mycorrhizal colonisation in *Thymus polytrichus* in northern England. Mycorrhiza 14:55–62

Wu QS, Xia RX (2006) Arbuscular mycorrhizal fungi influence growth, osmotic adjustment and photosynthesis of citrus under well-watered and water stress conditions. J Plant Physiol 163:417–442

Yao Q, Zhu HH, Zeng RS (2007) Role of phenolic compounds in plant defence: Induced by arbuscular mycorrhizal fungi. Allelopathy J 20:1–13

Yao Q, Lin FX, Chen JZ, Lei XT, Zhu HH (2008) Responses of citrus seedlings and a leguminous herb, *Stylosanthes gracilis*, to arbuscular mycorrhizal fungal inoculation. Proceedings of the international symposium on citrus and other tropical and subtropical fruit crops 773:63–67

Yokoyama K, Tateishi T, Saito M, Marumoto T (2005) Application of a molecular method for the identification of a *Gigaspora margarita* isolate released in a field. Soil Sci Plant Nutr 51: 125–128

# Chapter 11
# Enhancement of Rhizobia–Legumes Symbioses and Nitrogen Fixation for Crops Productivity Improvement

**Hamdi Hussein Zahran**

**Abstract** Rhizobia form a very interesting symbiotic relationship with leguminous plants. This relationship has attracted the attention of biologists all over the world due to the great impact of legumes for sustaining nutritional demands to humans and animals. Great efforts have been made to improve the symbiotic $N_2$-fixing ability and productivity of legumes. The first step to improve legume productivity is to select effective ($N_2$-fixing) rhizobia to be used as inoculants for the respective legumes. Recent studies reported about the wild-legume rhizobial ability to establish successful symbiosis with their original hosts, as well as with legume crops. In addition to the traditional approaches, modern strategies like genetic and biotechnological tools are adopted to unravel several molecular and genetic mechanisms controlling the rhizobia–legumes symbioses and to enhance $N_2$ fixation and productivity of the legumes. Extensive research work should be continued to improve the inoculation technology using modern approaches, especially in N-poor lands. Successful symbioses between the bacteria and the legumes are not sustained unless the effects of environmental stresses such as salinity and drought are modulated. The majority of arable lands of the globe experience one or more environmental stresses, therefore stress-tolerant and effective $N_2$-fixing legumes–rhizobia symbioses will be the only productive systems on these lands.

## 11.1 Introduction

Cultivation in the dry land of arid climates is increasingly advocated as a strategy to protect and reverse soil fertility decline, thus sustaining agricultural production and prevent serious nitrogen (N) deficits in many tropical agro-ecosystems.

H.H. Zahran
Faculty of Science, Beni-Suef University, Beni-Suef, 62511, Egypt
e-mail: hamdi_zahran@hotmail.com

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_11, 

Effective management of N in the environment is an essential element of agricultural sustainability. An essential source of N input into the soil is the biologically-fixed $N_2$ (Graham 2008), which is used directly by the plant, and so is less susceptible to volatilization, denitrification, and leaching. About 80% of this biologically-fixed $N_2$ comes from symbioses involving leguminous plants and various rhizobial species. Worldwide, legumes are grown on approximately 250 mha and fix about 90 million metric tons (Tg) of $N_2$ per year. $N_2$ fixation-based systems (e.g., legumes and trees) are the most promising and potentially profitable in extensive agricultural systems for coping with the N export problems (Zahran 1999, 2001; Graham and Vance 2000). The significance of $N_2$ fixation of rhizobium–legume symbiosis in global agriculture, in the light of modern biotechnological developments, was recently reviewed (Zahran 2005). The wide use of legumes as green manure or biofertilizer is mainly associated with their ability to establish symbiotic associations with $N_2$-fixing bacteria (NFB), collectively called rhizobia (Zahran 2006a). These bacteria are among the most studied group of microorganisms, mainly because of their potential to replace N-fertilizers, with emphasis on their key role in achieving sustainability of N-poor soils (Menna et al. 2006).

The significance of rhizobia–legume symbiosis for soil fertility improvement is well known. The root-nodule bacteria (rhizobia and other newly discovered NFB) establish substantial symbiotic associations with the leguminous plants to utilize atmospheric $N_2$. These diverse groups of bacteria form associations with crop (forage and grain), as well as with wild legumes (Zahran 2006b). The leguminosae is one of the most important and largest plant families and is composed of about 750 genera containing 16,000–19,000 species distributed worldwide and having major impacts on agriculture, the environment, animal/human nutrition, and health. Plants of the family leguminosae (Fabaceae) are able to form a symbiosis with bacteria (termed rhizobia), which leads to the development of a new organ on the roots (and sometimes stems) of the plant, the nodule, in which the bacteria fix $N_2$. The legume–*Rhizobium* symbiosis have tremendous ecological and agronomic importance and constitute a significant source of N, and consequently play an essential role in structure ecosystems and sustainable agriculture (Dita et al. 2006).

Current bacterial taxonomy divides a group of Gram-negative bacteria, the Proteobacteria, into a number of branches, α, β, γ, δ, and ε. The α-branch has seven orders, one of which is the rhizobiales, which has 11 families. Four of these families (Bradyrhizobiaceae, Methylobacteriaceae, Phyllobacteriaceae, and Rhizobiaceae) contain genera that can nodulate and fix $N_2$ in association with legumes (Sprent 2008). The β-proteobacterial branch also contains seven orders. One of these orders, Burkholderiales, is divided into five families, and one of these families, the Burkholderiaceae, contains two genera, *Burkholderia* and *Cupriavidus* having species known to nodulate legumes (Sprent 2008). Several species of *Burkholderia* have been shown to nodulate mimosoid legumes (Barrett and Parker 2005; Chen et al. 2005), with varying levels of host specificity, some of which are capable of fixing $N_2$ ex planta. However, it remains to be seen how these β-rhizobia become important in certain environments. The nodule-forming bacteria are currently divided into about 50–60 species within about 10–12 genera, with 8 genera

among the alphaproteobacteria: *Azorhizobium, Bradyrhizobium, Mesorhizobium, Methylobacterium, Rhizobium, Sinorhizobium (Ensifer), Devosia*, and *Blastobacter*, and 2 genera (*Burkholderia* and *Cupriavidus)* within the β-proteobacteria, in addition to some species in the γ-proteobacteria (Barrett and Parker 2005, 2006; Wang et al. 2006). However, the picture of rhizobia diversity is far from clear, especially considering the wide geographical distribution and the large number of leguminous species which might harbor novel root-nodule bacteria (Wei et al. 2007).

The desert areas are characterized by a water deficiency due to the atmospheric dryness, and very low rainfall, resulting in seriously-degraded vegetation and a progressive reduction of biodiversity in the ecosystem (Verdoy et al. 2006; Essendoubi et al. 2007). Rhizobia encountered in desert areas play an important role as root symbiotics involved in $N_2$ fixation of some leguminous plants (e.g., *Acacia*), which are pioneering species that contribute to soil fertility (Zahran 2005). Rhizobial bacteria occur naturally in most agricultural soils whose survival is affected directly both by drought and salinity (Zahran 1999). To make optimal use of the $N_2$-fixation process, the physical and physiological conditions that affect the survival of rhizobia during desiccation should be optimized (Vriezen et al. 2006).

The root-nodule bacteria from wild legumes are multipurpose bacteria with very interesting characteristics; this concept was recently reviewed (Zahran 2006a). They have a wide host range, a characteristic that offers these legumes an ecological advantage as they can form nodules with other wild or crop legumes (Zahran et al. 2003), and can be a source of genetic information to improve characteristics of other rhizobia. The significance of rhizobia from wild legumes is their symbiotic $N_2$-fixing activity, which improves soil fertility and plant productivity. Woody (trees) legumes like *Faidherbia albida, Inga oerstediana, Prosopis lavegata*, and *Pachycereus hollianus* host NFB, and occasionally arbuscular mycorrhizal fungi (AMF) may contribute to the soil organic carbon pool and soil fertility (Grossman et al. 2006; Bernatchez et al. 2008; González Ruiz et al. 2008). These leguminous trees usually create localized distributions of soil N, C, and P, through $N_2$ fixation and subsequent litter fall. The companion crops benefit from the $N_2$ fixation of trees by reabsorbing N mineralized from decomposing N-litter and prunings. Nitrogen may transfer directly by belowground processes from tree to grass, e.g., via root exudates and/or common mycorrhizal networks (Sierra and Nygren 2006). The nodulation of four tree legumes (*Calliandra calothyrsus, Gliricidia sepium, Leucaena leucocephala*, and *Sesbania sesban*) and the ecology of their native rhizobial populations in tropical soils has been examined (Bala et al. 2003). *C. calothyrsus* had the highest nodulation rate in the soils used; however, inoculation tests showed *L. leucocephala* to be the most promiscuous species, *G. sepium* to be the most effective symbiosis, and *S. sesban* to be the most specific for both nodulation and symbiotic effectiveness.

Salinity of soil is one of the most serious forms of land degradation in the world, especially in arid lands, where insufficient precipitation causes extensive reliance on irrigation. Globally, about 7% of all land areas is affected by soil salinity (Unni and Rao 2001). Of the total 1.5 billion ha of cultivated land, about 5% are affected

by excess salt content. Among the most common effects of soil salinity is growth inhibition by $Na^+$ and $Cl^-$. Elevated $Na^+$ in soil solution inhibits the uptake of other nutrients (e.g., P, K, Fe, Cu, and Zn) directly by interfering with various transporters in the root plasma membrane (Giri et al. 2007). Salinity has profound effects on crop production, therefore, reducing the spread of salinization and increasing the salt-tolerance of high-yielding crops, are becoming important global issues. Mineral nitrogen deficiency is another important limiting factor for plant growth in arid zones, and rhizobia–legume symbioses are the primary sources of fixed $N_2$ in these habitats (Verdoy et al. 2006). Salinated soil contains very little N and thus is not suitable for cultivation of most plants. An appropriate solution to this situation would be the cultivation of plants that are able to fix $N_2$ through symbiotic systems (Chen et al. 2000). Most leguminous plants, however, are sensitive to even low levels of salinity. In addition, most rhizobia are also sensitive to moderate and higher levels of salinity during both the free-living stage and the symbiotic process. Legumes used in the reclamation of degraded lands (e.g., salt-affected lands) include *P. juliflora, Acacia nilotica, A. auriculifonnis, Dalbergia sisso,* and *Glyricidia maculate*. However, both legume growth and the process of nodule formation are more sensitive to salinity than are rhizobia (Zahran 1999). For instance, *Sinorhizobium meliloti* tolerated up to 300 mM NaCl, while nodulation and $N_2$ fixation of its host (*M. sativa*) was inhibited at about 100 mM salt (Graham and Vance 2000).

Major advances in our understanding of the relations between legumes and rhizobia are being made using the legume models *Medicago truncatula and Lotus japonicus*. Despite the taxonomic diversity, all rhizobia establish symbioses using the same molecular mechanisms, involving signal molecule exchange between the two partners. Host plants excrete inducing compounds (mainly flavonoids) that activate regulatory NodD proteins and induce the expression of bacterial nodulation (*nod*) genes. *Nod* genes are involved in the biosynthesis of the lipochitooligosaccharides (nod factors) that are required for specific infection and nodulation of legumes (Ba et al. 2002). This chapter reviews several reports to assess the significance of symbiotic bacteria (rhizobia and other nodule bacteria) for the enhancement of $N_2$ fixation and the consequent effect on productivity improvement of the economically important crop plants, either legumes or nonlegume-associated crops. The recent developments in molecular genetics and their usefulness in improving *Rhizobium*–legume symbiosis are also reviewed and discussed.

## 11.2 Molecular and Genetic Mechanisms Controlling the Symbiosis

The soil bacteria (rhizobia) within the phylogenetic family Rhizobiaceae have the unique ability to infect and establish a $N_2$-fixing symbiosis on the roots or stems of leguminous plants. The establishment of the symbiosis involves complex interactions between the host and the rhizobia, resulting in the formation of a special

organ, the nodule, which the bacteria colonize as intracellular symbionts. Two general developments have contributed to the recent explosion of research progress in this area (Stacey et al. 2006): first, the adoption of two genetic model legumes, *M. truncatula* and *L. japonicus*, and second, the application of modern methods in functional genomics (transcriptomic, proteomic, and metabolomic analyses). The first *Rhizobium* genes for nitrogen fixation (*nif*) and for nodulation (*nod*) were cloned in the early 1980s (Long 2001), and soon many more *nif, nod*, and *fix* (symbiotic fixation) genes were discovered. The rules for genome organization are different for different rhizobia. Symbiotic genes are clustered or dispersed on plasmids and can spread at high frequency by conjugation, while in others, the genes are scattered among many chromosomes and plasmids. With all of this genomic diversity it is no wonder that systematists have had a field day classifying and reclassifying the root-nodule bacteria. Numerous genes coding for transporters (sugar, peptide, nitrate, and $H^+$-ATPase transporters) were shown to be upregulated in *L. japonicus* and *M. truncatula* nodules, suggesting an important role of these transporters in the exchange of carbon and nitrogenous compounds between legumes and rhizobia.

The DNA microarray and transcript profiling studies of nodulation have been considered only recently; however, the large numbers of expressed sequence tags (ESTs) available for legume plants, in addition to cDNA and oligonucleotides, have made large-scale transcriptomic studies on nodulation possible. Studying the gene expression using DNA microarray became possible because of the availability of the complete genomic sequence of one of the rhizobial strains (*S. meliloti* 1021). The DNA microarray technology has revolutionized gene expression studies by providing a powerful tool for parallel measurement of gene expression on a whole genome scale. In contrast to the labor-intensive gene fusion technique, this technology achieves a higher coverage and provides the direct determination of mRNA levels to monitor an expression profile (Rüberg et al. 2003). The microsatellites or simple sequence repeats (SSRs), which are ubiquitous in genomes of various organisms, can be used as genetic marker. The analysis of SSR in rhizobial genome provides useful information for a variety of applications in population genetics of rhizobia (Ya-mei et al. 2008). The occurrence, relative abundance, and relative densities of SSRs of *B. japonicum, M. loti*, and *S. meliloti* were analyzed and are available in the genomes sequenced database. The motif and distribution of SSRs among the three rhizobia genomes were similar, and the tetranucleotide, pentanucleotide and hexanucleotide repeats were predominant and indicated higher mutation rates in these species. This marker can be used to find the gene position quickly and exactly and then to scan, identify, and annotate the gene, and to study gene function. Thus, the SSR marker had an important effect on functional genome research of rhizobia.

Although rhizobia colonize roots in a way that is reminiscent of pathogenic microorganisms, no host plant defense reactions are triggered during successful symbiosis (Mithöfer 2002). The question is how defense responses in effective symbiosis might be suppressed? A prerequisite for understanding the molecular mechanisms underlying the control of defense in symbiosis is the identification of

receptors, as well as the elucidation of subsequent signal transduction events involved in the onset of plant defense for both rhizobial elicitors and suppressors (Mithöfer 2002). Some of the defense-related genes, e.g., those coding for enzymes of the phytoalexin biosynthesis pathway and genes coding for proteins that are involved in cell wall modifications, were also regulated during the nodulation process. Interestingly, the defense-related genes were upregulated during the early stages of nodulation (recognition and infection) and then decreased in later stages of nodule development, suggesting that the invading rhizobia suppress the host's defense to successfully colonize roots and so form nodules (Stacey et al. 2006).

Substantial progress has been made recently to understand how rhizobia enter into symbiotic relationships with legumes. The establishment of the legume–*Rhizobium* symbiosis requires recognition of the bacterial microsymbiont at the root epidermis followed by initiation of plant infection and nodule organogenesis. The symbiotic $N_2$-fixing rhizobia harbor a set of nodulation (*nod*) genes that control the synthesis of modified lipochitooligosaccharides, called nod factors; required for nodulation (Moulin et al. 2004). Studies on nod factor activities, coupled with the recent cloning of genes required for nodule initiation, are leading to an understanding of the first steps in the signaling pathways. Moreover, studies have shown that phytohormones (e.g., auxin and cytokinin) are involved in controlling or mediating symbiotic responses. The challenge for the future will be to establish how nod factor signaling integrates with phytohormone activities in the control of infection and nodulation in the establishment of symbiosis (Mulder et al. 2005). The *nod*A gene, which is essential for symbiosis, is responsible for the attachment of the fatty acid group to the oligosaccharide backbone. PCR amplification, sequencing and phylogenetic analysis of *nod*A gene sequences from a collection of diverse *Bradyrhizobium* strains, isolated from various legume species and geographical areas, revealed the monophyletic character of these strains. This study also revealed a large nucleotide diversity of *nod*A sequences within this genus, ranging up to 21%, which seems consistent with the overall genetic variability of bradyrhizobial strains and their ability to perform efficient symbiosis with many different plants (Moulin et al. 2004).

Plants, like humans, contain hemoglobin. Three distinct types of hemoglobin, with different functions exist in plants: symbiotic, nonsymbiotic, and truncated (Hoy and Hargrove 2008). Symbiotic hemoglobins (sHbs) are proteins found in millimolar quantities in legume root-nodules and sometimes in nonlegumes, for example, *Parasponia andersonii*–rhizobium symbiosis (Ott et al. 2005). The main function of sHbs in plants is well known: sHbS in the root-nodules are responsible for facilitating diffusion of the oxygen necessary for $N_2$ fixation, similar to the activity of mammalian myoglobin (MB) in muscles. While using an RNA interference approach to silence the expression of a three nodule-expressed leghemoglobin genes in the legume *L. japonicus*, it was found that leghemoglobins indeed are essential for $N_2$ fixation in nodules (Downie 2005). Leghemoglobins, however, can have a 20-fold higher affinity for oxygen than myoglobin. The oxygen-binding characteristics of leghemoglobins are unusual in that they have an extremely fast $O_2$ association rate and a relatively slow $O_2$ dissociation rate, and so can buffer the free

oxygen concentration at around 7–11 nM (Downie 2005). Legumes can select for greater mutualism, controlling nodule $O_2$ supply and reducing reproduction of rhizobia that fix less $N_2$ (Denison and Kiers 2004).

## 11.3 Selection of Effective Symbiotic Rhizobia

Because of the value of rhizobia in the environmental and agricultural fields, rhizobial analysis, based on symbiotic performance and cross inoculation range, is essential for selection for field applications. Selection of effective strains of rhizobia is mainly dependent on symbiotic properties of the bacteria. Symbiotic properties are coded by genetic elements (e.g., plasmids) that can be transmitted between different bacteria. Nevertheless, the symbiotic genes could be used as markers for complete rhizobial characterization. Common nodulation genes (e.g., *nod*ABC) or nitrogenase genes (e.g., *nif*HDK) have been useful in the study of interactions with host plants and to clarify relationships between rhizobial systematic and symbiotic properties (Villegas et al. 2006).

The establishment of new legumes in agriculture often requires the introduction of symbiotically competitive root-nodule bacteria as inoculants. However, prior to the introduction of new species, the symbiotic interactions between the new legume and the indigenous populations of root-nodule bacteria, and also the interaction of any new inoculants with legumes already in the ecosystem, must be carefully evaluated. The host recognizes the compatible and effective rhizobia from a soil that harbors effective and ineffective rhizobia. Therefore, optimization of the symbiosis between legume plants and their respective symbiotic rhizobia requires the presence in the rhizosphere of competitive and infective strains of rhizobia which are highly efficient at fixing $N_2$. The success of inoculated strains of *Rhizobium* in soil is largely dependent on their ability to colonize rapidly and out-compete coexisting strains for nodule occupancy by specific strains of rhizobia. Highly competitive strains for nodule formation need not always exhibit the highest level of saprophytic fitness (Duodu et al. 2005). The basal rhizobial cell density, rather than soil pH, is essential for colonization of the soil and selection by specific host (Yates et al. 2008). A legume introduced into a new area will only form nodules and fix $N_2$ if compatible rhizobia are present in the soil. Rhizobial population sizes estimated after trapping by the legume species, in soils from tropical areas of Africa, Asia and Latin America (Bala et al. 2003), ranged from undetectable numbers to $3.16 \times 10^4$ cells $g^{-1}$ of soil depending on the trap host species. Symbiotic effectiveness did not bear any close relationship with specific soil parameters, but rhizobial numbers have been found highly correlated with soil acidity, particle size, and exchangeable ions (Bala et al. 2003).

The traditional methods adopted for identifying the most effective rhizobial strains to be used as inoculant is a time-consuming process, involving the production of thousands of rhizobial cultures followed by greenhouse experiments and field trials. Selection of effective strains of nodulating bacteria for legumes should

consider the genetic characterization and taxonomic position (Menna et al. 2006). Rhizobia are known to vary widely in their ability to nodulate many species of legume host plants, ranging from species that appear to be highly specific (with a very limited host range), such as *Rhizobium galegae*, compared with *Rhizobium* sp. NGR 234 with a very wide host range and able to nodulate legumes from at least 112 genera, and some indigenous isolates of *Bradyrhizobium*, which nodulate promiscuous varieties of soybean (Musiyiwa et al. 2005). Identification of effective locally adapted strains with wide host ranges could be useful in the development of rhizobial inoculants which can survive longer in agricultural soil and, hence, reduce the need for regular inoculant applications.

It is essential to reclassify and evaluate the existing strains of rhizobia for their symbiotic and $N_2$-fixing abilities, using modern molecular genetic tools while selecting bacteria for raising inoculants. A comprehensive study consistent with this concept has been carried out in Brazil (Menna et al. 2006). The phylogeny of a collection of rhizobial strains from a wide range of legumes was examined based on the sequencing of the 16S rRNA genes. Host specificity of these strains was not related to 16S rRNA genes, suggesting the diverse evolution of ribosomal and symbiotic genes in such organisms (Menna et al. 2006). Similarly, strains of *R. leguminosarum* bv. *trifolii* showed a different symbiotic pattern with *Trifolium spp*., although their 16S rRNA gene sequences were identical (Yates et al. 2008). Strains of *R. leguminosarum* bv *viciae* from pea (*P. sativum* L.) displayed a relatively high degree of genetic diversity among both plasmid and chromosomal components (Vessey and Chemining'wa 2006). Rhizobial strains from *A. tortilis*, which are grouped in the genera *Mesorhizobium* and *Sinorhizobium*, exhibited very similar symbiotic characters despite their chromosomal diversity (Ba et al. 2002).

## 11.4 Selection of Symbiotic Rhizobia from Crop and Wild Legumes

### *11.4.1 Rhizobia from Crop Legumes*

Soils in cultivated areas are rich in rhizobia from the so-called "cowpea group" that effectively nodulate cowpea, peanut, and other tropical legumes. They are usually slow-growing bacteria belonging to the genus *Bradyrhizobium*. Bacteria in the genus *Bradyrhizobium* is a cosmopolitan and diverse group nodulating a variety of legumes, as well as the nonlegume *Parasponia*. Polyphasic characterization of these rhizobia from different hosts and from different geographic regions was done by analyzing the variability of 16S rRNA gene RFLP, 16S–23S rRNA gene intergeneric spacer (IGS) RFLP, G–C rich RAPD, and phenotype assays. The evolution of "cowpea group" isolates of *Bradyrhizobium* nodulating cowpea and peanut from different locations in South Africa were studied (Steenkamp et al. 2008). These bacteria are diverse, representing a number of distinct *Bradyrhizobum*

groups; horizontal gene transfer significantly influences the evolution of cowpea and peanut root-nodule bacteria. The identification of the *nod*Z, *noe*l and *noe*E genes in the isolates tested indicated that African *Bradyrhizobium* species may produce highly decorated nodulation factors, which represent an important adaptation enabling nodulation of a great variety of legumes inhabiting the African continent (Steenkamp et al. 2008). Based on these characteristics, mung bean (*Vigna radiata* L.) rhizobia from China (Yang et al. 2008) were found to have diverse slow-growing bacteria clustered into four groups, three related to *Bradyrhizobium* and the fourth group formed a miscellany of fast-growing isolates variously related to the genera *Mesorhizobium, Rhizobium* and *Sinorhizobium*. Studies of the native rhizobial populations associated with peanut *Arachis hypogaea* nodules in soils of Argentina, Morocco and Cameroon revealed that these populations are highly diverse and include slow-and fast-growing isolates (El-Akhal et al. 2008; Nkot et al. 2008). The bacteria isolated from Morocco were firstly characterized by restriction of the 16S rDNA region, and phylogeny was inferred from the 16S gene sequence. The isolates were grouped with species belonging to the *Bradyrhizobium* and *Rhizobium* genera (El-Akhal et al. 2008); a high degree of variability was detected among isolates in terms of their $N_2$-fixing ability. Strains from peanut (*A. hypogaea* L.) in Cameroon were examined after RFLP analysis of 16S–23S rDNA genes (Nkot et al. 2008) and a considerable level of genetic diversity was determined among those peanut isolates. Fast-growing *Rhizobium* isolates were obtained from root-nodules of the legumes belonging to the genera *Vicia, Lathyrus* and *Pisum* from different agro-ecological areas in Italy (Moschetti et al. 2005). Analysis of symbiotic properties showed a wide spectrum of nodulation. The majority of the isolates were presumptively identified as *R. leguminosarum* bv. *viciae* both by their symbiotic properties and by the specific amplification of the *nod*C gene, RFLP–PCR 16S rDNA analysis, and RAPD–PCR technique, which showed a high level of genetic polymorphism.

### *11.4.2 Rhizobia from Wild Legumes*

Wild (herb and tree) legumes harbor diverse and promiscuous root-nodule bacteria (Zahran 2006a), which are currently classified into several rhizobial or nonrhizobial genera. Further genetic and molecular studies are needed to elucidate the identification and classification of many new isolates of rhizobia from wild legumes. To develop new symbiotic legume species for agriculture, the genetic and symbiotic diversity of rhizobial isolates from various wild legumes are being investigated using numerical analysis, phenotypic characteristic, cross nodulation to selected legumes, and modern genetic tools, such as amplified 16S ribosomal DNA restriction analysis (ARDRA), RFLP analysis of 16S–23S rDNA intergeneric spacers (IGS), 16S rRNA gene sequencing, and multi-locus sequence analysis of the PCR-amplified *nod*C gene and DNA/DNA hybridization.

The rhizobial strains isolated from several wild forage legumes collected from various geographical regions were identified. For example, rhizobia from *Lathyrus, Vicia* and *Lotus* grown in China (Han et al. 2008) clustered into nine genomic species belonging to four genera: *Bradyrhizobium, Mesorhizobium, Rhizobium,* and *Sinorhizobium* (*Ensifer*). The $N_2$-fixing rhizobial isolates from root-nodules of *Medicago laciniata*, and from Mediterranean soils in Tunisia and France (Villegas et al. 2006), differed markedly from other *S. meliloti* or *S. medicae* isolates in their symbiotic traits, such as *nif*DK RFLP diversity, *nod*A sequences, and $N_2$-fixing efficiency with annual *Medicago* species (*M. truncatula, M. polymorpha and M. sauvagei*). The rhizobial isolates from root-nodules of *Astragalus, Lespedeza* and *Hedysarum* in China (Wei et al. 2007) were divided into two groups: the first was related to the *Rhizobium–Agrobacterium* group and the second was related to *R. galegae* and *R. huautlense*. The bacteria isolated from nodules of *Lotononis angolensis* were fast-growing, highly mucoid, and pink pigmented, and related to genera other than rhizobia based on 16S rRNA phylogeny, while those isolated from *L. bainesii and L. listii* were slow-growing, pink pigmented and attributed to *Methylobacterium* (Yates et al. 2007). Sequencing of the 16S rRNA region of *Biserrula pelecinus* (a new forage legume in Australia with high quality feed for cattle and sheep and having many agronomic attributes) root-nodule bacterial isolates revealed a close relationship between these bacteria and with both *M. loti* and *M. ciceri* (Nandasena et al. 2004). As *B. pelecinus* can be nodulated by *Mesorhizobium* spp. isolated from other legumes, particularly *Lotus,* there is an opportunity to utilize this trait in cultivar development.

The leguminous trees have an important role in maintaining soil fertility. Many legume trees were recently screened for their ability to form effective $N_2$-fixing nodules. Large numbers of the diverse root-nodule bacteria were isolated and identified, and their symbiotic activities were assessed by using traditional methods, molecular methods, and genetic tools. Tree legumes such as *Acacia, Prosopis, Sesbania, Albizia, Leucaena*, etc. have received increasing attention because of their uses as fodder and wood, and are included in ambitious reforestation programs in arid environments (Zakhia et al. 2004; Zahran 2006a). Some of the legume trees have records of good $N_2$ fixation capacities with their symbiotic bacteria. The high diversity (rhizobial genotypes, host affinities and symbiotic effectiveness) of the isolates require rigorous selection for host × strain × site combinations in order to obtain effective and competitive inoculants for legume trees and crops in rehabilitation and agro-forestry programs. A collection of rhizobial isolates from *A. tortilis* subsp. *raddiana*, from various sites in the north and south of Sahara (Africa), was very diverse (Ba et al. 2002). Based on whole cell protein (SDS-PAGE) and 16S rDNA sequence analysis, most strains were related to *Mesorhizobium* and *Sinorhizobium* genera, but with similar symbiotic characters (Ba et al. 2002). The rhizobia isolated from root-nodules of *Acacia* species native to Mexico constitute a diverse group of bacteria distinguished from other *Sinorhizobium* species by their levels of DNA–DNA hybridization and the sequence of 16S rRNA and *nif*H genes. Therefore, a new species, *S. americanus*, was described (Toledo et al. 2003). The rhizobial strains isolated

from *A. abyssinica, A. seyal, A. tortilis, F. albida, S. sesban, P. vulgaris* and *V. unguiculata* grown in southern Ethiopia was assessed using the Biologe™ system and AFLP finger printing technique (Wolde-Meskel et al. 2004). The rhizobia constituted metabolically and genomically diverse groups that are not linked to reference species. The root-nodule bacteria from tree legumes (*Acacia, Prosopis, Sesbania and Faidherbia*) and other legumes (*Macroptilium, Phaseolus* and *Vigna),* growing in ecologically diverse sites in Kenya (Odee et al. 2002), were analyzed by PCR–RFLP of the 16S rRNA gene and sequence analysis of a 230-bp fragment of this gene. The bacteria were assigned to four rhizobial genera (*Bradyrhizobium, Mesorhizobium, Rhizobium, and Sinorhizobium*) and the *Agrobacterium*. An analysis of the PCR-amplified 16S rDNA gene digestion profiles, using 5-endonucleases, indicated the presence of different lineages among the taxa associated with *P. juliflora* nodules in arid soils of Morocco (Benata et al. 2008). Nucleotide sequencing of the small subunit rRNA gene and blast analysis showed that *P. juliflora* hosted bacteria belong to *Rhizobium*, *Sinorhizobium* and *Achromobacter xylosoxidans*; the latter species is from β-proteobacteria.

Diversity of root-nodule bacteria (and sometime stem-nodule bacteria) from the legume shrub *Sesbania* has received increasing interest. The symbiotic properties, LPS profiles, sym plasmid and rhizobiophage sensitivity of bacterial isolates of three *Sesbania* species (*S. sesban, S. aegyptica* and *S. rostrata*) of the semiarid Delhi region of India were analyzed (Sharma et al. 2005). The isolates differed in their symbiotic efficiency, but 16S rDNA sequences revealed that root-nodule isolates of *Sesbania* belonged to diverse rhizobial taxa (*S. saheli, S. meliloti, R. huautlense*) whereas stem-nodule isolates were strictly *Azorhizobium caulinodans*. Molecular systematics of rhizobia based on phylogenies inferred from *rrs*, *atp*D, *rec*A and *nif*H sequences were used for classification of *Sesbania* isolates from Venezuelan wetlands (Vinuesa et al. 2005). The isolates were consistently identified as *M. plurifarium* or *R. huautlense*. DNA–DNA hybridization and 16S rRNA sequences support the inclusion of the rhizobial strains isolated from root-nodules of *Sesbania virgata* in southeast Brazil in the genus *Azorhizobium* but not in the species *A. caulinodans*, and hence the name *A. doebereinerae* was proposed (Moreira et al. 2006). Rhizobial isolates from *Albizia* trees grown in China were identified employing PCR–RFLP and sequencing of 16S rRNA genes, SDS-PAGE of whole cell proteins, and clustering of phenotypic characters (Wang et al. 2006). *Albizia* trees are nodulated by a wide range of rhizobial species within the genera *Rhizobium, Bradyrhizobium* and *Mesorhizobium.* The bacterial isolates from root-nodules of the woody legumes *Wisteria sinensis, Cercis racemosa and Amorpha fruticosa* grown in China were characterized with phenotypic analysis, PCR-based 16S and 23S rRNA gene RFLP, Box PCR and 16S rRNA gene sequencing (Liu et al. 2005) and identified into three rhizobial genera (*Rhizobium, Bradyrhizobium* and *Mesorhizobium*). The diversity of *Retama raetam* root-nodule bacteria, grown in arid regions of Tunisia, was determined by 16S rRNA gene sequencing and phenotypic analysis (Mahdhi et al. 2008). The bacteria were assigned to the genera *Agrobacterium*, *Rhizobium* and *Sinorhizobium*, those of the latter genus showed a large diversity in their symbiotic properties and $N_2$-fixing ability.

## 11.5 Selection of Symbiotic Rhizobia Tolerant to Various Abiotic Stresses

### *11.5.1 The Problem of Soil Salinity*

One of the most severe and widespread problems facing agriculture is the degradation of soil quality due to desiccation and salinity. Almost 40% of the world's land surface is affected by salinity-related problems (Zahran 1999; Vriezen et al. 2007). Salinity may have a detrimental effect on soil microorganisms and, in general, results in a decreased productivity of crop plants. Harsh environmental conditions negatively affect the activity of most endogenous soil bacteria including rhizobia. The crops grown in 90% of arable lands are subjected to one or more environmental stresses. To face the threat of these stresses, several genetic improvement strategies are available from classical plant breeding to more direct physiological and genetic approaches. However, understanding the mechanisms underlying a specific stress is essential (Dita et al. 2006). Various soil problems (such as extreme pH, salinity, drought, and high temperature) hinder agriculture in arid areas. Some legumes are relatively tolerant to such stress conditions, but nodule formation and $N_2$ fixation are greatly impaired by stress conditions. Therefore, for legume cultivation in arid areas under environmentally friendly and economically attractive conditions (i.e., without applying fertilizer-N), the availability of stress-tolerant rhizobial strains is an absolutely required precondition (Priefer et al. 2001).

Cultivation of agricultural crops in soil is limited by salt stress, which arises from the excessive uptake of salt by plants and is an unavoidable consequence of high ion concentrations. A higher salt amount in a soil, most commonly NaCl, has detrimental effects on plant growth and productivity. In this regard, salinity affects the activities of enzymes involved in nitrogen metabolism, but the mechanism underlying this phenomenon still remains unclear. It is well known that the establishment of legume–*Rhizobium* symbiosis is susceptible to the effects of salinity. The responses of plant genotypes to salinity differ widely, depending in the form and dosage of salt and the stage of plant growth (Zilli et al. 2008).

### *11.5.2 Free-Living and Symbiotic Rhizobia Under Abiotic Stress*

The symbiotic interactions between rhizobia and their host legumes have strongly driven the investigation and application of biotechnology tools for legumes. Stress parameters such as soil acidity, salinity and desiccation affect rhizobial persistence and nodulation efficiency. The responses of various rhizobia to desiccation were recently reviewed (Vriezen et al. 2007). An adaptation to increased salt or osmotic stress elevates the ability of rhizobia to survive desiccation by accumulation of osmoprotectants, desiccation protectants, and heat-shock proteins (Vriezen et al. 2006).

Four strains of rhizobia nodulating *Acacia* were isolated from the Moroccan desert soil by trapping seedlings of *A. gummifera* and *A. raddiana*, and were studied for their ability to tolerate high salinity and dry conditions (Essendoubi et al. 2007). Three strains were halotolerant, and grew at 1 M NaCl and accumulated glutamate and mannosucrose. Such strains showed high resistance to desiccation, whose tolerance to dryness was stimulated by osmotic pretreatment. The accumulation of solutes and sugars in these rhizobia represents both an osmoadaptive response and a part of a desiccation tolerance mechanism (Essendoubi et al. 2007). High salt concentrations may have detrimental effects on rhizobial populations as a result of direct toxicity, as well as through osmotic stress. Howieson and Ballard (2004) suggested that stressful environments limit rhizobial communities to less than 100 cells $g^{-1}$ soil, at some time during the season. One of the salt-induced responses in rhizobia is changes in cell morphology and size, and modifications in the pattern of polysaccharides (Zahran 1999). The latter responses may have an impact on the symbiotic interaction between rhizobia and their legumes because extracellular polysaccharides (EPS) and lipo-polysaccharides (LPS) are essential factors for the development of root nodules. A *Rhizobium* mutant (EPS deficient, $exo^{-}$), showed decreased levels of LPS in the presence of 350 mM NaCl (Unni and Rao 2001). The response and adaptation of rhizobia to environmental stresses are probably complex phenomena involving many physiological and biochemical processes that likely reflect changes in gene expression and consequently the activity of enzymes and transport proteins (Wei et al. 2004; Domínguez-Ferreras et al. 2006). Some species of rhizobia adapt to saline conditions by intracellular accumulation of low-molecular-weight solutes called osmolytes, such as glutamate, glycine betaine and polyamines, or by accumulation of $K^{+}$. As salt tolerance of rhizobia is a phenotype, in which many regulatory mechanisms are involved, it is necessary to study salt-tolerant rhizobial genes. Different genes involved in salt tolerance of *S. meliloti* were identified using random Tn5-1063 insertional mutagenesis (Wei et al. 2004). The adaptation of *S. meliloti* to salt is a complex multilevel regulatory process in which many different genes can be involved.

The rhizobia–legume symbioses are more sensitive to salt or osmotic stress than free-living rhizobia. Salt stress may inhibit the initial steps of the symbiosis (root colonization and infection), but it also has a depressive effect on $N_2$ fixation. Nevertheless, salt-tolerant rhizobia are important to establish a salt-tolerant symbiotic system. Selection of adapted strains of rhizobia under stress conditions is possible and they can be used as inoculants for successful lupin growth (Raza et al. 2001). Objectives of genomic research on rhizobia are the identification of genes and regulatory networks relevant for symbiosis and the consideration of the free-living status, especially adaptations to fluctuated soil environmental conditions (e.g., temperature, osmolarity, pH, and nutrient supply). DNA microarrays have been used to examine gene expression in response to various abiotic stresses in rhizobia (Domínguez-Ferreras et al. 2006). Based on the complete *S. meliloti* genome sequence, Rüberg et al. (2003) established DNA microarrays as a comprehensive tool for systematic genome-wide gene expression analysis in *S. meliloti* 1021. Gene expression in *S. meliloti* in response to an osmotic upshift, imposed by

the addition of 0.38 M NaCl, was monitored using the DNA microarray tool. About 137 genes showed significant changes in gene expression resulting from the osmotic upshift. From these genes, 52 were induced and 85 were repressed (Rüberg et al. 2003). DNA microarrays were used to investigate genome-wide transcriptional responses of *S. meliloti* to a sudden increase in external osmolarity elicited by addition of either NaCl or sucrose to exponentially-growing cultures (Domínguez-Ferreras et al. 2006). The genetic data correlated well with the microarray results and suggested that pSymB contains a large number of genes upregulated after an osmotic upshift, which may play an active role in the osmoadaptation of *S. meliloti*. A well-characterized promoter is now available to drive expression of rhizobial stress-tolerance genes of interest under acidic conditions. Selection of stress-adapted symbioses, or elite rhizobial strains or legume genotypes, can overcome these stress factors. *Rhizobium* mutants whose adaptation to high salinity is affected have deficiencies in their symbiotic capacity (Nogales et al. 2002). Salt-tolerance genes of *S. fredii* (a halotolerant rhizobium strain grow at 0.6 M NaCl) were identified by the construction and screening of a transposon Tn5-1063 library containing over 30,000 clones (Jiang et al. 2004). This strain could have developed sophisticated mechanisms to maintain its intracellular steady osmotic and ionic state. Several genes involved in osmotic tolerance of this strain were identified, which are important to improve salt tolerance of salt-sensitive rhizobia.

Bacterial isolates from *Canavalia rosea*, an indigenous leguminous halophyte grown in the seaside area of southern Taiwan, were effective symbionts for the original host and able to grow at NaCl concentrations up to 3–3.5% (w/v). Based on SDS-PAGE of proteins, pulsed-field gel electrophoresis (PFGE) analysis and ARDRA results, the 12 isolates were highly diverse. The 16S rRNA and *nif*H gene sequences were determined for isolates with distinct ARDRA patterns and compared with other members of the rhizobial species. The isolates were proposed to be classified into the genus *Sinorhizobium* and distinguished from the current species of the genus (Chen et al. 2000). Rhizobia were isolated from the root-nodules of *Glycyrrhiza glabra* and *G. uralensis*, growing in the arid and semiarid regions of northwestern China (Ge-Hong et al. 2008). The taxonomic position and stress tolerance were determined to select the promising putative inoculant strain. Based on physiological and biochemical characteristics, the isolates were clustered into three groups. One isolate was found to have high tolerance to NaCl, pH and temperature. Based on the sequence analysis of 16S rDNA and *nod*A gene, this isolate was placed in the genus *Mesorhizobium*.

### *11.5.3 Symbiotic $N_2$ Fixation of Legumes Under Abiotic Stresses*

Optimum performance of the $N_2$-fixing symbiosis depends upon preselection of both symbiotic partners for adaptation to the target environment, which may in some cases present a challenge to rhizobial survival or nodulation. Symbiotic $N_2$ fixation in legumes is limited, especially in semiarid conditions. Legumes are

classified as salt-sensitive crops, and their limitation in productivity is associated with poor development of symbiotic root-nodule bacteria, reduction in the $N_2$ fixation capacity and lower growth of the host plant. $N_2$ fixation of legumes is usually affected by abiotic stress; the response of root-nodules of legumes to various kinds of stress was examined extensively at the physiological and biochemical levels. However, improvement of $N_2$ fixation of these legumes under abiotic stressed environments requires a better knowledge of the key elements acting at the molecular level.

The response of *Rhizobium–Phaseolus* symbiosis to salt stress was investigated, and each of the symbiotic partners was examined separately for their salt tolerance (Bouhmouch et al. 2005). Plant cultivars of *Phaseolus* showed genotypic variations with respect to salt tolerance. *Rhizobium* strains (e.g., *R. tropici* and *R. giardinii*), were tolerant up to 250 mM NaCl but failed to nodulate *Phaseolus* under saline conditions (50 mM NaCl). The unsuccessful nodulation could be related to the inhibition of one or more steps of the early events of the infection process under salt stress (Zahran and Sprent 1986). In contrast, common bean plants (*P. vulgaris*) inoculated with a salt-tolerant strain (wild-type *R. tropici*) formed more active symbiosis than did its decreased salt-tolerant mutant derivatives (Tejera et al. 2004). The growth, nodule weight and nitrogenase activity of salinized common bean (*P. vulgaris*) plants were improved by abscisic acid (ABA) pretreatment (Khadri et al. 2007). ABA treatment limited $Na^+$ translocation to shoots, which is a strategy of bean to limit $Na^+$ toxicity. When water-stressed *P. vulgaris* was inoculated with salt-tolerant rhizobia, the nodulation, shoot dry weight and grain yield were significantly increased under nonirrigated conditions (Mnasri et al. 2007). The improvement of the cultivation of *P. vulgaris* under arid conditions can take place by enhancing stress tolerance of $N_2$-fixing rhizobia (Priefer et al. 2001).

Alterations of plant growth, nitrogenase activity and nutrient concentration as a consequence of salt treatment was studied in five chickpea (*Cicer arietinum*) cultivars from Spain and Syria, in symbiosis with *Mesorhizobium ciceri* (Tejera et al. 2006). One cultivar (ILC1919) was moderately salt tolerant at 100 mM NaCl, and exhibited less $N_2$ fixation inhibition, a higher root-to-shoot ratio, normalized nodule weight and shoot K/Na ratio, and a reduced foliar accumulation of $Na^+$. The results revealed the effectiveness of these nutritional and physiological indicators in the selection of salinity-tolerant and symbiotic $N_2$-fixing chickpea plants. Since nitrogenase, the enzyme responsible for $N_2$ fixation, is $O_2$ labile, nodules have evolved mechanisms to regulate their permeability to $O_2$ and maintain the infected-cell $O_2$ concentration at the lowest level (about 5–50 nM) compared with approximately 250 μM for cells in equilibrium with air (Aydi et al. 2004). Nodule conductance to $O_2$ diffusion is a major factor of the inhibition of $N_2$ fixation by soil salinity. However, salinity did not significantly change the nodule conductance and nodule permeability of the plant, and the salt tolerance of this variety appears to be associated with stability in nodule conductance and the capacity to form nodules under salt constraint (L'taief et al. 2007). Salinity stress (75 mM NaCl) significantly increased the nodule conductance of *M. truncatula* plants inoculated with

*S. meliloti* (Aydi et al. 2004). The sensitivity to salinity appears to be associated with an increase in nodule conductance that supports the increased respiration of $N_2$-fixing nodules under salinity (Aydi et al. 2004). Nodule conductance (oxygen uptake) in chickpea inoculated with *M. ciceri* was measured under salt (25 mM NaCl) treatment (L'taief et al. 2007). The symbiotic performance of three strains of rhizobia (*M. ciceri, M. mediterraneum* and *S. medicae*) with chickpea was investigated under salt stress (25 mM NaCl) conditions (Mhadhbi et al. 2004). *M. ciceri*, the most efficient strain, seemed to allow the best tolerance to chickpea plants under salt stress. The activity of enzymes involved in nitrogen metabolism, as well as oxidative stress generation and heme oxygenase (HO) gene and protein expression and activity, were analyzed in soybean (*Glycine max* L.) nodules exposed to 50, 100 and 200 mM NaCl (Zilli et al. 2008). Nitrogenase activity and leghemoglobin content were diminished while ammonium content increased only at 200 mM NaCl. The synthesis of several enzymes involved in nitrogen metabolism was only changed at higher levels of salt (200 mM NaCl). HO activity, protein synthesis and gene expression were significantly increased under 100 mM NaCl treatment. The data demonstrated that the upregulation of HO, as part of an antioxidant defense system, could protect $N_2$ fixation and assimilation under saline stress conditions. Under osmotic stress conditions, plants accumulate some compatible osmolytes (e.g., proline, glycine betaine, etc.) to protect major processes such as cell respiration, photosynthetic activity, nutrient transport, and nitrogen and carbon metabolism. *L. japonicus* and *M. truncatula*, as legume models, provided a convenient system to study plant–*Rhizobium* interactions (López et al. 2008). Plant growth parameters, nitrogenase activity, and activities of trehalose-6-phosphate synthase and trehalase of *L. japonicus* and *M. truncatula* decreased with NaCl treatments (25 and 50 mM NaCl). Legume root-nodule $N_2$-fixing activity was severely affected by osmotic stress (100 mM NaCl) in *M. truncatula* (Verdoy et al. 2006). High proline accumulation level was found in the transgenic *M. truncatula* plants under osmotic stress. $N_2$ fixation in the transgenic *M. truncatula* plants was significantly less affected by salt treatment compared to the wild-type plants. This is the first time that a produced transgenic legume displayed $N_2$-fixing activity with enhanced tolerance to osmotic stress, and that the essential role of proline in the maintenance of $N_2$-fixing activity under osmotic stress was ascertained (Verdoy et al 2006).

Under stress treatments, some genes of nodules are downregulated while others are upregulated as an adaptive response to abiotic stress. To identify molecular markers of drought stress in nodules of soybean, the suppression subtractive hybridization (SSH) was chosen as a method to isolate rare but differentially expressed genes (Clement et al. 2008). In this work, 56 cDNA fragments were validated as drought stress-induced genes by reverse northern hybridization, and analysis was focused on two genes, encoding respectively a ferritin and a metallothionein, which are involved in homeostasis (cellular metal balance) and detoxification of metals. These two genes showed high accumulation of transcripts restricted to infected cells of nodules in response to drought. The growth of *Lotus creticus*, a major forage legume in the arid climate of Tunisia, was studied under

salt stress (Rejili et al. 2007). The presence of salt (50–400 mM) negatively affected plant growth, such negative effects being more obvious on aerial organs than on roots. The content of $Na^+$ in both parts (above and below ground) increased; however, plants probably expressed a certain mechanism of compartmentation. Under salt stress, salt-tolerant plants maintain a high concentration of $K^+$ and a low concentration of $Na^+$ in the cytosol (Zhu 2003; Zahran et al. 2007). They do this by regulating the expression and activity of $K^+$ and $Na^+$ transporters and of $H^+$ pumps that generate the driving force for transport. The effect of salinity (EC up to 3.8 dS $m^{-1}$) on yield and nitrogen uptake of four grain legumes (broad bean, chickpea, lentil and soybean) was examined (Van Hoorn et al. 2001). Salinity affected crop yield, crop total nitrogen uptake, and the nitrogen contribution of the soil. A salinity effect on $N_2$ fixation explains, at least partly, the salt sensitivity of grain legumes. Exposure of mungbean to water deficit results in variability in grain yield, nitrogen accumulation, and grain quality (Thomas et al. 2004). Nodule activities are dramatically affected in most legumes under several environmental constrains, an effect which results in crop yield loss. A negative response to drought stress effects on $N_2$ fixation by root-nodules of *P. sativum* is mainly controlled in nodules rather than by a systemic nitrogen signal (Marino et al. 2007).

Wild forage legumes are the most potential forage legumes grown in salt-affected soils (Ashraf and Bashir 2003; Zahran et al. 2007) which play a vital role in long-term maintenance of soil productivity because of their high $N_2$-fixing ability. The effects of salt stress on growth and metabolic changes in nodules and other plant parts of two leguminous species, *P. vulgaris* (salt sensitive) and *S. aculeata* (salt tolerant), were investigated (Ashraf and Bashir 2003). Plants of *P. vulgaris* were subjected to 3.5 dS $m^{-1}$ of NaCl and those of *S. aculeata* to 13 dS $m^{-1}$ of NaCl. *S. aculeate* showed a small reduction in the number of root-nodules, high proline content in leaves, high glycine betaine content in all plant parts, a high photosynthetic rate, and low uptake of $Cl^-$ in the leaves. The nodules of *S. aculeata* seemed to have played an active role in the high salt tolerance of the species because the nodules had lower concentrations of both $Na^+$ and $Cl^-$ and a higher level of glycine betaine as compared to those found in *P. vulgaris*. Many legumes form symbioses with both rhizobia and AM- fungi. Dual inoculation with both microorganisms results in a tripartite mutualistic symbiosis and generally increases plant growth to a greater extent than inoculation with only one organism. Enhanced acquisition of P by the host and effects on molecular signaling between the three symbionts may explain the synergism of AMF and rhizobia. De Varennes and Goss (2007) investigated how the rate of colonization by indigenous AM fungi (AMF) affects the interaction between *S. meliloti* and *M. truncatula* and AMF. The effects of dual inoculation of *V. faba* plants with NFB such as *Azospirillum brasilense* and AMF were investigated at five levels (0–6.0 dS $m^{-1}$) of NaCl using irrigating water (Rabie and Al Madini 2005). The AMF infection significantly increased tolerance to salinity, mycorrhizal dependency, P level, phosphatases, nodule number, nitrogen level, protein content, and nitrogenases in all salinized *V. faba* plants, in comparison with control and non-AMF-colonized plants, either in the absence or presence of NFB. The study provides evidence for benefits of NFB to

AMF in the protection of host plants against the detrimental effects of salt. The bacterial–AMF–legume tripartite symbioses could be a new and interesting approach for increasing tolerance of legumes to salinity conditions (Rabie and Al Madini 2005). The effects of a mycorrhizal fungus (*Glomus fasciculatum*) on growth and mineral acquisition by *A. nilotica* seedlings over a wide range of soil salinity was investigated (Giri et al. 2007). Mycorrhizal plants maintained greater root and shoot biomass and higher P, Zn, and Cu concentrations than uninoculated plants at all salinity levels. Mycorrhizal fungus alleviated deleterious effects of salinity on plant growth that could be primarily related to improved P nutrition. The improved K/Na ratios in root and shoot tissues of mycorrhizal plants may help in protecting disruption of K-mediated enzymatic processes under salt stress conditions.

## 11.6 Improvement of Inoculation Technology

Many of the microbial inoculants applied worldwide are based on solid peat formulations. This has been mostly true for well-developed legume inoculants due to peat bacterial protection properties. Commercial legume inoculant formulations include powder or granular carriers, and broth cultures or liquid formulations, for agricultural applications. Due to unavailability of peat, more readily available alternative carriers for inoculant production should be investigated. An appropriate material for carrying microorganisms must offer special properties, such as water-holding capacity, chemical and physical uniformity, and lack of compounds toxic to microbial strains, and be environmentally safe. However, osmotic stress, nutrient deficiencies (especially phosphorous), and the lack of efficient strains of rhizobia are major factors limiting symbiotic $N_2$ fixation and yield of legumes. Ideally, inoculation is required in the absence of compatible resident rhizobia, where the native rhizobial population density is very low, or where the resident rhizobia are less infective than alternative (inoculant) strains. Soils lacking in compatible rhizobia are found in areas where indigenous legumes are absent or where levels of pH, osmotic stress, high temperature, and heavy metals are detrimental to rhizobial populations. Chemining'wa and Vessey (2006) found that effective strains of *R. leguminosarum* bv *viciae* occur broadly in agricultural soils; nevertheless, there is a tendency for increased symbiotic efficiency with the use of commercial inoculation.

Effective rhizobial inoculants, inoculation carriers able to support viable rhizobia, and appropriate methods of storage, are required for successful symbiosis. Rhizobial density and the period for which these are maintained are highly variable. Even where strains are carefully evaluated, the lack of competitiveness with indigenous rhizobia and inconsistency in their performance can lead to a decline in nitrogen-fixing efficiency under field environments. And, hence, before applying rhizobia, the establishment and persistence of such strains must be considered in inoculation studies. Perlite and compost from the cork industry were superior to

peat in maintaining survival of different rhizospheric bacteria. Similarly, some culture media maintained more than $10^9$cfu $mL^{-1}$ of *S. fredii* or *B. japonicum* after 3 months of storage. Soybean plants inoculated with two highly effective rhizobia, using the carriers cork compost, perlite and liquid formulations, produced seed yields that were not significantly different to those produced by peat-based inoculants (Albareda et al. 2008). Responses to bean rhizobia inoculation and P fertilization were investigated (Zaman-Allah et al. 2007), and a significant effect of inoculation and P supply on nodulation, N content and growth was found. It is concluded that inoculation with suitable rhizobia with supply of additional P is a technology that may improve symbiotic $N_2$ fixation and yield in the common bean.

Rhizobial inoculation of legume seeds or soils has been well studied. However, much less work has been done regarding the association and growth-promoting activities of rhizobia with nonlegumes. Efforts at extending $N_2$-fixing ability to important nonleguminous crops such as cereals has long been a major goal of workers in the field of biological $N_2$ fixation and would be a useful technology for increasing crop yields (Matiru and Dakora 2004). Although some inoculation attempts have resulted in nodule formation in cereal plants, there was no evidence of $N_2$ fixation. Nevertheless, associated rhizobia produce molecules such as auxins, cytokinins, abscisic acids, IAA, lumichrome, riboflavin, lipochitooligosaccharides and vitamins that promote plant growth. Therefore, rhizobial colonization and infection of cereal roots would be expected to increase plant development and grain yield (Matiru and Dakora 2004). Roots of sorghum (*Sorghum bicolour)* and millet (*Pennisetum americanum)* were easily infected by rhizobia from five unrelated legume genera. Plant growth and P uptake, in sorghum in particular, were significantly increased by rhizobial inoculation, suggesting that field selection of suitable rhizobia/cereal combinations could increase yields and produce fodder for livestock production (Matiru and Dakora 2004). Inoculation of rice (*Oryza sativa*) seedlings with *R. leguminosarum* bv. *trifolii* and *Rhizobium* sp. stimulated rice growth and increased grain and straw yields (Biswas et al. 2000). The tested rhizobial strains produced IAA in vitro, and some of them reduced acetylene to ethylene in association with rice under laboratory growth conditions. Enhancement of legume $N_2$ fixation by coinoculation of rhizobia with plant growth-promoting bacteria is thus important for improving nitrogen availability in sustainable agriculture.

Endophytes recovered from the surface-sterilized root-nodules of pigeonpea (*Cajanus cajan*), designated as nonrhizobial isolates (Rajendran et al. 2008), facilitated plant growth and increased the fresh weight and symbiotic traits when coinoculated with the rhizobial bioinoculant strain, isolated from nodules of the same plant. The nonrhizobial isolates were not able to nodulate pigeonpea and nor did they show significant plant growth promotion when inoculated alone without *Rhizobium* spp. The nonrhizobial isolates were identified as *Bacillus megaterium* following amplified ribosomal DNA restriction analysis and partial sequences of 16S rRNA genes studies. Several papers have reported on dual inoculation with rhizobia and NFB (e.g., *Azospirillum*) and P-solubilizing bacteria (e.g., *Pseudomonas*). The legume growth, yield, and P and N uptake, could be increased by coinoculation

(Graham and Vance 2000). A general positive effect of *Azospirillium–Rhizobium* coinoculation on the expression of *nod* genes by *R. tropici* and *R. etli*, and on nodulation, was observed in the presence of root exudates (Dardanelli et al. 2008). The negative effects obtained under salt stress on *nod* gene expression and on nod factor appearance were relieved in coinoculated plants. The effect of dual inoculation of P-solubilizing bacteria (*Pseudomonas putida*) on the symbiosis of *S. meliloti and B. japonicum* with alfalfa and soybean, respectively, was evaluated (Rosas et al. 2006). Modification of shoot and root dry weights occurred in soybean but not in alfalfa in the presence of *P. putida*.

## 11.7 Breeding and Selection for Enhanced $N_2$ Fixation in Crop Legumes

Biotechnology is a powerful tool that has potential to contribute to sustainable agriculture. Over the years, biotechnology has emerged as a promising tool to overcome stresses in plants, but to date, progress has been limited in legumes. However, molecular approaches, such as marker-assisted breeding, genetic transformation, tissues cultures, gene expression, transcriptomics, transcription factors, proteomics, etc., can contribute to speed-up classical breeding. Dita et al. (2006) reviewed many of the modern biotechnological techniques and evaluated their applications to improve tolerance of legumes to abiotic stress. The use of genetic and genomic analysis to help identifying DNA regions tightly linked to agronomic traits in crops, the so-called molecular markers, can facilitate breeding strategies for crop improvement. Numerous molecular marker-related techniques have been used in legumes in relation to abiotic stress. As a result, genetic maps for many species have been established and quantitative trait loci (QTLs) have been located in some legumes, as reported for soybean, under salt stress (Lee et al. 2004). This improved the knowledge of genetic control of specific resistance and/or tolerance in many legumes by providing information on the chromosomal number and location, and individual or interactive effects of the QTLs involved. In plants, there is a poor understanding of most abiotic stress responses. Thus, the successful use of genetic transformation requires a better physiological and molecular understanding of these stresses. Enhancement of resistance against both hyperosmotic stress and $Na^+$ toxicity is necessary for successful molecular breeding of salt-tolerant plants. Introduction of genes for osmolyte biosynthesis is useful to increase hyperosmotic tolerance of plant cells (Yoshida 2002). Overexpression of a single-gene controlling vacuolar or plasma membrane $Na^+/H^+$ antiport protein in plants (e.g., *M. intertexta*; Zahran et al. 2007) provided them with a high level of salt tolerance. Identification of differentially expressed genes is particularly important to understand stress responses in plants. With respect to abiotic stress, gene expression analyses have been mainly based on studies with cloned genes. Transcriptomic tools are a good option for legume breeding to environmental stresses. Using a modified cDNA-AFLP

technique in soybean, 140 differentially expressed cDNA fragments were obtained by comparing control and osmotic-treated plants. Some of the responsive genes encoded ion transporters, transcription factors and redox enzymes (Umezawa et al. 2002). Further, recent technological developments have allowed the establishment of valuable methods for quantitative and qualitative protein profiling. This approach is very important in evaluating stress responses because mRNA levels do not always correlate with protein accumulation (Dita et al. 2006).

Graham and Vance (2000) reviewed the developments and research areas which are critical for improvement of $N_2$ fixation. Genetic improvement in nodulation and nitrogen fixation can be done by indirect selection for growth and seed yield in plants grown under N limitation conditions, and by direct selection for nodule mass, acetylene reduction activity and xylem ureide content. For all legumes, there is great potential to increase the percent of legume N derived from $N_2$ fixation as well to enhance the total $N_2$ fixed through improved management and genetic modification of the plant. Herridge and Rose (2000) reviewed strategies and operational frameworks for conducting selection and breeding programs to enhance $N_2$ fixation, and methods for measuring $N_2$ fixation that have been used in such programs. Three general strategies for increasing legume $N_2$ fixation through breeding. Firstly, there is the maximizing of legume (biomass) and seed yield under constraints imposed by agronomic management and the environment. This approach assumes a capacity for $N_2$ fixation sufficient to satisfy increased N demand of larger plants, but has particular application to lower-yielding plants such as common bean, lentil (*L. esculentum*), mungbean, and chickpea. Secondly, legume ability to nodulate and fix $N_2$ in the presence of soil nitrate is enhanced. Variation for symbiotic nitrate tolerance exists in natural plant populations and has also been created through mutagenesis. Thirdly, legume nodulation is optimized through specific nodulation traits (e.g., mass and duration). Crop improvement through genetic engineering has become a reality, and it is now possible to transform many grain legumes using *A. tumefaciens* as a vector for legume transformation; the inserted DNA can be either a specific gene with a specific biochemical function, a regulatory gene that controls a network of other genes, or multiple genes to generate long-term durable resistance. Rhizobia were examined for their ability to be used as potential vehicles for plant transformation. Several species of rhizobia were successfully transformed with broad host-range plasmids of different replicons. A genetic binary vector (pPZP211) was maintained in *M. loti* and stably inherited during nodulation (Vincze and Bowra 2006).

## 11.8 Conclusion

The information available in the literature highlights the significance of $N_2$-fixing rhizobia–legumes symbioses for improving soil fertility and, consequently, plant productivity. The symbiotic bacteria that colonize nodules of these plants are very diverse, with prominent ecological characteristics and specific genetic traits.

These bacteria are classified into more than 70 genomic species belonging to several genera. With all of this genomic diversity, it is no wonder that the systematics of root-nodule bacteria are changing every day. Recent developments in the field of *Rhizobium* biology has taken place due to the advances in the molecular and genetic techniques. Such techniques provide an effective tool to characterize and identify the bacterial isolates vis-a-vis examining the physiological responses of the host plant. Molecular identification of rhizobia depends on the symbiotic and ribosomal genes. However, in some cases, host specificity of rhizobial strains was not related to ribosomal genes (16S rRNA). The evolution of ribosome and symbiotic genes may have been diverse. The symbiotic and $N_2$-fixing genes appear to be spread in various bacterial taxa and not restricted to what is known as rhizobium bacteria of the alphaproteobacteria. In recent years, plenty of new strains of symbiotic bacteria were classified in nonrhizobial genera, namely *Burkholderia* and *Cupriavidus*, in the β-proteobacteria.

Symbiotic characteristics are some of the basic criteria for selecting compatible rhizobia. Numerous species of the newly isolated and identified nodule bacteria have shown substantial symbiotic activities and proved that legumes exhibit biodiversity with regards to symbiotic bacteria and also the host plants. Enhancement of $N_2$ fixation of the existing symbiotic systems, and those newly discovered symbiotic systems, is possible through the inoculation of legumes with compatible and effective strains of bacteria. However, inoculation technology and delivery system is needed to be improved. Successful symbiosis can be obtained by coinoculation or mixed inoculation with other plant-growth promoting rhizobacteria including $N_2$ fixers. Legumes' $N_2$ fixation enhancement is likely to improve the productivity of associated nonlegume plants by providing them with N and growth-promoting substances. Suitable rhizobia–cereal combinations could increase yields and produce fodder for livestock. Bacterial–AMF–legume tripartite symbioses could also be viable alternatives to enhance $N_2$ fixation of legumes especially under salt stress conditions.

The $N_2$-fixing activity of root-nodules is severely affected by osmotic stress. However, elucidation of genes involved in the regulation of ion transport and their expression has increased our understanding about how legumes circumvent the stress conditions. One of the strategies adopted to combat the problem of soil salinity is the usage of transgenic leguminous plants. The transgenic plants in certain cases are more salt tolerant than wild-type plants. For example, transgenic plants (e.g., *M. truncatula*) accumulated higher levels of proline and fixed $N_2$ actively more than wild-type under osmotic stress, and is the first transgenic legume that displayed $N_2$-fixing activity with enhanced tolerance to osmotic stress. However, selection of a salt-tolerant effective *Rhizobium*–legume symbiosis is of paramount importance. The symbiotic bacteria have shown a variable response to abiotic stresses such as salinity, acidity, and aridity. Some strains of symbiotic bacteria exhibit substantial stress-tolerant abilities. Understanding the mechanisms of osmoadaptation of rhizobia at the molecular level would help to rationally design and engineer better strains for field applications.

## References

Albareda M, Rodríguez-Navarro DN, Camacho M, Temprano FJ (2008) Alternatives to peat as a carrier for rhizobia inoculants: solid and liquid formulations. Soil Biol Biochem 40:2771–2779

Ashraf M, Bashir A (2003) Salt stress induced changes in some organic metabolites and ionic relations in nodules and other plant parts of two crop legumes differing in salt tolerance. Flora 198:486–498

Aydi S, Drevon J-J, Abdelly C (2004) Effect of salinity on root-nodule conductance to the oxygen diffusion in the *Medicago truncatula-Sinorhizobium meliloti* symbiosis. Plant Physiol Biochem 42:833–840

Ba S, Willems A, De Lajudie P, Roche P, Jeder H, Quatrini P, Neyra M, Ferro M, Promé J-C, Gillis M, Boivin-Masson C, Lorquin J (2002) Symbiotic and taxonomic diversity of rhizobia isolated from *Acacia tortilis* subsp: *raddiana* in Africa. Syst Appl Microbiol 25:130–145

Bala A, Murphy PJ, Osunde AO, Giller KE (2003) Nodulation of tree legumes and the ecology of their native rhizobial populations in tropical soils. Appl Soil Ecol 22:211–223

Barrett CF, Parker MA (2005) Prevalence of *Burkholderia* sp. nodule symbionts on four mimosoid legumes from Barro Colorado Island, Panama. Syst Appl Microbiol 28:57–65

Barrett CF, Parker MA (2006) Coexistence of *Burkholderia, Cupriavidus,* and *Rhizobium* sp. nodule bacteria on two *Mimosa* spp. in Costa Rica. Appl Environ Microbiol 72:1198–1206

Benata H, Mohammed O, Noureddine B, Abdelbasset B, Abdelmoumen H, Muresu R, Squartini A, El Idrissi MM (2008) Diversity of bacteria that nodulate *Prosopis juliflora* in the eastern area of Morocco. Syst Appl Microbiol 31:378–386

Bernatchez F, Jeannotte R, Begg CBM, Hamel C, Whalen JK (2008) Soil fertility and arbuscular mycorrhizal fungi related to trees growing on smallholder farms in Senegal. J Arid Environ 72:1247–1256

Biswas JC, Ladha JK, Dazzo FB, Yanni YG, Rolfe BG (2000) Rhizobial inoculation influences seedling vigor and yield of rice. Agron J 92:880–886

Bouhmouch I, Souad-Mouhsine B, Brhada F, Aurag J (2005) Influence of host cultivars and *Rhizobium* species on the growth and symbiotic performance of *Phaseolus vulgaris* under salt stress. J Plant Physiol 162:1103–1113

Chemining'wa GN, Vessey JK (2006) The abundance and efficacy of *Rhizobium leguminosarum* bv. *viciae* in cultivated soils of the eastern Canadian prairie. Soil Biol Biochem 38:294–302

Chen W-M, Lee T-M, Lan C-C, Cheng C-P (2000) Characterization of halotolerant rhizobia isolated from root nodules of *Canavalia rosea* from seaside areas. FEMS Microbiol Ecol 34:9–16

Chen W-M, James EK, Chou J-H, Sheu S-Y, Yang S-Z, Sprent JI (2005) β-rhizobia from *Mimosa pigra*, a newly discovered invasive plant in Taiwan. New Phytol 168:661–675

Clement M, Lambert A, Herouart D, Boncompagni E (2008) Identification of new up-regulated genes under drought stress in soybean nodules. Gene 426:15–22

Dardanelli MS, Fernández De Córdoba FJ, Espuny MR, Rodríguez Carvajal MA, Soria Díaz ME, Gil Serrano AM, Okon Y, Megías M (2008) Effect of *Azospirillum brasilense* coinoculated with *Rhizobium* on *Phaseolus vulgaris* flavonoids and nod factor production under salt stress. Soil Biol Biochem 40:2713–2721

De Varennes A, Goss MJ (2007) The tripartite symbiosis between legumes, rhizobia and indigenous mycorrhizal fungi is more efficient in undisturbed soil. Soil Biol Biochem 39:2603–2607

Denison RF, Kiers ET (2004) Why are most rhizobia beneficial to their plant hosts, rather than parasitic? Microb Infect 6:1235–1239

Dita MA, Rispail N, Prats E, Rubiales D, Singh KB (2006) Biotechnology approaches to overcome biotic and abiotic stress constraints in legumes. Euphytica 147:1–24

Domínguez-Ferreras A, Pérez-Arnedo R, Becker A, Olivares J, Soto MJ, Sanjuan J (2006) Transcriptome profiling reveals the importance of plasmid pSymB for osmoadaptation of *Sinorhizobium meliloti*. J Bacteriol 188:7617–7625

Downie JA (2005) Legume haemoglobins: symbiotic nitrogen fixation needs bloody nodules. Curr Biol 15:196–198

Duodu S, Bhuvaneswari TV, Gudmundsson J, Svenning MM (2005) Symbiotic and saprophytic survival of three unmarked *Rhizobium leguminosarum* biovar *trifolii* strains introduced into the field. Environ Microbiol 7:1049–1058

El-Akhal MR, Rincon A, Arenal F, Lucas MM, El Mourabit N, Barrijal S, Pueyo JJ (2008) Genetic diversity and symbiotic efficiency of rhizobial isolates obtained from nodules of *Arachis hypogaea* in northwestern Morocco. Soil Biol Biochem 40:2911–2914

Essendoubi M, Brhada F, Eljamali JE, Filali-Maltouf A, Bonnassie S, Georgeault S, Blanco C, Jebbar M (2007) Osmoadaptative responses in the rhizobia nodulating *Acacia* isolated from south-eastern Moroccan Sahara. Environ Microbiol 9:603–611

Ge-Hong W, Xue-Ying Y, Zhi-Xin Z, Ya-Zhen Y, Lindström K (2008) Strain *Mesorhizobium* sp. CCNWGX035: a stress-tolerant isolate from *Glycyrrhiza glabra* displaying a wide host range of nodulation. Pedosphere 18:102–112

Giri B, Kapoor R, Mukerji KG (2007) Improved tolerance of *Acacia nilotica* to salt stress by arbuscular mycorrhiza, *Glomus fasciculatum* may be partly related to elevated K/Na ratios in root and shoot tissues. Microb Ecol 54:753–760

González Ruiz T, Rodríguez Zaragoza S, Ferrera Cerrato R (2008) Fertility islands around *Prosopis laevigata* and *Pachycereus hollianus* in the drylands of Zapotitlán Salinas, México. J Arid Environ 72:1202–1212

Graham PH (2008) Ecology of the root-nodule bacteria of legumes. In: Dilworth MJ, James EK, Sprent JI, Newton WE (eds) Nitrogen-fixing leguminous symbiosis. Springer, Dordrecht, The Netherland, pp 23–58

Graham PH, Vance CP (2000) Nitrogen fixation in perspective: an overview of research and extension needs. Field Crops Res 65:93–106

Grossman JM, Sheaffer C, Wyse D, Bucciarelli B, Vance C, Graham PH (2006) An assessment of nodulation and nitrogen fixation in inoculated *Inga oerstediana,* a nitrogen-fixing tree shading organically grown coffee in Chiapas, Mexico. Soil Biol Biochem 38:769–784

Han TX, Wang ET, Han LL, Chen WF, Sui XH, Chen WX (2008) Molecular diversity and phylogeny of rhizobia associated with wild legumes native to Xinjiang, China. Syst Appl Microbiol 31:287–301

Herridge D, Rose I (2000) Breeding for enhanced nitrogen fixation in crop legumes. Field Crops Res 65:229–248

Howieson J, Ballard R (2004) Optimizing the legume symbiosis in stressful and competitive environments within southern Australia – some contemporary thoughts. Soil Biol Biochem 36:1261–1273

Hoy JA, Hargrove MS (2008) The structure and function of plant hemoglobins. Plant Physiol Biochem 46:371–379

Jiang JQ, Wei W, Du BH, Li XH, Wang L, Yang SS (2004) Salt-tolerance genes involved in cation efflux and osmoregulation of *Sinorhizobium fredii* RT19 detected by isolation and characterization of TN5 mutants. FEMS Microbiol Lett 239:139–146

Khadri M, Tejera NA, Lluh C (2007) Sodium chloride-ABA interaction in two common bean (*Phaseolus vulgaris*) cultivars differing in salinity tolerance. Environ Exp Bot 60:211–218

Lee GJ, Boerma HR, Villagarcia MR, Zhou X, Carter TE Jr, Li Z, Gibbs MO (2004) A major QTL conditioning salt tolerance in S-100 soybean and descendent cultivars. Theor Appl Genet 109:1610–1619

Liu J, Wang ET, Chen WX (2005) Diverse rhizobia associated with woody legumes *Wisteria sinensis, Cercis racemosa* and *Ammorpha fruticosa* grown in the temperate zone of China. Syst Appl Microbiol 28:465–477

Long SR (2001) Genes and signals in the *Rhizobium*-legume symbiosis. Plant Physiol 125:69–72

López M, Herrera-Cervera JA, Iribarne C, Tejera NA, LLuch C (2008) Growth and nitrogen fixation in *Lotus japonicus* and *Medicago truncatula* under NaCl stress: nodule carbon metabolism. J Plant Physiol 165:641–650

L'taief B, Sifi B, Zaman-Allah M, Drevon J-J, Lachaâl M (2007) Effect of salinity on root-nodule conductance to the oxygen diffusion in the *Cicer arietinum-Mesorhizobium ciceri* symbiosis. J Plant Physiol 164:1028–1036

Mahdhi M, Nzoué A, DeLajudie P, Mars M (2008) Characterization of root-nodulating bacteria on *Retama raetam* in arid Tunisian soils. Prog Nat Sci 18:43–49

Marino D, Frendo P, Ladrera R, Zabalza A, Puppo A, Arrese-Igor C, González EM (2007) Nitrogen fixation control under drought stress. Localized or systemic? Plant Physiol 143:1968–1974

Matiru VN, Dakora FD (2004) Potential use of rhizobial bacteria as promoters of plant growth for increased yield in landraces of African cereal crops. Afr J Biotechnol 3:1–7

Menna P, Hungria M, Barcellos FG, Bangel EV, Hess PN, Martínez-Romero E (2006) Molecular phylogeny based on the 16S rRNA gene of elite rhizobial strains used in Brazilian commercial inoculants. Syst Appl Microbiol 29:315–332

Mhadhbi H, Jebara M, Limam F, Aouani ME (2004) Rhizobial strain involvement in plant growth, nodule protein composition and antioxidant enzyme activities of chickpea-rhizobia symbioses: modulation by salt stress. Plant Physiol Biochem 42:717–722

Mithöfer A (2002) Suppression of plant defence in rhizobia-legume symbiosis. Trends Plant Sci 7:440–444

Mnasri B, Aouani ME, Mhamdi R (2007) Nodulation and growth of common bean (*Phaseolus vulgaris*) under water deficiency. Soil Biol Biochem 39:1744–1750

Moreira FMD, Cruz L, De Faria SM, Marsh T, Martínez-Romero E, Pedrosa FD, Pitard RM, Young JPW (2006) *Azorhizobium doebereinerae* sp. nov. microsymbiont of *Sesbania virgata* (Caz.) Pers. Syst Appl Microbiol 29:197–206

Moschetti G, Peluso A, Protopapa A, Anastasio M, Pepe O, Defez R (2005) Use of nodulation pattern, stress tolerance, *nod*C gene amplification, RAPD-PCR and RFLP-16S rDNA analysis to discriminate genotypes of *Rhizobium leguminosarum* biovar *viciae*. Syst Appl Microbiol 28:619–631

Moulin L, Béna G, Boivin-Masson C, Stępkowski T (2004) Phylogenetic analysis of symbiotic nodulation genes support vertical and lateral gene co-transfer within the *Bradyrhizobium* genus. Mol Phylogenet Evol 30:720–732

Mulder L, Hogg B, Bersoult A, Cullimore JV (2005) Integration of signalling pathways in the establishment of the legume-rhizobia symbiosis. Physiol Plant 123:207–218

Musiyiwa K, Mpepereki S, Giller KE (2005) Symbiotic effectiveness and host ranges of indigenous rhizobia nodulating promiscuous varieties in Zimbabwean soils. Soil Biol Biochem 37:1169–1176

Nandasena KG, O'Hara GW, Tiwari RP, Yates RJ, Kishinevsky BD, Howieson JG (2004) Symbiotic relationships and root nodule ultrastructure of the pasture legume *Biserrula pelecinus* L. – a new legume in agriculture. Soil Biol Biochem 36:1309–1317

Nkot LN, Krasova-Wade T, Etoa FX, Sylla SN, Nwaga D (2008) Genetic diversity of rhizobia nodulating *Arachiss hypogaea* L. in diverse land use systems of humid forest zone in Cameroon. Appl Soil Ecol 40:411–416

Nogales J, Campos R, Ben Abdelkhalek H, Olivares J, LLuch C, Sanjuan J (2002) *Rhizobium tropici* genes involved in free-living salt tolerance are required for the establishment of efficient-nitrogen-fixing symbiosis with *Phaseolus vulgaris*. Mol Plant Microb Interact 15:225–232

Odee DW, Haukka K, McInroy SG, Sprent JI, Sutherland JM, Young JPW (2002) Genetic and symbiotic characterization of rhizobia isolated from tree and herbaceous legumes grown in soils from ecologically diverse sites in Kenya. Soil Biol Biochem 34:801–811

Ott T, Van Dongen JT, Günther C, Krusell L, Desbrosses G, Vigeolas H, Bock V, Czechowski T, Geigenberger P, Udavardi MK (2005) Symbiotic leghemoglobins are crucial for nitrogen fixation in legume root nodules but not for general plant growth and development. Curr Biol 15:531–535

Priefer UB, Aurag J, Boesten B, Bouhmouch I, Defez R, Filali-Maltouf A, Miklis M, Moawad H, Mouhsine B, Prell J, Schlüter A, Senatore B (2001) Characterization of *Phaseolus* symbionts isolated from Mediterranean soils and analysis of genetic factors related to pH tolerance. J Biotechnol 91:223–236

Rabie GH, Al Madini AM (2005) Role of bioinoculants in development of salt-tolerance of *Vicia faba* plants under salinity stress. Afr J Biotechnol 4:210–222

Rajendran G, Sing F, Desai AJ, Archana G (2008) Enhanced growth and nodulation of pigeon pea by co-inoculation of *Bacillus* strains with *Rhizobium* spp. Bioresource Technol 99:4544–4550

Raza S, Jørnsgård B, Abou-Taleb H, Christiansen JL (2001) Tolerance of *Bradyrhizobium* sp. (*Lupini)* strains to salinity, pH, $CaCO_3$ and antibiotics. Lett Appl Microbiol 32:379–383

Rejili M, Vadel AM, Guetet A, Neffatti M (2007) Effect of NaCl on the growth and the ionic balance $K^+/Na^+$ of two populations of *Lotus creticus* (L.) (Papilionaceae). South Afr J Bot 73:623–631

Rosas SB, Andrés JA, Rovera M, Correa NS (2006) Phosphate-solubilizing *Pseudomonas putida* can influence the rhizobia-legume symbiosis. Soil Biol Biochem 38:3502–3505

Rüberg S, Tian Z-X, Krol E, Linke B, Meyer F, Wang Y, Pühler A, Weidner S, Becker A (2003) Construction and validation of a *Sinorhizobium meliloti* whole genome DNA microarray: genome-wide profiling of osmoadaptive gene expression. J Biotechnol 106:255–268

Sharma RS, Mohammed A, Mishra V, Babu CR (2005) Diversity in a promiscuous group of rhizobia from three *Sesbania* spp. colonizing ecologically distinct habitats of the semi-arid Delhi region. Res Microbiol 156:57–67

Sierra J, Nygren P (2006) Transfer of N fixed by a legume tree to the associated grass in a tropical silvopastrol system. Soil Biol Biochem 38:1893–1903

Sprent JI (2008) 60Ma of legume nodulation: what's new? what's changing? J Exp Bot 59:1081–1084

Stacey G, Libault M, Brechenmacher L, Wan J, May GD (2006) Genetics and functional genomics of legume nodulation. Curr Opin Plant Biol 9:110–121

Steenkamp ET, Stępkowski T, Przymusiak A, Botha WJ, Law IJ (2008) Cowpea and peanut in Southern Africa are nodulated by diverse *Bradyrhizobium* strains harboring nodulation genes that belong to the large pantropical clade common in Africa. Mol Phylogenet Evol 48:1131–1144

Tejera NA, Campos R, Sanjuan J, Lluch C (2004) Nitrogenase and antioxidant enzyme activities in *Phaseolus vulgaris* nodules formed by *Rhizobium tropici* isogenic strains with varying tolerance to salt stress. J Plant Physiol 161:329–338

Tejera NA, Soussi M, LLuch C (2006) Physiological and nutritional indicators of tolerance to salinity in chickpea plants growing under symbiotic conditions. Environ Exp Bot 58:17–24

Thomas RMJ, Fukai S, Peoples MB (2004) The effect of timing and severity of water deficit on growth, development, yield accumulation and nitrogen fixation of mungbean. Field Crops Res 86:67–80

Toledo I, Lloret L, Martínez-Romero E (2003) *Sinorhizobium americanus* sp. Nov., a new *Sinorhizobium* species nodulating native *Acacia* spp. in Mexico. Syst Appl Microbiol 26:54–64

Umezawa T, Mizuno K, Fujimura T (2002) Discrimination of genes expressed in response to the ionic or osmotic effect of salt stress in soybean with c-DNA-AFLP. Plant Cell Environ 25:1617–1625

Unni S, Rao KK (2001) Protein and lipopolysaccharide profiles of salt-sensitive *Rhizobium* sp. and exopolysaccharide deficient mutant. Soil Biol Biochem 33:111–115

Van Hoorn JW, Katerji N, Hamdy A, Mastrorilli M (2001) Effect of salinity on yield and nitrogen uptake of four grain legumes and on biological nitrogen contribution from the soil. Agric Water Manage 51:87–98

Verdoy D, De La Peña TC, Redondo FJ, Lucas MM, Pueyo JJ (2006) Transgenic *Medicago truncatula* plants that accumulate proline display nitrogen-fixing activity with enhanced tolerance to osmotic stress. Plant Cell Environ 29:1913–1923

Vessey JK, Chemining'wa GN (2006) The genetic diversity of *Rhizobium leguminosarum* bv. *viciae* in cultivated soils of the eastern Canadian prairie. Soil Biol Biochem 38:153–163

Villegas MDC, Rome S, Mauré L, Domergue O, Gardan L, Bailly X, Cleyet-Marel J-C, Brunel B (2006) Nitrogen-fixing sinorhizobia with *Medicago laciniata* constitute a novel biovar (bv. *medigaginis*) of *S. meliloti*. Syst Appl Microbiol 29:526–538

Vincze E, Bowra S (2006) Transformation of rhizobia with broad-host range plasmids by using a freeze-thaw method. Appl Environ Microbiol 72(2290):2293

Vinuesa P, Silva C, Lorite MJ, Izaguirre-Mayoral ML, Bedmar EJ, Martínez-Romero E (2005) Molecular systematics of rhizobia based on maximum likelihood and Bayesian phylogenies inferred from *rrs*, *atp*D, *rec*A and *nif*H sequences, and their use in the classification of *Sesbania* microsymbionts from Venezuelan wetlands. Syst Appl Microbiol 28:702–716

Vriezen JAC, De Bruijn FJ, Nüsslein K (2006) Desiccation responses and survival of *Sinorhizobium meliloti* USDA 1021 in relation to growth phase, temperature, chloride and sulfate availability. Lett Appl Microbiol 42:172–178

Vriezen JAC, De Bruijn FJ, Nüsslein K (2007) Response of rhizobia to desiccation in relation to osmotic stress, oxygen and temperature. Appl Environ Microbiol 73:3451–3459

Wang FQ, Wang ET, Zhang YF, Chen WX (2006) Characterization of rhizobia isolated from *Albizia* spp. in comparison with microsymbionts of *Acacia* spp. and *Leucaena leucocephala* grown in China. Syst Appl Microbiol 29:502–517

Wei W, Jiang J, Li X, Wang L, Yang SS (2004) Isolation of salt-sensitive mutants from *Sinorhizobium meliloti* and characterization of gene involved in salt tolerance. Lett Appl Microbiol 39:278–283

Wei GH, Zhang ZX, Chen C, Chen WM, Ju WT (2007) Phenotypic and genetic diversity of rhizobia isolated from nodules of the legume genera *Astragalus, Lespedeza* and *Hedysarum* in northwestern China. Microbiol Res 163:651–662

Wolde-Meskel E, Terefework Z, Lindström K, Frostegård A (2004) Rhizobia nodulating African *Acacia* spp. and *Sesbania sesban* trees in Southern Ethiopian soils are metabolically and genomically diverse. Soil Biol Biochem 36:2013–2025

Ya-mei G, Yi-qiang H, Hui T, Dong-mei S, Yan-jie W, Wei-dong W (2008) Analysis of simple sequence repeats in genomes of rhizobia. Agric Sci China 7:1189–1195

Yang JK, Yuan TY, Zhang WT, Zhou JC, Li YG (2008) Polyphasic characterization of mung bean (*Vigna radiata* L.) rhizobia from different geographical regions of China. Soil Biol Biochem 40:1681–1688

Yates RJ, Howieson JG, Reeve WG, Nandasena KG, Law IJ, Bräu L, Ardley JK, Nistelberger HM, Real D, O'Hara GW (2007) *Lotononis angolensis* forms nitrogen-fixing, lupinoid nodules with phylogenetically unique, fast-growing, pink-pigmented bacteria, which do not nodulate *L. bainesii* or *L. listii*. Soil Biol Biochem 39:1680–1688

Yates RJ, Howieson JG, Reeve WG, Bräu L, Speijers J, Nandasena KG, Real D, Sezmis E, O'Hara GW (2008) Host-strain mediated selection for an effective nitrogen-fixing symbiosis between *Trifolium* spp. and *Rhizobium leguminosarum* biovar *trifolii*. Soil Biol Biochem 40:822–833

Yoshida K (2002) Plant biotechnology–genetic engineering to enhance plant salt tolerance. J Biosci Bioeng 94:585–590

Zahran HH (1999) *Rhizobium*-legume symbiosis and nitrogen fixation under severe conditions and in an arid climate. Microbiol Mol Biol Rev 63:968–989

Zahran HH (2001) Rhizobia from wild legumes: diversity, taxonomy, ecology, nitrogen fixation and biotechnology. J Biotechnol 91:143–153

Zahran HH (2005) Rhizobial nitrogen fixation in agriculture: biotechnological perspectives. In: Ray RC (ed) Microbial biotechnology in agriculture and aquaculture, vol 1. Science, Enfield, USA, pp 71–100

Zahran HH (2006a) Wild-legume rhizobia: biodiversity and potential as biofertilizers. In: Rai MK (ed) Handbook of microbial biofertilizers. Haworth, New York, pp 203–222

Zahran HH (2006b) Nitrogen ($N_2$) fixation in vegetable legumes: biotechnological perspectives. In: Ray RC, Ward OP (eds) Microbial biotechnology in horticulture, vol 1. Science, Enfield, USA, pp 49–82

Zahran HH, Sprent JI (1986) Effects of sodium chloride and polyethylene glycol on root-hair infection and nodulation of *Vicia faba* L. plants by *Rhizobium leguminosarum*. Planta 167:303–309

Zahran HH, Abdel-Fattah M, Ahmad MS, Zaki AY (2003) Polyphasic taxonomy of symbiotic rhizobia from wild leguminous plants gowing in Egypt. Folia Microbiol 48:510–520

Zahran HH, Marin-Mansano MC, Sánchez-Raya AJ, Bedmar EJ, Venema K, Rodríguez-Rosales MP (2007) Effect of salt stress on the expression of NHX-type ion transporters in *Medicago intertexta* and *Melilotus indicus*. Physiol Plant 131:122–130

Zakhia F, Jeder H, Domergue O, Willems A, Cleyet-Marel J-C, Gillis M, Dreyfus B, De Lajudie P (2004) Characterization of wild legume nodulating bacteria (LNB) in the infra-arid zone of Tunisia. Syst Appl Microbiol 27:380–395

Zaman-Allah M, Sifi B, L'taief B, El Aouni MH, Drevon J-J (2007) Rhizobial inoculation and P fertilization response in common bean (*Phaseolus vulgaris*) under glasshouse and field conditions. Exp Agric 43:67–77

Zhu J-K (2003) Regulation of ion homeostasis under salt stress. Curr Opin Plant Biol 6:441–445

Zilli CG, Balestrasse KB, Yannarrelli GG, Polizio AH, Santa-Cruz DM, Tomaro ML (2008) Heme oxygenase up-regulation under salt stress protects nitrogen metabolism in nodules of soybean plants. Environ Exp Bot 64:83–89

# Chapter 12
# Monitoring the Development of Nurse Plant Species to Improve the Performances of Reforestation Programs in Mediterranean Areas

**R. Duponnois, M. Hafidi, J. Thioulouse, A. Galiana, L. Ouahmane, B. Dreyfus, and Y. Prin**

**Abstract** In the Mediterranean basin, a millenarian history of overexploitation has lead to the loss of most primeval forests and an increase of the surface area covered by shrublands that represent stages of degradation of mature forests. In this situation, and since environmental characteristics act as barriers to succession, human intervention is usually necessary to improve recovery of woodlands. Reafforestation is a common practice in Mediterranean areas to achieve this aim but its performances are very low with high rates of early mortality making this practice unprofitable in ecological as well as in economic terms.

In degraded semiarid ecosystems, shrub and tall-grass species grow following a patchy distribution. Traditionally, shrubs growing near to newly planted trees are considered heavy competitors, and are consequently removed before planting. However, the vegetation patches usually constitute "fertility islands" or "resource islands" which could promote the tree species development. It has previously been assessed that some native plant species could act as "nurse plants" through their positive impacts on soil abiotic characteristics (i.e., soil nutrient contents), but they also exhibit a positive influence on soil microbiota, especially on symbiotic

R. Duponnois(✉), B. Dreyfus
IRD. Laboratoire Commun de Microbiologie IRD/ISRA/UCAD, Centre de Recherche de Bel Air, BP1386, Dakar, Sénégal
e-mail: robin.duponnois@ird.sn

M. Hafidi and L. Ouahmane
Equipe Ecologie et Environnement associée au CNRST, Faculté des sciences Semlalia, Université Cadi Ayyad, Marrakech, Maroc

J. Thioulouse
Université Lyon 1; CNRS; UMR 5558 Laboratoire de Biométrie et Biologie Evolutive, 43 boulevard du 11 novembre 1918, VilleurbanneF-69622, France

A. Galiana and Y. Prin
CIRAD. UMR 113 CIRAD/INRA/IRD/AGRO-M/UM2, Laboratoire des Symbioses Tropicales et Méditerranéennes (LSTM), Montpellier, France

L. Ouahmane
Centre Régional de Recherche Foresti're, Marrakech, Maroc

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_12, 

microorganisms including rhizobia and mycorrhizal fungi. In this chapter, an attempt is made to assess the beneficial effects of plant nurses on the growth of Mediterranean tree species like *Cupressus* species, and on the bio-functioning of soils. Furthermore, the potential benefits of native plant species in the rehabilitation of degraded areas especially in stressful conditions is reviewed and discussed.

## 12.1 Introduction

Shrub and tall-grass species growth following a patchy distribution are characteristic of the plant communities in semiarid ecosystems and more particularly in Mediterranean areas. The vegetation patches usually form "fertility islands" (Garner and Steinberger 1989) or "resource islands" (Schlesinger et al. 1996) that could be involved in the development of native plant species (Callaway 1995, 1997). It has been previously reported that some native plants species improve their own environment by self-promoting changes in water infiltration, organic matter, etc. (Bochet et al. 1999; Valladares and Pugnaire 1999), and could act as "nurse plants" through their positive impacts on the survival of other native plant species (Carrillo-Garcia et al. 2000). It is now well accepted that the spatial proximity among plants could be beneficial in environments such as Mediterranean-type ecosystems that are characterized by abiotic stress (Boucher et al. 1982; Callaway and Walker 1997; Gomez-Aparicio et al. 2004).

In semiarid Mediterranean ecosystems, desertification processes result from scarce and irregular rainfall, long dry and hot summers, and man-mediated degradative activities like deforestation, overgrazing, non-regulated cultivation techniques, etc. (Francis and Thornes 1990). Desertification generally alters natural plant communities including population structure, succession pattern, and species diversity (Barea and Jeffries 1995). In addition, these disturbances are often accompanied by degradation of physico-chemical and biological soil properties, such as nutrient availability, microbial activity, soil structure, etc., that largely determine soil quality and fertility (Garcia et al. 1997a, b; Albaladejo et al. 1998; Requena et al. 2001). For instance, it is well known that land degradation is usually linked with reductions in the belowground microbial diversity and/or activity (Kennedy and Smith 1995).

Among components of soil microbiota, arbuscular mycorrhizal (AM) fungi are known to be key components of natural systems in semiarid ecosystems and particularly important in counteracting desertification of Mediterranean ecosystems (Carpenter and Allen 1998; Brundrett 1991). The mycorrhizal symbiosis mobilizes and transports nutrients to roots (Smith and Read 1997), reduces water stress (Augé 2001), and improves soil aggregation in eroded soils (Caravaca et al. 2002). AM fungal symbiosis also changes root functions (e.g., root exudation) (Graham et al. 1981; Marshner et al. 1997), modifies carbohydrate metabolism of the host plant (Shachar-Hill et al. 1995), and synergistically interacts with rhizosphere populations (Andrade et al. 1997; 1998). The structure and functionalities of these microbial communities surrounding AM roots differ from those of the rhizosphere

(Duponnois et al. 2005), and this microbial compartment has been named "mycorrhizosphere" (Linderman 1988). It has also been reported that AM fungi affect the diversity of plant communities (van der Hejden et al. 1998; Klironomos et al. 2000; O'Connor et al. 2002) and influence competitive relationships between plants (West 1996; Marler et al. 1999; van der Heijden et al. 2003).

It is well known that the AM fungal activity is generally low in degraded semiarid Mediterranean ecosystems (Maremammani et al. 2003). However, an increased activity of fungal inoculum is needed in both natural and artificial processes of re-vegetation. It has already been shown that AM inoculation of plants is very effective in establishing plants on disturbed soils (Estaun et al. 1997). In order to increase and maintain high populations of infective AM propagules in soil, two main cultural practices can be considered: (1) screening of AM fungal isolates (native or exotic isolates) for their effect on the plant growth under controlled conditions and a cultural substrate inoculation with the most efficient AM strains, and (2) adoption of field practices to manage and improve the inoculum potential of indigenous AM fungi. The inoculation practice is generally used in tree nurseries to help tree establishment in field conditions (Plenchette 2000; Franson and Bethlenfalvay 1989). However, previous studies have reported that, although AM inoculation improved plant growth, it could also strongly modify soil microbial activities (Dabire et al. 2007). In some conditions, to overcome the negative influences of AM inoculation on soil microbiota, it is possible to increase AM soil potential through the management of highly mycotrophic plants (Azcon and Barea 1997). In the following section, focus is placed on highlighting the impacts of some Mediterranean shrubs on microbial functionalities and AM potential and evaluating their significant use in forestry practices to enhance the performance of afforestation program. In this context, most of the reported results will be directed to *Cupressus* spp. and *Lavendula* species. In Morocco, the area of natural and introduced cypress stands, one natural species, *Cupressus atlantica*, and two introduced species, *Cupressus sempervirens* and *C. arizonica*, has declined, and numerous reports indicate a complete absence of natural regeneration. Although attempts have been made to replant these species, the rate of success has been very low. *Lavandula* species are representative plant species in Mediterranean shrublands and belong to the natural succession in semiarid Mediterranean ecosystems (Barea et al. 1992). They have been classified as "obligatory mycorrhizal" (Brundrett 1991) or as "highly dependent on mycorrhiza" (Habte and Manjunath 1991).

## 12.2 Impacts of "Nurse Plant Species" on Soil Microbial Functionalities and AM Fungus Communities

A study has been conducted in the N'Fis valley (Haut Atlas mountains, Morocco) at the Idni station (8°17′02″ W, 31°54′34″N, 1,700 m above sea level) in a natural stand of *C. atlantica* (Ouahmane et al. 2006). This area was covered by a sparse

and degraded vegetation mainly composed of grasses (i.e., *Stipa nitens* Ball.) and various shrub species, such as *Cistus salviifolus* L., *Lavandula dentata* L., *L. stoechas* L., *Thymus pallidus* Coss., *Polygala balansae* Coss., *Globularia alypum* L. and *Thymus satureioides* Coss. Soil samples were collected from the rhizosphere of *L. dentata*, *L. stoechas*, and *T. satureioides* (abundant shrub species and always recorded in the vicinity of *C. atlantica* adult trees) and of *C. atlantica*. Control samples were collected from bare soil sites, away from plant influence. The soil carbon and nitrogen contents were higher under *C. arizonica* than under the other sampling areas due to the leaf litter formation and the fact that, in forest ecosystems, most of the soil nitrogen is in organic form (Kaye and Hart 1997) (Table 12.1), while soluble phosphorus contents were significantly lower under the targeted plant species than in the bare soil (Table 12.1). Therefore, the shrub species did not demonstrate greater effects on soil chemical characteristics and, in contrast, decreased soil P content. The main impacts of these shrub species were recorded on soil functionalities and AM soil potential.

Microbial functional diversity in rhizosphere and in bare soil was assessed by measuring the patterns of in situ catabolic potential (ISCP) of microbial communities (Degens and Harris 1997). Organic compounds comprising of a range of amino acids, carbohydrates, organic acids, and amides were screened for differences in substrate-induced respiration (SIR) between soil treatments. The results show that microbial communities are very different according to the soil origin (Fig. 12.1). In particular, SIR responses to carboxylic acids were significantly higher in the *C. atlantica* and *T. satureioides* rhizosphere soils than in the soils of other origins (Fig. 12.1). Soluble organic acids are involved in plant nutrient acquisition and they particularly act as biological weathering agents of minerals in soils. They could be of high molecular weight (HMW) (i.e., humic substances) or low molecular weight (LMW) produced by plant roots and soil microorganisms (Ochs 1996). In the process of P acquisition from minerals, among the identified carboxylic acids, dicarboxylic (oxalic, tartaric, malic, fumaric, malonic acids) and tricarboxylic acids (citric acid) have been found very effective in P mobilization (Ryan et al. 2001). Since the highest SIR responses with most of these organic acids have been measured under *C. atlantica* and one of the targeted shrub species (*T. satureioides*), it suggests that these plant species and their associated microflora

**Table 12.1** Chemical characteristics of the rhizosphere soils collected from *Lavandula dentata*, *L. stoechas*, *Thymus satureioides*, *Cupressus atlantica* and the bare soil (control) in the *C. atlantica* stand located in the N'Fis valley (Haut Atlas mountains, Morocco)

| | Control | Plant species | | | |
|---|---|---|---|---|---|
| | | *L. dentata* | *L. stoechas* | *T. satureioides* | *C. atlantica* |
| pH | 7.5 a | 7.0 a | 7.5 a | 7.4 a | 7.7 a |
| Total carbon (%) | 1.58 a | 1.60 a | 1.73 a | 1.80 a | 3.15 b |
| Total nitrogen (%) | 0.09 a | 0.10 ab | 0.12 ab | 0.10 a | 0.14 b |
| C/N | 17.2 a | 15.7 a | 14.7 a | 18.8 a | 22.9 a |
| Soluble P (mg $kg^{-1}$) | 19.7 d | 7.9 a | 9.8 ab | 11.8 bc | 13.1 c |

Data in the same line followed by the same letter are not significantly different ($p < 0.05$) according to one-way analysis of variance. Adapted from Ouahmane et al. (2006)

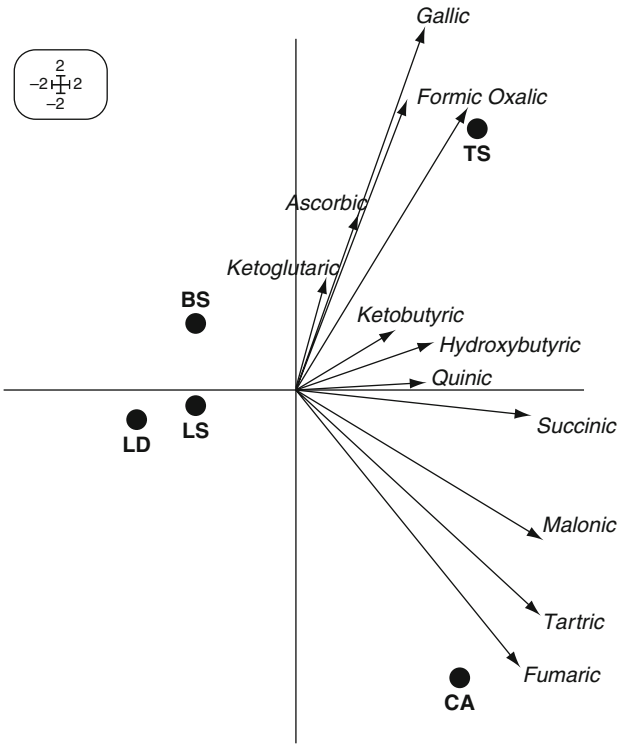



**Fig. 12.1** Between-group analysis (BGA) of the substrate-induced respiration (SIR) responses with respect to the rhizosphere and the bare soils. *LS*, *LD*, *TS*, *CA* and *BS* represent *Lavandula stoechas, L. dentata*, *Thymus satureioides*, *Cupressus atlantica* and bare soils, respectively (Ouahmane et al. 2006)

(AM fungi and mycorrhizosphere microbiota) excreted higher amounts of such organic acids which could exert a selective influence on soil microbial communities through a multiplication of microorganisms that catabolize organic acids.

Beside the positive effects of "nurse" plant species on soil microbial functionalities, they also increase the mycorrhizal soil infectivity. Three *Lavandula* species (*L. multifida*, *L. dentata*, *L. stoechas*) have been studied for their AM dependencies (Ouahmane et al. 2007). After inoculating with the AM fungus, *Glomus intraradices*, the mycorrhizal dependency of *L. stoechas* was 33% whereas those recorded for *L. dentata* and *L. multifida* were 63 and 58%, respectively (Ouahmane et al. 2007). These results confirmed the high mycorrhizal dependency of these plant species (Azcon and Barea 1997). Lavender plants are very mycotrophic and enrich their cultural soil in AM fungal propagules. The *L. multifida* have been found to increase the mycorrhizal soil infectivity (Table 12.2) in six different soils as determined by MPN (most probable number) method (Ouahmane et al. 2007). In addition, this positive contribution is linked with the total soil P contents since the highest increases were recorded in soils with the lowest soil total P contents. Hence,

**Table 12.2** Responses of soil mycorrhizal infectivity to *Lavandula multifida* plantation in six sandy soils after 5 months culturing

| Soils | pH ($H_2O$) | Total C (g C $kg^{-1}$) | Organic matter (%) | Total N (g $kg^{-1}$) | Total P (g $kg^{-1}$) | MSI[a] |
|---|---|---|---|---|---|---|
| 1 | 5.8 | 3.99 | 0.7 | 0.26 | 68.5 | 533.3 |
| 2 | 4.9 | 2.85 | 0.5 | 0.19 | 78.7 | 1121.9 |
| 3 | 5.4 | 6.62 | 1.1 | 0.46 | 119.2 | 144.9 |
| 4 | 5.7 | 3.17 | 0.5 | 0.12 | 48.4 | 443.0 |
| 5 | 6.1 | 7.29 | 1.3 | 0.60 | 200.7 | 87.2 |
| 6 | 5.7 | 3.09 | 0.5 | 0.47 | 90.0 | 131.3 |

[a] (MSI of soil planted with *L. multifida*) – (MSI of soil unplanted with *L. multifida*) after 5 months culture in each soil. Adapted from Ouahmane et al. (2007)

as lavender plants have a patchy distribution in *Cupressus* stands in Haut Atlas Mountains in Morocco, this *Lavandula* species could act as a "nurse plant" for natural regeneration of *Cupressus* young seedlings by (1) enhancing soil microbial activities (in particular those involved in P mobilization), and (2) enhancing the mycorrhizal soil infectivity.

## 12.3 Response of *Cupressus* sp. Growth to the "Nurse" Plant Effects

Recognizing the benefits recorded for lavender plants on soil diversity and functioning, several studies have been undertaken to determine their potential impacts on the early growth of *Cupressus* sp. To test the potential benefits from the association between *Cupressus* sp. and "nurse plant" on the growth of each plant partner, Ouahmane et al. (2007) conducted an experiment in controlled conditions combining the following treatments: *C. arizonica*+*L. multifida*, *L. multifida* alone and *C. arizonica* alone in 20-l pots filled with non-disinfected sandy soil. The results show that the height of plant species was increased in the dual cultivation treatment (Fig. 12.2). In addition, after 4 months culturing, the growth of *L. multifida* and *C. arizonica* was generally higher when they were cultured together than those recorded for singly cultured treatment (Fig. 12.2). A similar effect was observed for the mycorrhizal colonization of the plants (Table 12.3). Other studies have also been conducted to evaluate the capacity of the "fertility islands" created by the nurse plants to facilitate the early growth of *Cupressus* spp.. Soil samples were collected under *L. dentata*, *L. stoechas* and *T. satureioides* rhizosphere and from an area free of cover plant (control). Soils were then packed in 1-$dm^3$ pots and one *C. arizonica* seedling was planted per pot. After 6 months of culturing, the height, stem diameter, N and P foliar contents, AM colonization, and shoot and root biomass were significantly higher in the soils originating from the nurse plants than in the bare soil (Table 12.4). These results suggest that *C. atlantica* has to be mycorrhizal in order to reach its optimal growth and that this Mediterranean tree species is "highly dependent on mycorrhizas" (Habte and Manjunath 1991). These

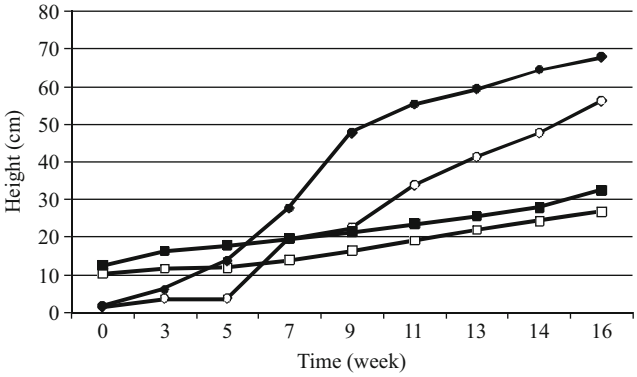



**Fig. 12.2** Time course changes in plant height (in cm) of *Cupressus arizonica* and *Lavandula multifida* seedlings in the mono and dual cultivation treatments. Height growth of *C. arizonica* seedlings: *empty square C. arizonica* alone; *filled square C. arizonica*+*L. multifida* – height growth of *L. multifida* seedlings; *empty circle L. multifida* alone; *filled circle L. multifida*+ *C. arizonica* (adapted from Ouahmane et al. 2007)

**Table 12.3** Growth and mycorrhizal colonization of *Cupressus arizonica* and *Lavandula multifida* after 4 months culturing in a non-disinfected sandy soil

| Plant species | Treatment | Shoot biomass (mg dry weight/plant) | Root biomass (mg dry weight/plant) | Mycorrhizal colonization (%) |
|---|---|---|---|---|
| *C. arizonica* | Alone | 3,393 a | 735 a | 4 a |
| | + *L. Multifida* | 3,456 b | 1,499 b | 92 b |
| *L. multifida* | Alone | 1,020 a | 1,102 a | 10 a |
| | + *C. arizonica* | 1,032 a | 2,120 b | 50 b |

For each plant species, data in the same column followed by the same letter are not significantly different ($p<0.05$) according to one-way analysis of variance. Adapted from Ouahmane et al. (2007)

**Table 12.4** Growth and mycorrhizal colonization of *Cupressus atlantica* seedlings planted in the rhizosphere soils collected from *Lavandula dentata*, *L. stoechas*, *Thymus satureioides*, *C. atlantica* and the bare soil (control) after 6 months culturing in greenhouse conditions

| | Control | Soil origin | | | |
|---|---|---|---|---|---|
| | | *L. dentata* | *L. stoechas* | *T. satureioides* | *C. atlantica* |
| Height (cm) | 14.2 a | 18.6 b | 21.0 cd | 23.0 d | 19.4 bc |
| Stem diameter (mm) | 2.02 a | 2.72 bc | 2.72 bc | 2.94 c | 2.54 b |
| Shoot biomass (mg dry weight/plant) | 330 a | 634 bc | 738 c | 666 bc | 486 ab |
| Root biomass (mg dry weight/plant) | 76 a | 176 c | 157 bc | 115 abc | 104 ab |
| N (mg/plant) | 0.79 a | 1.56 b | 1.82 c | 2.03 d | 1.48 b |
| P (mg/ plant) | 0.033 a | 0.107 c | 0.115 c | 0.147 d | 0.090 b |
| AM colonization (%) | 35 b | 48 b | 50 b | 75 c | 54 b |

Data in the same line followed by the same letter are not significantly different ($p<0.05$) according to one-way analysis of variance. Adapted from Ouahmane et al. (2007)

studies confirmed that plant nurses mainly act on *Cupressus* seedling growth through their impact on AM fungus communities. AM fungi are important agents in promoting plant co-existence (Allen and Allen 1990). Moreover, the abundance and diversity of AM fungi are known to have a strong effect on the direction of succession (Medve 1984). This AM fungal effect is mainly important in early successional ecosystems where plant and soil have been severely disturbed and where AM fungi are absent or are in low abundance and patchily distributed (Hart et al. 2003). Hence, the use of plant nurses as promoting agent of mycorrhizal soil infectivity could be of great interest in restoring a self-sustaining vegetation cover in order to act against desertification.

## 12.4 Conclusion

The use of nurse shrubs facilitates seedling establishment in many different ecological settings in Mediterranean mountains (Gomez-Aparicio et al. 2004) suggesting that the removal of shrubs is not an appropriate practice for reforestation in Mediterranean mountains. Therefore, a new paradigm for the science of restoration of Mediterranean forests emerges from all these ecological studies (Maestre et al. 2001, 2002; Gomez-Aparicio et al. 2004). It is based on the natural spatial patterns of regeneration of woody vegetation with shrubs as micro-sites for recruitment. Furthermore, since the benefits of positive interactions between plant species is widely recognized (Callaway et al. 2002), this innovative forestry practice might be more relevant under the predicted rise in temperatures, dryness, and rainfall variability for the Mediterranean region under global warming (IPCC 2001). Since the primary limitation of plant fitness is generally represented by the severity of the physical environment, the enhancement of environmental conditions by nurse shrubs can be of crucial importance in many stressful environments.

## References

Albaladejo J, Martinez-Mena M, Roldan A, Castillo V (1998) Soil degradation and desertification induced by vegetation removal in a semiarid environment. Soil Use Manage 14:1–5

Allen EB, Allen MF (1990) The mediation of competition by mycorrhizae in successional and patchy environments. In: Grace JB, Tilman D (eds) Perspectives in plant competition. Academic Press, New York, pp 367–389

Andrade G, Mihara KL, Linderman RG, Bethlenfalvay GJ (1997) Bacteria from rhizosphere and hyphosphere soils of different arbuscular mycorrhizal fungi. Plant Soil 192:71–79

Andrade G, Mihara KL, Linderman RG, Bethlenfalvay GJ (1998) Soil aggregation status and rhizobacteria in the mycorrhizosphere. Plant Soil 202:89–96

Augé RM (2001) Water relations, drought and vesicular arbuscular mycorrhizal symbiosis. Mycorrhiza 11:3–42

Azcon R, Barea JM (1997) Mycorrhizal dependency of a representative plant species in Mediterranean shrublands (*Lavandula spica* L.) as a key factor to its use for revegetation strategies in desertification-threatened areas. Appl Soil Ecol 7:83–92

Barea JM, Jeffries P (1995) Arbuscular mycorrhizas in sustainable soil plant systems. In: Hock B, Varma A (eds) Mycorrhiza structure, function, molecular biology and biotechnology. Springer, Heidelberg, pp 521–559

Barea JM, Azcon R, Azcon-Aguilar C (1992) The use of $^{15}N$ to assess the role of VA mycorrhiza in plant N nutrition and its application to evaluate the role of mycorrhiza in restoring Mediterranean ecosystems. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ (eds) Mycorrhizas in Ecosystems. Structure and Function. CAB International, Wallingford, UK, pp 190–197

Bochet E, Rubio JL, Poesen J (1999) Modified topsoil islands within patchy Mediterranean vegetation in SE Spain. Catena 38:23–44

Boucher DH, James S, Keeler KH (1982) The ecology of mutualism. Ann Rev Ecol Syst 13:315–347

Brundrett MC (1991) Mycorrhizas in natural ecosystems. In: Macfayden A, Begon M, Fitter AH (eds) Advances in ecological research, vol 21. Academic Press, London, pp 171–313

Callaway RM (1995) Positive interactions among plants. Bot Rev 61:306–349

Callaway RM (1997) Positive interactions in plant communities and the individualistic-continuum concept. Oecologia 112:143–149

Callaway RM, Walker LR (1997) Competition and facilitation: a synthetic approach to interactions in plant communities. Ecology 78:1958–1965

Callaway RM, Brooker RW, Choler P, Kikvidze Z, Lortie CJ, Michalet R, Paolini L, Pugnaire FI, Newingham B, Aschehoug ET, Armas C, Kikidze D, Cook BJ (2002) Positive interactions among alpine plants increase with stress. Nature 417:844–848

Caravaca F, Barea JM, Figueroa D, Roldan A (2002) Assessing the effectiveness of mycorrhizal inoculation and soil compost addition for reafforestation with *Olea europaea* subsp. *sylvestris* through changes in soil biological and physical parameters. Appl Soil Ecol 20:107–118

Carpenter AT, Allen MF (1998) Responses of *Hedysarum boreale* Nutt. to mycorrhizas and *Rhizobium*: plant and soil nutrient changes in a disturbed shrub-steppe. New Phytol 109:125–132

Carrillo-Garcia A, Bashan Y, Bethlenfalvay GJ (2000) Resource island soils and the survival of the giant cactus, cardon, of Baja California Sur. Plant Soil 218:207–214

Dabire AP, Hien V, Kisa M, Bilgo A, Sangare KS, Plenchette C, Galiana A, Prin Y, Duponnois R (2007) Responses of soil microbial catabolic diversity to arbuscular mycorrhizal inoculation and soil disinfection. Mycorrhiza 17:537–545

Degens BP, Harris JA (1997) Development of a physiological approach to measuring the catabolic diversity of soil microbial communities. Soil Biol Biochem 29:1309–1320

Duponnois R, Colombet A, Hien V, Thioulouse J (2005) The mycorrhizal fungus *Glomus intraradices* and rock phosphate amendment influence plant growth and microbial activity in the rhizosphere of *Acacia holosericea*. Soil Biol Biochem 37:1460–1468

Estaun V, Save R, Biel C (1997) AM inoculation as a biological tool to improve plant re-vegetation of a disturbed soil with *Rosmarinus officinalis* under semi-arid conditions. Appl Soil Ecol 6:223–229

Francis DF, Thornes JB (1990) Matorral: erosion and reclamation. In: Albaladejo J, Stocking MA, Diaz E (eds) Soil degradation and rehabilitation in Mediterranean environmental conditions. CSIC, Murcia, Spain, pp 87–115

Franson RI, Bethlenfalvay GJ (1989) Infection unit method of vesicular arbuscular mycorrhizal propagule determination. Soil Sci Soc Am J 53:754–756

Garcia C, Hernandez T, Costa F (1997a) Potential use of dehydrogenase activity as an index of microbial activity in degraded soils. Commun Soil Sci Plan Anal 28:123–124

Garcia C, Hernandez T, Roldan A, Albaladejo L (1997b) Biological and biochemical quality of a semiarid soil after induced revegetation. J Environ Qual 26:1116–1122

Garner W, Steinberger Y (1989) A proposed mechanism for the formation of fertile islands in the desert ecosystem. J Arid Environ 16:257–262

Gomez-Aparicio L, Zamora R, Gomez JM, Hodar JA, Castro J, Baraza E (2004) Applying plant facilitation to forest restoration: a meta-analysis of the use of shrubs as nurse plants. Ecol Appl 14:1128–1138

Graham JH, Leonard RT, Menge JA (1981) Membrane-mediated decrease in root exudation responsible for phosphorus inhibition of vesicular-arbuscular mycorrhiza formation. Plant Physiol 68:548–552

Habte M, Manjunath A (1991) Categories of vesicular–arbuscular mycorrhizal dependency of host species. Mycorrhiza 1:3–12

Hart MM, Reader RJ, Klironomos JN (2003) Plant coexistence mediated by arbuscular mycorrhizal fungi. Trends Ecol Evol 18:418–423

IPCC [Inter-Government Panel on Climate Change] (2001) In: Watson RT, The Core Writing Team (eds) Climate change 2001: third assessment report of the Intergovernmental Panel on Climate Change(WG I & II). Cambridge University Press, Cambridge, UK

Kaye JP, Hart SC (1997) Competition for nitrogen between plants and soil microorganisms. Tree 12:139–143

Kennedy AC, Smith KL (1995) Soil microbial diversity and the sustainability of agriculture soils. Plant Soil 170:75–86

Klironomos JN, McCune J, Hart M, Neville J (2000) The influence of arbuscular mycorrhizae on the relationship between plant diversity and productivity. Ecol Lett 3:137–141

Linderman RG (1988) Mycorrhizal interactions with the rhizosphere microflora: the mycorrhizosphere effect. Phytopathology 78:366–371

Maestre FT, Bautista S, Cortina J, Bellot J (2001) Potential for using facilitation by grasses to establish shrubs on a semiarid degraded steppe. Ecol Appl 11:1641–1655

Maestre FT, Bautista S, Cortina J, Diaz G, Honrubia M, Vallejo R (2002) Microsite and mycorrhizal inoculums effects on the establishment of *Quercus coccifera* in a semiarid degraded steppe. Ecol Eng 19:289–295

Maremammani A, Bedini S, Matosevic I, Tomei PE, Giovannetti M (2003) Type of mycorrhizal associations in two coastal nature reserves of Mediterranean basin. Mycorrhiza 13:33–40

Marler MJ, Zabinski CA, Callaway RM (1999) Mycorrhizae indirectly enhance competitive effects of an invasive forb on a native bunchgrass. Ecology 80:1180–1186

Marshner P, Crowley DE, Higashi M (1997) Root exudation and physiological status of a root-colonizing fluorescent pseudomonad in mycorrhizal and non-mycorrhizal pepper (*Capsicum annuum* L.). Plant Soil 189:11–20

Medve RJ (1984) The mycorrhizae of pioneer species in disturbed ecosystems in western Pennsylvania. Am J Bot 71:787–794

O'Connor PJ, Smith SE, Smith FA (2002) Arbuscular mycorrhizas influence plant diversity and community structure in a semiarid herbland. New Phytol 154:209–218

Ochs M (1996) Influence of humidified and non-humidified natural organic compounds on mineral dissolution. Chem Geol 132:119–124

Ouahmane L, Hafidi M, Kisa M, Boumezouch A, Thioulouse J, Plenchette C, Duponnois R (2006) Some Mediterranean plant species (*Lavandula* spp. and *Thymus satureioides*) act as "plant nurses" for the early growth of *Cupressus atlantica*. Plant Ecol 185:123–134

Ouahmane L, Hafidi M, Kisa M, Boumezouch A, Thioulouse J, Duponnois R (2007) *Lavandula* species as a source of arbuscular mycorrhizal propagules facilitating the early development of *Cupressus arizonica*. Appl Soil Ecol 34:190–199

Plenchette C (2000) Receptiveness of some tropical soils from banana fields in Martinique to the arbuscular fungus *Glomus intraradices*. Appl Soil Ecol 15:253–260

Requena N, Perez-Solis E, Azcon-Aguilar C, Jeffries P, Barea JM (2001) Management of indigenous plant–microbe symbioses aids restoration of desertified ecosystems. Appl Environ Microbiol 67:495–498

Ryan PR, Delhaise E, Jones DL (2001) Function and mechanism of organic anion exudation from plant roots. Annu Rev Plant Physiol 52:527–560

Schlesinger WH, Raikes JA, Hartley AE, Cross AF (1996) On the spatial pattern of soil nutrients in desert ecosystems. Ecology 7:364–374

Shachar-Hill Y, Pfeffer PE, Douds D, Osman SF, Doner LW, Ratcliffe RG (1995) Partitioning of intermediary carbon metabolism in vesicular arbuscular mycorrhizal leeks. Plant Physiol 108:7–15

Smith SE, Read DJ (1997) Mycorrhizal symbiosis, 2nd edn. Academic Press, London

Valladares F, Pugnaire FI (1999) Tradeoffs between irradiance capture and avoidance in semiarid environments assessed with a crown architecture model. Ann Bot 83:459–469

van der Heijden MGA, Wiemken A, Sanders IR (2003) Different arbuscular mycorrhizal fungi alter coexistence and resource distribution between co-occuring plant. New Phytol 157:569–578

van der Hejden MGA, Klironomos JN, Ursic M, Moutoglis P, Streitwolf-Engel R, Boller T, Wiemken A, Sanders IR (1998) Mycorrhizal fungal diversity determines plant biodiversity ecosystem variability and productivity. Nature 396:69–72

West HM (1996) Influence of arbuscular mycorrhizal infection on competition between *Holcus lanatus* and *Dactylis glomerata*. J Ecol 84:429–438

# Chapter 13
# Pea Cultivation in Saline Soils: Influence of Nitrogen Nutrition

**Etelvina Figueira**

**Abstract** Salinity is one of the most important abiotic factors limiting plant growth and productivity. This is especially acute in arid and semiarid regions of the world. In this chapter, attention is paid to evaluate the influence of different forms of nitrogen on the tolerance and the yield of pea (*Pisum sativum* L.) plants cultivated under different saline conditions. Results show that the nitrogen form influenced pea growth, yield and ionic content, both under the presence and absence of salinity. Under nonsaline conditions, the highest growth and yields were obtained by $NO_3NH_4$, whereas under salinity, $NO_3^-$ displayed the highest values. The inoculation with a salt-tolerant strain allowed the nodule formation process to remain unaffected by salt. Nevertheless, these plants had lower yields than $NO_3^-$-fed plants, although similar values to $NH_4^+$-supplemented plants, indicating that, by relying solely on biological $N_2$ fixation or $NH_4^+$ fertilization, pea yields under saline conditions will be compromised.

## 13.1 Introduction

Salinity stress is of great importance in arid and semiarid areas of the world, since most crops are sensitive to salinity, evidencing sharp yield decreases in the presence of moderate salt concentrations (Subbarao and Johansen 2004). More than 6% of the total land area of the world is salt affected. Most of these salt-affected areas have arisen from natural causes, by the accumulation of salts over long periods of time in arid and semiarid zones (Rengasamy 2002). Apart from natural salinity, a significant proportion of recently cultivated agricultural land has become saline owing to land clearing or irrigation, both of which cause water tables to rise and

E. Figueira
Centre for Cell Biology, Departamento de Biologia, Universidade e Aveiro, Campus de Santiago, 3810-193, Aveiro, Portugal
e-mail: efigueira@ua.pt

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_13, 

concentrate the salts in the root zone. Of the 1,500 million ha of land farmed by dry land agriculture, 32 million ha (2%) are affected by secondary salinity of varying degrees. Of the current 230 million ha of irrigated land, 45 million ha (20%) are salt affected (Munns and Tester 2008).

Tolerance to salinity may have different meanings according to the objectives by which it is considered. If tolerance is viewed from an ecological perspective, the most relevant is that species colonizing a habitat can complete their life cycle and create conditions for their permanence. From an agronomic point of view, the most important factor is productivity. If salinity causes toxic effects and osmotic and nutritional imbalances, a large part of photosynthates will be used as osmotic solutes or in ion exclusion, reducing growth and productivity and putting their cultivation in salt-affected agricultural soils at risk.

The focus of this chapter is to evaluate the influence that different nitrogen sources have on salt tolerance of pea (*Pisum sativum)*, during its growth and productivity. To achieve this goal, growth, productivity, seed protein content, and ion contents were determined and compared between *Pisum sativum* plants grown for 75 days under the presence (90 mM NaCl) or absence (0 mM NaCl) of salinity and four types of nitrogen nutrition ($NO_3^-$, $NH_4^+$, $NH_4NO_3$, or symbiotically fixed $N_2$ by an halotolerant isolate of *Rhizobium leguminosarum*).

## 13.2 Nitrogen Nutrition Under Salt Stress

### *13.2.1 Cell Ionic Status*

As the boundary between the outside and inside, the plasma membrane is one of the most important structures that cells have to adjust their composition. Plasmalemma proteins control which solutes will be accumulated and which will be excluded, creating gradients through the membrane. This process is important in many aspects of plant growth and development, including mineral nutrition (Ward 1997). Some ions and solutes, such as $H^+$, $Na^+$, and $Ca^{2+}$, are excluded from the cytosol of cells, while others, such as $K^+$, $NH_4^+$, $NO_3^-$, $Cl^-$, $SO_4^{2-}$, $HPO_4^-$, sugars, organic acids, and amino acids are accumulated. One of the fundamental characteristics of membrane transport in plants is that protons are actively excluded from the cytosol to the cell outside. This process creates a pH gradient, usually between 1.5 and 2 units (Marschner 1995; Ward 1997), and a voltage gradient, usually between 100 and 200 mV (Marschner 1995; Ward 1997) in the plasmalemma. Thus, in electrophysiological terms the absorption of cations can occur passively, while anion absorption uses up energy.

As the selectivity of ion channels and transporters is based on the ionic charge it leads to competition between ions of the same electrical charge. The competition between $Cl^-$ and $NO_3^-$, between $K^+$ and $Na^+$, or between $Ca^{2+}$ and $Na^+$ (Marschner 1995; Tyerman 1992; Fox and Guerinot 1998) results in severe nutritional imbalances

to plants when the intracellular ratios of these ions are very different from those in the soil solution. In the presence of saline conditions, $Na^+$ and $Cl^-$ are often the most abundant ions. Therefore, it is not surprising that these soils are characterized by low activities of nutrient ions and high ratios of $Na^+/Ca^{2+}$, $Na^+/K^+$, and $Cl^-/NO_3^-$. In these circumstances, nutritional disorders may develop and plant growth be reduced.

### *13.2.2 Nitrogen as a Plant Macronutrient*

Nitrogen is the fourth most abundant element in organisms, constituting about 1.5–5% of the plant dry weight (Below 1995). Due to the high requirements of this element, about 70% of the ions absorbed by plants are nitrogen-containing compounds (Marschner 1995). Among the mineral nutrients, nitrogen often limits the growth and productivity of many crops (Marschner 1995). Nitrogen is incorporated in numerous organic compounds that include proteins, nucleic acids, chlorophylls, and growth regulators, all playing crucial roles in plant growth and development.

#### 13.2.2.1 Inorganic Nitrogen Nutrition

Nitrogen is available to plants in the soil in a variety of forms including ammonium ($NH_3$ and $NH_4^+$), nitrate, amino acids, soluble proteins, and other nitrogen-containing compounds, but plants absorb nitrogen primarily in an inorganic form as $NO_3^-$ or ammonium (Williams and Miller 2001). Under natural conditions, the nitrogen present in the soil originates from biological fixation, or animal and plant decomposition. The vast majority (over 90%) of nitrogen present in the soil is contained in organic matter, which is relatively stable and is not directly available to plants (Below 1995). However, part of the organic nitrogen may become available through mineralization by microorganisms in the soil (Marschner 1995). The form of nitrogen provided, cationic or anionic, affects their availability to plants due to differences in the mobility of the two ions in the soil solution. The cation $NH_4^+$ binds to the negatively charged particles in the soil and becomes relatively immobile. In contrast, the $NO_3^-$ anion is repelled, which increases its bioavailability for root uptake. However, this form of nitrogen is easily lost by leaching and denitrification (Bray 1983; Below 1995).

Plants acquire the vast majority of their nitrogen through the root system. This process involves the movement of inorganic nitrogen, $NO_3^-$ or $NH_4^+$, through the plasma membrane. The high-affinity transport systems in roots are able to scavenge $NH_4^+$ and $NO_3^-$ from the soil at concentrations between 1 μM and 1 mM, whereas the activity of low-affinity transport systems becomes evident when these ions are above 5 mM (Jackson et al. 2008). All $NO_3^-$ transport systems need energy to overcome an unfavorable electrochemical gradient between the outside solution and the symplast of root cells. Ammonium transport can be passive if uptake occurs

through low-affinity systems or active through high-affinity systems (Williams and Miller 2001; Grossman and Takahasi 2001). Whereas $NH_4^+$ can be directly used in the synthesis of amino acids, $NO_3^-$ must first be reduced to $NH_4^+$. This reduction is a process that uses up energy and may be the reason why $NH_4^+$ inhibits $NO_3^-$ uptake (Gazzarrini et al. 1999; Gessler et al. 1998; Siddiqi et al. 2002).

#### 13.2.2.2 $N_2$ Fixation and Plant Nutrition

Eukaryotic cells do not possess the biochemical "machinery" to react with diatomic nitrogen. Only a few prokaryotes, including species of the genera *Azotobacter*, *Azotococcus*, *Clostridium*, *Klebesiella*, *Bacillus*, *Azopirillum*, *Nostoc*, *Anabaena*, *Rhizobium*, *Bradyrhizobium*, and *Frankia*, have enzymes that reduce $N_2$ (Bray 1983; Marschner 1995). Some of them establish endosymbiosis, such as the association between plants of the family Fabaceae and bacteria of the genera *Rhizobium*, *Bradyrhizobium, Azorhizobium*, *Mesorhizobium*, and *Ensifer*. Free-living rhizobia do not fix $N_2$, this function is accomplished only in association with the host plants. Bacteria colonize legume roots at the beginning of the vegetative growth and leads to the formation of nodules. In nodules, bacteria undergo structural (such as loss of cell wall) and metabolic (as the expression of nitrogenases) changes that enable them to fix the $N_2$, turning into bacteroids (Brewing et al. 1993). Individually or in small groups, each bacteroid is surrounded by a membrane, forming a structure called the symbiosome which is the site of $N_2$ fixation. The $NH_4^+$ resulting from the reduction of $NO_3^-$, from the symbiotic fixation of $N_2$, or absorbed directly, is assimilated into glutamate and glutamine. Ammonium assimilation usually occurs at root level, whereas nitrate assimilation can occur both in roots and leaves. Glutamate and glutamine are the precursors of other amino acids, generating all the amino acids required for protein synthesis. They are also the raw material responsible for generating a series of other compounds, such as chlorophylls, plant growth regulators, and nucleic acids (Below 1995).

#### 13.2.2.3 $N_2$ Fixation in Plants Under Salinity Stress

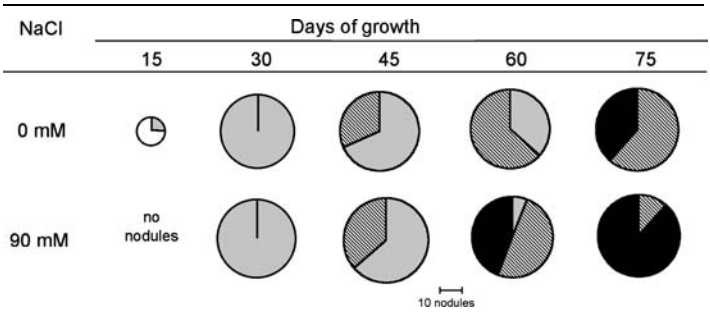
Many studies have reported the inhibitory effects of salinity in the fixation of $N_2$ (Lauter and Munns 1986; Cordovilla et al. 1994, 1996, 1999a, 1999b; Tu 1981; Salehi et al. 2008; Tawfik 2008). In saline environments, the nodulation reduction may be due to the decrease in rhizobial populations, because of its sensitivity to salt, affecting the survival and distribution of rhizobia in the soil and rhizosphere of plants (Singleton et al. 1982; Craig et al. 1991). For example, Pereira et al. (2008) found that 50 mM NaCl strongly inhibited the growth of three of the five *Rhizobium* populations compatible with pea plants, when grown in YEM medium supplemented with salt. Elsheikh and Wood (1995) selected strains of *Rhizobium* and *Bradyrhizobium* for their salt tolerance and reported that one of them only grew in the absence of NaCl, and the others showed different tolerances but were not able

to grow above 340 mM. However, Zharan et al. (1994) isolated strains of *Rhizobium* with different salt tolerances; some grew at 1.7 M NaCl. Pereira et al. (2008) also recorded isolates that grew at the same level of halotolerance (1.6 M NaCl).

In the nodulation process, salt inhibition is regarded more as an effect on the establishment of nodules than on the reduction of *Rhizobium* population. There are several studies showing that the most sensitive process to salinity is the establishment of the nodules (Lauter and Munns 1986; Cordovilla et al. 1999a). For example, Singleton and Bholool (1983) found a strong reduction (50%) in the number of nodules in the roots of soybean [*Glycine max* (L.) Merr] growing under 26.6 mM NaCl. In a similar study, Elsheikh and Wood (1990) observed that nodulation in chickpea (*Cicer arietinum* L.) was completely inhibited by 61.6 mM NaCl, even when inoculated with a strain tolerant to salt, while Abd-Alla et al. (1998) observed that salinity (30 and 60 mM NaCl) inhibited the number of nodules and the biomass of four cultivars of *G. max* inoculated with a salt-tolerant strain of *Bradyrhizobium*. The reduction in the number of nodules has been associated with the effect of salinity on cell expansion inhibition, and to root hairs number and curling (Sprent and Zahran 1988; Tu 1981). In *Pisum sativum* plants growing in the presence (90 mM) and absence of NaCl, it was observed that salinity delayed nodule formation. In contrast to control plants, 15-day-old salt-stressed plants had no nodules. However, salinity had no effect on nodule number at later stages, although nodule senescence started earlier (Table 13.1). These results show that the susceptibility of the nodulation process to salinity is not always verified and that the selection of a halotolerant *Rhizobium* strain with good $N_2$-fixing ability is important to legume nodulation in saline conditions.

The effects of salinity on the symbiosis between rhizobia and host may also be on the $N_2$-fixation process directly. Several hypotheses aim to explain the negative

**Table 13.1** Number and color of root nodules produced on halotolerant *Rhizobium*-inoculated *Pisum sativum* plants grown in soils treated with (90 mM NaCl) or without (0 mM NaCl) salts




Circle diameter reflects the number of nodules present in roots: less than 30 (small circles); and higher than 30 (big circles); nodule absence is also registered. The relative abundance of different nodule colors is also presented: *white* white nodules, *gray* pink nodules, *stripes* green nodules, *black* brown nodules

effects of salt on $N_2$ fixation: a decline in the supply of photosynthates to the nodules (Bekki et al. 1987; Georgiev and Atkins 1993); lower availability of reduced compounds to bacteroids (Delgado et al. 1993, 1994); changes in the oxygen diffusion barrier (Delgado et al. 2006; Serraj et al. 1994; Wahab and Zahran 1981); or inhibition of several enzymes important to $N_2$ assimilation. Serraj et al. (1994) showed that exposure of *G. max* plants to 0.1 M NaCl resulted in a rapid decrease in nitrogenase activity. Abd-Alla et al. (1998) found that 30 mM was enough to cause a sharp decline in the activity of nitrogenase in three of the four cultivars of *G. max* used in the study. Bourgeais-Chaillou et al. (1992) described reductions in the activity of glutamine synthetase (GS) and glutamate synthase (GOGAT) in the root nodules of plants under salt stress. Cordovilla et al. (1996, 1999a, 1999c) reached the same conclusion and showed that GOGAT was more inhibited than GS; thus concluding that GOGAT limited the assimilation of ammonium in the nodules of *Vicia faba* L. plants under salt stress. In other works, the same authors (Cordovilla et al. 1994, 1999c) concluded that $N_2$ fixation was more sensitive to salinity than the assimilation of ammonium.

The influence of salt stress in $N_2$ fixation is higher than on the absorption of inorganic nitrogen due to the higher energy costs to fix $N_2$, leaving less energy available for growth and for salinity coping. The accumulation of intracellular organic solutes in the nodules, such as proline and glycine betaine, has been correlated with salt tolerance (Robson and Bottomley 1991; Botsford and Lewis 1990). A more efficient way to acquire salt tolerance will be to compartmentalize subcellularly inorganic compounds in the vacuoles, thus avoiding ion toxicity (Zahran 1991). The improvement of ion partitioning in the nodule cells could promote sustainable legume growth by means of $N_2$ fixation in the presence of salinity. According to Zahran (1991), this seems to be a good criterion for selecting legumes for cultivation under salt conditions. Thus, although the importance that salt-tolerant plant species and cultivars may have on the establishment of an effective symbiosis under salt stress, the need for salt-tolerant rhizobia cannot be ignored. Over time, only the rhizobia tolerant to salt can survive and persist in a saline soil. It is imperative that soils contain salt-tolerant populations of rhizobia compatible with the legume crops to be cultivated, in order to establish an effective symbiosis and to promote good growth and yield in saline soils.

## 13.3 Plant Growth Under Salt Stress

### *13.3.1 Growth in Saline Conditions*

Plants absorb most of the mineral nutrients from the soil. That is why they are greatly affected by soil characteristics. Most crops are glycophytes and evolved under conditions of low salinity in the soil. Therefore, they possess mechanisms for nutrient absorption in nonsaline soils and are often unable to grow in this type of environment without evidencing damage. Plant growth and productivity involve the

integrated effect of diverse environmental factors and metabolic processes that act with different intensities throughout the plant life cycle. The first problem that plants have to overcome when growing in an adverse environment is to establish. Establishment is only possible if plants build up a good photosynthetic capacity to produce enough biomass to support their vegetative growth and reproduction. Maintenance of the photosynthetic capacity can become a problem when plants grow under stressful conditions, such as those propitiated by salinity. Salinity is characterized by an excess of ions, frequently $Na^+$ and $Cl^-$, in whose presence most plants grow poorly (Figueira and Caldeira 2005; Dudley1994; Marschner 1995).

### *13.3.2 Nitrogen Nutrition and Growth Under Salinity*

Under saline conditions, the three main constraints imposed by salinity on growth are (1) osmotic stress that originates with the increase of the soil osmotic potential, (2) ion toxicity which is associated to excessive uptake of $Cl^-$ and $Na^+$; and (3) nutritional imbalances due to the decrease of absorption and/or transport to shoots as well as to an altered distribution of mineral nutrients (Marschner 1995; Munns and Tester 2008). The decrease in the turgor pressure caused by the defective osmotic regulation of the shoot is regarded as the main factor causing the inhibition of leaf elongation, leading to a reduction in leaf area and to the partial closure of stomata. The prolonged stomata closure causes considerable reduction in the $CO_2$ fixation per leaf area and per plant (Marschner 1995). The higher respiration rates and the shorter leaf duration also contribute to reduction in photosynthetic rates (Yeo 1998; Munns and Tester 2008). In fact, many studies report reductions of the photosynthetic capacity in plants subjected to salt stress (Below 1995; Marschner 1995; Yeo 1998). The decrease of photosynthates and the additional energy costs with the exclusion, partitioning, excretion, osmotic adjustment, and repair of cellular damage necessary to cope with salinity, contribute to the decline of plant growth under saline conditions (Marschner 1995; Munns and Tester 2008). In pea plants grown under different salt concentrations (0 and 90 mM NaCl) and nitrogen nutrition ($NO_3^-$, $NH_4^+$, $NO_3NH_4$, *Rhizobium* inoculation), it was observed that both salinity and N form affected plant growth (Fig. 13.1). Root growth was reduced by salinity and irrespective to the duration of salt exposure the N form also affected root growth. Under saline conditions, $N_2$-fixing plants had a higher root biomass than plants fed with mineral N (Fig. 13.1a, b). In the absence of salinity, shoot biomass was lower in $N_2$-fixing than in mineral N-fed plants, and $NO_3NH_4$ produced the highest shoot biomass (Fig. 13.1c). The enhanced biomass of shoots may certainly be related to the effect that a combined nitrogen nutrition of anions and cations has on the maintenance of cell pH, through the rates of $H^+$ production (assimilation of $NH_4^+$) and consumption (assimilation of $NO_3^-$), with lower energy costs in the maintenance of the citosolic pH homeostasis, usually between 7.3 and 7.6 (Marschner 1995). Under salt stress, however, the response pattern changed. Although biomass was reduced in all N treatments, $N_2$-fixing plants were less and

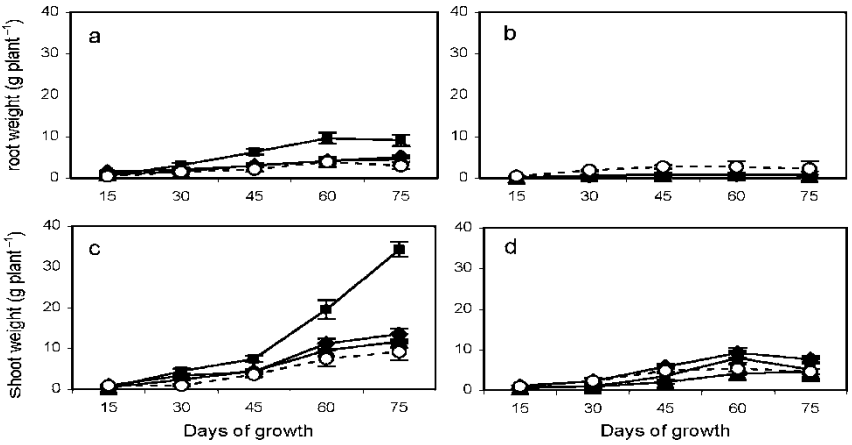



**Fig. 13.1** Root and shoot weight of *Pisum sativum* grown during 75 days at two levels of salinity: (**a** roots without NaCl supply, **b** roots with 90 mM NaCl supply, **c** shoots without NaCl supply, **d** shoots with 90 mM NaCl supply) and with different N forms (*filled diamond* $NO_3^-$, *filled triangle* $NH_4^+$, *filled square* $NO_3NH_4$, *open circle Rhizobium* inoculation). Root and shoot fresh weight (g plant$^{-1}$) values are means ± standard errors of 16 replicates

$NH_4^+$-nourished plants more affected by salt. Other authors also observed that $NH_4^+$ was the N form causing the highest decreases in plant growth: Leidi et al. (1992) in cotton (*Gossypium hirsutum* L.) and peanut (*Arachis hypogaea* L.), Lewis et al. (1989) in maize (*Zea mays* L.), and Feigin (1990) in muskmelon (*Cucumis melo* L.). Among the different N forms, $NO_3^-$ induced the highest growth of pea plants under moderate salt conditions (Fig. 13.1d), as also reported by Leidi et al. (1992) and Silberbush and Lips (1991a, 1991b).

The high concentrations of inorganic solutes, especially $Na^+$ and $Cl^-$ absorbed by roots from the salinized media, directly affect plant growth. Above certain levels, the accumulation of ions are toxic to cells and will have a negative impact on plant metabolism and growth (Munns and Tester 2008). In severe cases of toxicity, plants show not only growth decreases but also marginal chlorosis and necrosis in mature leaves. Later on or with increasing salinity, the damaged areas will increase and organ or plant death may occur.

## 13.4 Salinity and Nitrogen Nutrition Influence on Productivity

### *13.4.1 The Importance of Seeds*

Seeds perpetuate species over time and space, and are especially important in those species that do not propagate vegetatively. Perhaps for this reason, plants spend abundant resources to ensure that enough energy and nutrients exist for germination

and establishment. In *Pisum sativum*, cotyledons contribute actively to the growth of plantlets in the first 2 weeks following germination, losing 88% of their weight during this time (Lovell 1977). The richness of the seeds in minerals and organic compounds such as sugars, starch, fats, and proteins explain their excellent nutritional value and, perhaps for that reason, quickly resulted in their use as a food. Thus, besides the ecological importance that seeds have in the perpetuation of the species, the economic significance related to productivity maximization also became an important issue.

### *13.4.2 Seed Production*

Legumes are generally considered either sensitive or moderately tolerant to salinity (Läuchli 1984; Zahran 1991). For crops, salinity tolerance is normally expressed in terms of the yield decrease associated with a given level of salinity. A yield decrease of 50% is usually considered a critical level for evaluating the relative salt tolerance of crops (Maas and Hoffman 1977). Pea is one of the most tolerant crop legumes, with 50% yield reduction in the presence of 100 mM NaCl (Subbarao and Johansen 1994). When pea plants were grown under different forms of N nutrition and salt conditions, it was observed that, under nonsaline conditions, productivity was two to four times higher when the source of nitrogen was $NO_3NH_4$. The highest seed weight in plants grown under this form of N is mainly due to the production of a higher number of seeds per plant, as a result of the increase in pod number (results not shown). Salinity decreased productivity, markedly in $NO_3NH_4$ and $NH_4^+$ and only slightly in $NO_3^-$-nourished plants, where salinity did not change productivity significantly (Fig. 13.2). Doré et al. (1998) also

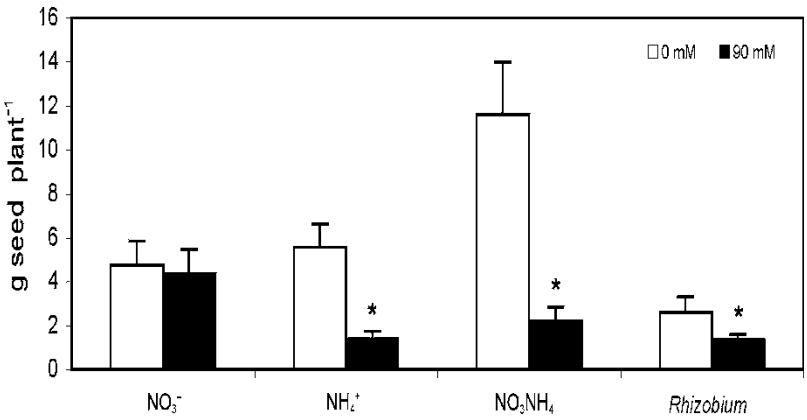



**Fig. 13.2** Seed production of *Pisum sativum* grown at two levels of salinity (0 and 90 mM NaCl) and with different N forms ($NO_3^-$, $NH_4^+$, $NO_3NH_4$, *Rhizobium* inoculation). Seed fresh weight (g plant$^{-1}$) values are means ± standard errors of 12 replicates. Significantly ($P < 0.001$) different values are marked (*)

observed variability in the productivity of pea plants cultivated under different growth conditions, and these authors also found that changes in productivity were related to the number and not the size of the seeds.

The productivity of plants exclusively dependent on $N_2$ fixation are generally smaller than those fed with inorganic nitrogen, due to the higher energy costs associated initially to anabolic processes leading to the formation of nodules and to the metabolic "machinery" necessary for $N_2$ fixation, and later to energy costs with the $N_2$ fixation process itself and with the maintenance of conditions that enable $N_2$ fixation. The high energy cost of $N_2$ fixation explains why, in *Pisum sativum* plants grown under different forms of nitrogen nutrition ($NO_3^-$, $NH_4^+$, $NO_3NH_4$, *Rhizobium* inoculation), those dependent on symbiotic fixation showed lower productivity (Fig. 13.2). Since inorganic nitrogen uptake is less costly to the plant than maintaining the capacity to reduce atmospheric $N_2$ (Lee et al. 2003), under conditions of increased soil nitrogen (e.g., fertilization), $N_2$ fixation is inhibited. $NO_3^-$ generally has a greater inhibitory effect on $N_2$ fixation than $NH_4^+$ (Hartwig 1998; Lee et al. 2003). However, fixation has the advantage of freeing plants from the dependence of available nitrogen for their growth and development in N-limited soils, allowing sustainable legume production.

### *13.4.3 Seed Protein Content*

A deficient nitrogen nutrition not only impacts significantly on productivity but also on the seed protein levels, especially in pulses, due to their high protein content (25% of *P. sativum* seed dry weight), indicating that seeds could be an appropriate organ to assess the nutritional status of plants (Atta et al. 2004). Protein synthesis has long been identified as one of the processes strongly affected by salinity (Gibson et al. 1984; Yeo 1998), due to the requirement of ion homeostasis that favors $K^+$ over $Na^+$ (Leigh and Wyn-Jones 1984), even in plants with as high salt tolerance as halophytes (Yeo 1998; Greenway and Munns 1980). In most cases, salinity decreases the protein level as a result of reduced protein synthesis. However, in some cases, an increase in the levels of proteins is reported (Dubey and Runi 1987; Joshi 1987; Dubey 1994). *Pisum sativum* plants grown under different nitrogen sources ($NO_3^-$, $NH_4^+$, $NO_3NH_4$, *Rhizobium* inoculation) and salinity conditions ( 0 and 90 mM NaCl) showed that both factors influenced protein levels in peas. Under nonsaline conditions, nutrition with $NH_4^+$ propitiated the accumulation of 64% more proteins than the other nitrogen treatments (Fig. 13.3a). However, the interaction of salinity with the nitrogen source changed this scenario. The amount of protein increased significantly in $NO_3^-$- and $NO_3NH_4$-treated plants to levels similar to those previously provided by $NH_4^+$, but the seed protein content of plants dependent on $N_2$ remained low (Fig. 13.3a). However, when calculating the total seed protein per plant, the value was lower under salt than under control conditions for all treatments except for $NO_3^-$, since in the other treatments ($NH_4^+$, $NO_3NH_4$ and *Rhizobium* inoculation) salinity caused a massive reduction in

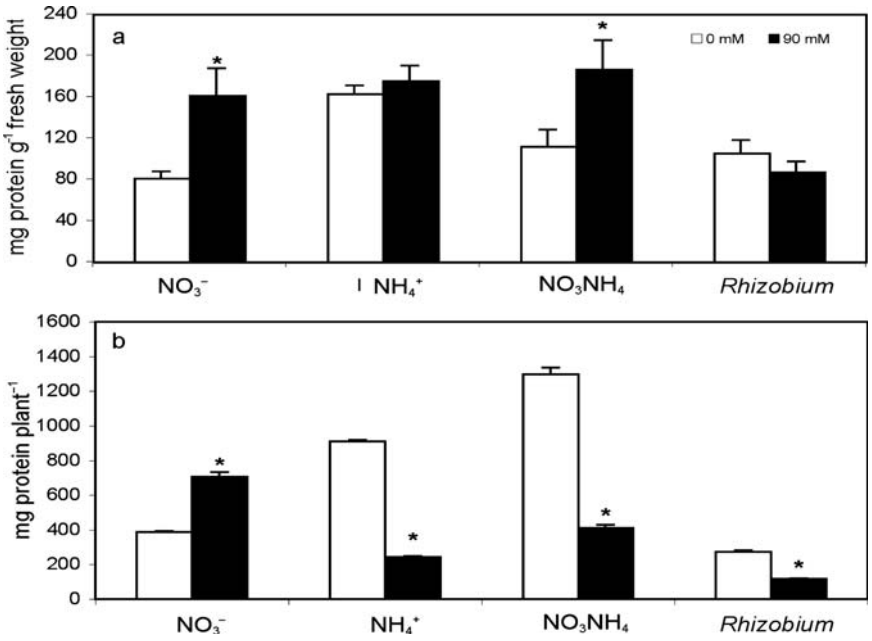



**Fig. 13.3** Seed proteins of *Pisum sativum* grown for 75 days under different conditions of salinity (0 and 90 mM NaCl) and nitrogen sources ($NO_3^-$, $NH_4^+$, $NO_3NH_4$, *Rhizobium* inoculation): **a** expressed in mg proteins per gram of seed fresh weight, **b** expressed in mg proteins per plant. Values are means of 12 replicates. Significantly ($P < 0.001$) different values are marked (*)

productivity (seed weight per plant) that protein increment did not compensate for (Fig. 13.3b). From a food production perspective, the amount of total protein produced by plants is more important than the protein concentration in the seed. Results clearly show that salinity has a strong impact in pea productivity, except for the $NO_3^-$ nutrition, suggesting that this form of N should be used as a source of N nutrition for pea cultivation in salt-affected areas.

## 13.5 Salinity Influence and Nitrogen Nutrition on Ion Accumulation

### *13.5.1 Vegetative Organs*

Among the various factors contributing to plant growth, the availability of nutrients plays a vital role. Inorganic elements regulate plant metabolism by integrating organic molecules or acting as cofactors or activators of a large number of enzymes. Their availability for plant growth is influenced by biotic and abiotic factors, by synergistic and antagonistic interactions, and by uptake rates. Many of the

elements are absorbed or excreted actively, using energy supplied mostly by root aerobic respiration at the expense of carbohydrates and $O_2$ (Velagaleti and Schweitzer 1994).

The form of nitrogen supplied to plants ($NO_3^-$, $NH_4^+$ or fixed $N_2$) has a vast impact on the relationships between anions and cations. Plants nourished with $NH_4^+$ are characterized by a high ratio of absorption of cations over anions. The opposite situation occurs in plants absorbing $NO_3^-$, due to the higher consumption of protons used in the cotransport through plasma membrane and to the accumulation of anions within the cell. Thus, in plants supplied with $NH_4^+$, absorption of anions is favored over cations. In *Pisum sativum* plants grown in the presence of salt (90 mM NaCl) and different N sources ($NO_3^-$, $NH_4^+$, $NO_3NH_4$, fixed $N_2$), the lower salt tolerance of plants under $NH_4^+$ nutrition may be related to the lower absorption of $K^+$ and to the higher accumulation of $Na^+$ and $Cl^-$ in the shoot (Figs. 13.4, 13.5, 13.6). Other authors also reported a similar influence of $NH_4^+$ nutrition in plants grown under saline and nonsaline conditions. For instance, Smart and Bloom (1998) observed that exposure of tomato (*Lycopersicon esculentum* L.) plants to ammonium led to a lower absorption of $K^+$ and an increase in the efflux of protons to the apoplast. Martínez and Cerdá (1989) observed that the addition of $NH_4^+$ to the growing medium decreased the accumulation of $K^+$ in cucumber (*Cucumis sativus* L.) plants growing in saline conditions. These authors also found that the increase of the ratio $NH_4^+/NO_3^-$ induced plants to accumulate more $Cl^-$ and $Na^+$ and less $K^+$ in the leaves. Plants dependent on symbiotically fixed $N_2$ accumulated lower concentrations of any of the ions analyzed (Figs. 13.4, 13.5, 13.6) than plants grown in inorganic nitrogen nutrition ($NO_3^-$, $NH_4^+$, $NO_3NH_4$). Thus, the organs

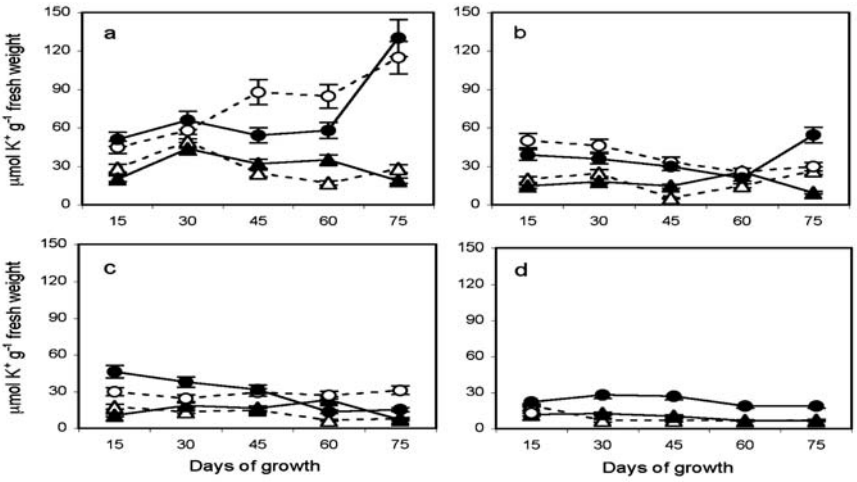



**Fig. 13.4** Potassium concentration in roots and shoots of *Pisum sativum* grown for 75 days at two salinity levels (0 and 90 mM NaCl) and with four nitrogen nutrition sources: (**a** $NO_3^-$, **b** $NH_4^+$, **c** $NO_3NH_4$, **d** *Rhizobium* inoculation): *filled circle* shoots with 90 mM NaCl, *open circle* shoots with 0 mM NaCl, *filled triangle* roots with 90 mM NaCl, *open triangle* roots with 0 mM NaCl. Values are means ± standard errors of six replicates

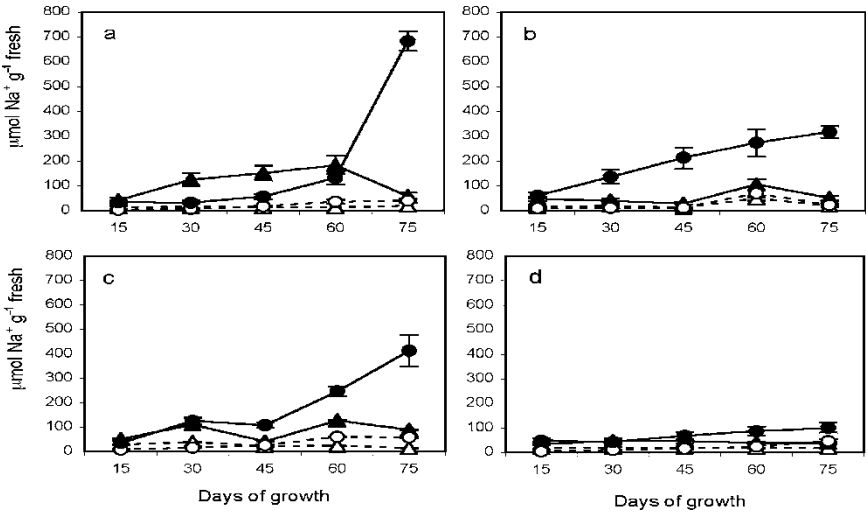



**Fig. 13.5** Sodium concentration in roots and shoots of *Pisum sativum* grown for 75 days at two salinity levels (0 and 90 mM NaCl) and with four nitrogen nutrition (**a** $NO_3^-$, **b** $NH_4^+$, **c** $NO_3NH_4$, **d** *Rhizobium* inoculation): *filled circle* shoots with 90 mM NaCl, *open circle* shoots with 0 mM NaCl, *filled triangle* roots with 90 mM NaCl, *open triangle* roots with 0 mM NaCl. Values are means ± standard errors of six replicates

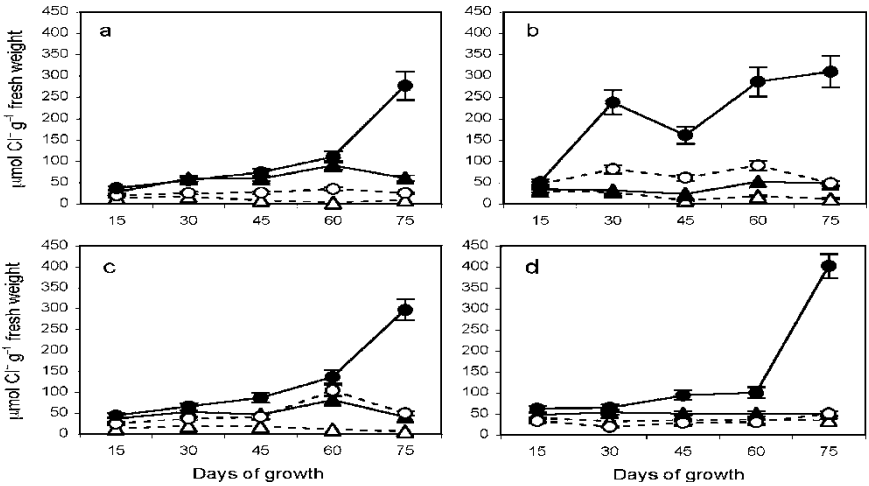



**Fig. 13.6** Chloride concentration in roots and shoots of *Pisum sativum* grown for 75 days at two salinity levels (0 and 90 mM NaCl) and with four nitrogen nutrition (**a** $NO_3^-$, **b** $NH_4^+$, **c** $NO_3NH_4$, **d** *Rhizobium* inoculation): *filled circle* shoots with 90 mM NaCl, *open circle* shoots with 0 mM NaCl, *filled triangle* roots with 90 mM NaCl, *open triangle* roots with 0 mM NaCl. Values are means ± standard errors of six replicates

of these plants must have experienced lower toxicity, and in turn a smaller inhibition would be caused by salt on vegetative and reproductive growth compared to plants nourished with $NO_3NH_4$ and $NH_4$. However, plants tolerated better salt stress when $NO_3^-$ was the N form provided. In plants nourished with $NO_3^-$, pH increases, enhancing cations absorption and inhibiting the absorption of other anions, and thus these plants frequently have higher $K^+$ contents than plants nourished with $NH_4^+$ (Marschner 1995). In *Pisum sativum* plants grown under different nitrogen sources, plants nourished with $NO_3^-$ showed a higher allocation of $Na^+$ in the root (Fig. 13.5), and higher $K^+$/ $Na^+$ ratios and higher $Ca^{2+}$ concentrations (results not shown) in the shoot compared to plants nourished with other forms of N. The first recorded response to an increase in $Na^+$ around roots is an increase in cytosolic-free $Ca^{2+}$. (Munns and Tester 2008). The best characterized signaling pathway specific to salinity stress involves increases in cytosolic-free $Ca^{2+}$ (Zhu 2002). Calcium ions play a role in maintaining the integrity of the plasma membrane and the activation effect that $Ca^{2+}$ has on plasmalemma P-ATPases can contribute to facilitate the absorption of $K^+$ at low pH values (Marschner 1995). The influence that $Ca^{2+}$ has on the maintenance of high $K^+/Na^+$ rates highlights the important role that $Ca^{2+}$ plays in the maintenance of $K^+$ concentrations, simultaneously restricting the $Na^+$ uptake in salt-stressed plants. Martínez and Cerdá (1989) also observed thatm when $NO_3^-$ was the only source of nitrogen available, the accumulation of $K^+$ in plants increased under saline conditions compared to plants supplied with mixed nitrogen nutrition. A study conducted by Subbarao et al. (1990) showed that the increase in saline tolerance of three different species of *Atylosa* was linked to a higher efficiency in the regulation of $Na^+$ and $Cl^-$ acropetal transport and to the maintenance of $K^+$ selectivity. The shoot exclusion mechanism observed in plants of *Pisum sativum* under $NO_3^-$ nutrition seems to have lost much of its effectiveness in older plants over a 15-day period (between 60 and 75 days), the concentration of $Na^+$ increased about five times in the shoot and decreased three times in the root (Fig. 13.5). The $Cl^-$ ratio between shoots and roots is kept close to the unit, however, at the end of the growth increases six times (Fig. 13.6). Thus, for most of the growth period, plants dependent on $NO_3^-$ accumulated 20–50% less $Cl^-$, 30–70% less $Na^+$, and 0.4–3.6 times more $K^+$ in the shoots than plants nourished with other forms of N, which certainly contributed to the higher salt tolerance observed in plants nourished with nitrate.

In most situations, $Cl^-$ influx requires energy and is probably catalyzed by a $Cl^-/2H^+$ cotransporter (Felle 1994; Sanders 1980). The cytosolic $Cl^-$ concentration is likely in the range of 10–20 mM, but may be higher in saline conditions (Munns and Tester 2008). $Cl^-$ tissue concentrations as high as 400 mM are tolerated by most species, and even sensitive species can tolerate 250 mM (Munns and Tester 2008). The absorption of $Cl^-$ is often inhibited by $NO_3^-$ (Marschner 1995; Grattan and Grieve 1994), not only because of the influence of pH, but also due to the competition between the two anions (Caldwell et al. 1986; Glikey and Staehelin 1989; Marschner 1995; Tyerman 1992). This competition is of great importance in the tolerance to salinity. Rogers et al. (1997) observed that the difference in salinity

tolerance shown by two populations of white clover (*Trifolium repens* L.) was due to the lower absorption of $Cl^-$ and to an increase of its restriction efficiency from the shoot. Läuchli and Wieneke (1979) recorded that some varieties of *G. max* exhibited different salt tolerances and that these differences could be related to the restriction of $Cl^-$ transport in the xylem. Rogers et al. (1997) and Läuchli and Wieneke (1979) concluded that within these species salt tolerance seems to be related to $Cl^-$ exclusion, in particular from the shoot. Chloride concentration differences in *Pisum sativum* plants grown under different forms of nitrogen nutrition and NaCl concentrations (Fig. 13.6) showed that $Cl^-$ accumulation is not only genotypically fixed but is also dependent on the N source.

### *13.5.2 Seeds*

In pea plants grown in the presence of NaCl, $Na^+$ concentrations in seeds are about ten times lower than those found in vegetative organs, with the N form supplied

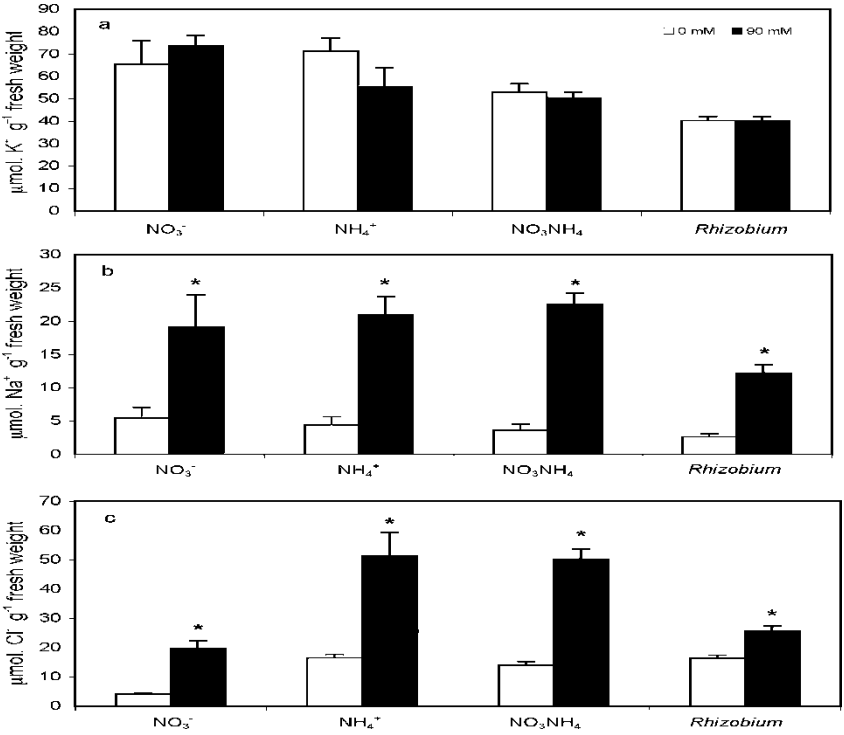



**Fig. 13.7** Potassium (**a**) sodium (**b**) and chloride (**c**) concentrations in seeds of *Pisum sativum* plants grown for 75 days under different conditions of salinity (0 and 90 mM NaCl) and nitrogen sources ($NO_3^-$, $NH_4^+$, $NO_3NH_4$, *Rhizobium* inoculation). Values are means ± standard errors of 16 replicates. Significantly ($P < 0.001$) different values are marked (*)

influencing $Na^+$ levels (Fig. 13.7b), as also reported by Greenway and Munns (1980), Hajibagheri et al. (1987), and Marschner (1995), who suggested that $Na^+$ is excluded from reproductive and young vegetative organs. Chloride, that was preferentially accumulated in the shoot, was also excluded from seeds, although less effectively than $Na^+$, and concentrations varied with the N form supplied to plants (Fig. 13.7c). The large acropetal translocation of ions that occurred at the later stages of *Pisum sativum* plants growth was also described by Pate and Flinn (1977), and was explained by these authors as a physiological process that mobilizes organic nutrients and minerals to the growing seeds. Although this process is advantageous for seeds, it also changes the mechanisms of salt tolerance, with decreases in the efficiency of $Na^+$ and $Cl^-$ exclusion from the shoot, leaving this organ more vulnerable to salinity, but maintaining low $Na^+$ and $Cl^-$ concentrations in the reproductive organs and thus not compromising seeds viability.

## 13.6 Conclusion and Future Prospects

In this chapter, a better knowledge of *Pisum sativum* responses to salinity as well as some of the factors that influence these responses is provided. Plants nourished with $NO_3^-$ experienced lower toxicity in the shoots than those of other N treatments, due to lower accumulations of $Na^+$ and $Cl^-$ and to higher $K^+$ concentrations. Thus, it is possible to grow *Pisum sativum* in moderate salinity levels without significant decreases in productivity if plants are provided with adequate levels of nitrogen in the form of nitrate. Plants dependent on symbiotically-fixed nitrogen had lower productivity, but were not as affected by salinity as plants dependent on ammonium or ammonium nitrate.

This chapter also raised some issues that require urgent attention. Can the study of salt tolerance of different *Pisum sativum* cultivars extend the known range of this species tolerance? Would the screening of new *Rhizobium* strains increase the nodulation efficiency in *Pisum sativum* growing in saline and nonsaline conditions? Which are the organic solutes that cells of *Pisum sativum* accumulate in order to adjust the cytoplasm osmotically? What are the concentration gradients of sodium and chloride through tonoplast in each of the plant organs? How are xylem loading and acropetal transport of ions regulated? What will be the growth and yield responses of plants under salt stress to a mixed nutrition of symbiotic nitrogen and low levels of nitrate?

The answer to these questions could be approached in future in order to reach a better understanding of the factors determining salt tolerance in legumes, and allow more efficient pulses cropping in salinized areas without massive reductions in productivity or high inputs of inorganic N fertilization.

**Acknowledgments** I thank Georgina Hodge for her help and suggestions. This work was supported by the Centre for Cell Biology.

## References

Abd-Alla MH, Vuong TD, Harper JE (1998) Genotypic differences in dinitrogen fixation response to NaCl stress in intact and grafted soybean. Crop Sci 38:72–77

Atta S, Maltese S, Cousin R (2004) Protein content and dry weight of seeds from various pea genotypes. Agronomie 24:257–266

Bekki A, Trinchant JC, Rigaut J (1987) Nitrogen fixation ($C_2H_2$ reduction) by *Medicago* nodules and bacteroides under sodium chloride stress. Physiol Plantarum 71:61–67

Below FE (1995) Nitrogen metabolism and crop productivity. In: Pessarakli M (ed) Handbook of plant and crop physiology. Marcel Dekker, New York, pp 275–301

Binzel ML, Hess FD, Bressan RA, Hasegawa PM (1988) Intracellular compartmentation of ions in salt adapted tobacco cells. Plant Physiol 86:607–614

Bohnert HJ, Su H, Shen B (1999) Molecular mechanisms of salinity tolerance. In: Shinozaki K, Yamaguchi-Shinozaki K (eds) Molecular responses to cold, Drought, heat and salt stress in higher plants. University of Arizona, Arizona, pp 29–60

Botsford JL, Lewis TA (1990) Osmoregulation in *Rhizobium meliloti*: production of glutamic acid in response to osmotic stress. Appl Environ Microbiol 56:488–494

Bourgeais-Chaillou P, Alfocea FP, Guerrier G (1992) Comparative effects of N-sources on growth and physiological responses of soybean exposed to NaCl-stress. J Exp Bot 43:1225–1233

Bray CM (1983) Nitrogen metabolism in plants. Longman, New York

Brewing NJ, Ambrose MJ, Downie JA (1993) Root nodules, *Rhizobium* and nitrogen fixation. In: Casey R, Davies DR (eds) Peas: genetics, molecular biology and biothechnology. Cab International, Wallingford, pp 237–290

Caldwell JH, Van Brunt J, Harold FM (1986) Calcium-dependent anion channel in the water mold, *Blastocladiella emersonii*. J Membrane Biol 39:85–97

Cordovilla MP, Ligero F, Lluch C (1994) The effect of salinity, N fixation and assimilation in *Vicia faba*. J Exp Bot 279:1483–1488

Cordovilla MD, Ligero F, Lluch C (1996) Growth and nitrogen assimilation in nodules in response to nitrate levels in *Vicia faba* under salt stress. J Exp Bot 47:203–210

Cordovilla MD, Ligero F, Lluch C (1999a) Effect of salinity on growth, nodulation and nitrogen assimilation in nodules of faba bean (*Vicia faba* L.). Appl Soil Ecol 11:1–7

Cordovilla MD, Berrido SI, Ligero F, Lluch C (1999b) *Rhizobium* strain effects on the growth and nitrogen assimilation in *Pisum sativum* and *Vicia faba* plant growth under salt stress. Plant Sci 140:127–136

Cordovilla MP, Ligero F, Lluch C (1999c) Effects of NaCl on nitrogen fixation and assimilation of inoculated and $KNO_3^-$ fertilized of *Vicia faba* L. and *Pisum sativum* L. Plant Sci 140:127–136

Craig GF, Atkins CA, Bell DT (1991) Effect of salinity on growth of four strains of *Rhizobium* and their infectivity and effectiveness on two species of *Acacia*. Plant Soil 133:253–262

Delgado MJ, Ligero F, Lluch C (1993) Nitrogen fixation and carbon metabolism by nodules and bacteroids of pea plants under sodium chloride stress. Physiol Plantarum 89:824–829

Delgado MJ, Ligero F, Lluch C (1994) Effect of salt stress on growth and nitrogen fixation by pea, faba bean, common bean and soybean plants. Soil Biol Biochem 26:371–376

Delgado MJ, Garrido JM, Ligero F, LIuch C (2006) Nitrogen fixation and carbon metabolism by nodules and bacteroids of pea plants under sodium chloride stress. Physiologica plantarum 89:824–829

Doré T, Meynard JM, Sebillotte M (1998) The role of grain number, nitrogen nutrition and stem number in limiting pea crop (*Pisum sativum*) yields under agricultural conditions. Eur J Agron 8:29–37

Dubey RS (1994) Protein synthesis by plants under stressful conditions. In: Pessarakli M (ed) Handbook of plant and crop stress. Marcel Dekker, New York, pp 277–299

Dubey RS, Runi M (1987) Proteases and proteins in germinating rice seeds in relation to salt tolerance. Plant Physiol Biochem 12:9–15

Elsheikh EAE, Wood M (1990) Effect of salinity on growth, nodulation and nitrogen yield of chickpea (C*icer arietinum* L.). J Exp Bot 231:1263–1269

Elsheikh EAE, Wood M (1995) Nodulation and $N_2$ fixation by soybean inoculated with salt-tolerant rhizobia or salt-tolerant bradyrhizobia in saline soil. Soil Biol Biochem 27:657–661

Feigin A (1990) Interactive effects of salinity and ammonium/nitrate ratio on growth and chemical composition of melon plants. J Plant Nutr 13:1257–1269

Felle H (1994) The $H^+/Cl^-$ symporter in root-hair cells of *Sinapis alba*. An electrophysiological study using ion-selective microelectrodes. Plant Physiol 106:1131–1136

Figueira EMDAP, Caldeira GCN (2005) Effect of nitrogen nutrition on salt tolerance of *Pisum sativum* during vegetative growth. J Plant Nutr Soil Sci 168:359–363

Fox TC, Guerinot ML (1998) Molecular biology of cation transport in plants. Annu Rev Plant Physiol Plant Mol Biol 49:669–696

Gazzarrini S, Lejay T, Gojon A, Ninnemann O, Frommer WB, Von Wiren N (1999) Three functional transporters for constitutive, diurnally regulated, and starvation induced uptake of ammonium into *Arabidopsis* roots. Plant Cell 11:937–947

Georgiev GI, Atkins CA (1993) Effects of salinity on $N_2$ fixation, nitrogen metabolism, export and diffusive conductance of cowpea root nodules. Symbiosis 15:239–255

Gessler A, Schneider S, Von Sengbusch D, Weber P, Hanemann U (1998) Field and laboratory experiments on net uptake of nitrate and ammonium by the roots of spruce (*Picea abies*) and beech (*Fagus sylvatica*) trees. New Phytol 138:275–285

Gibson TS, Speirs J, Brady CJ (1984) Salt tolerance in the plants. II. In vivo translaction of m-RNAs from salt-tolerant and salt-sensitive plants on wheat germ ribosomes: responses to ions and compatible solutes. Plant Cell Environ 7:579–587

Glikey JC, Staehelin LA (1989) A new organelle related to osmoregulation in ultrarapidly frozen *Pelvetia* embryos. Planta 178:425–435

Grattan SR, Grieve CM (1994) Mineral nutrient acquisition and response by plants grown in saline environments. In: Pessarakli M (ed) Handbook of plant and crop physiology. Marcel Dekker, New York, pp 203–226

Greenway H, Munns R (1980) Mechanisms of salt tolerance in nonhalophyes. Annu Rev Plant Physiol 31:149–190

Grossman A, Takahasi H (2001) Macronutrient utilization by photosynthetic eukaryotes and the fabric of interactions. Annu Rev Plant Physiol Plant Mol Biol 52:163–210

Hajibagheri MA, Harvey DMR, Flowers TJ (1987) Quantitative ion distribution within root cells salt-sensitive and salt-tolerant maize varieties. New Phytol 105:367–379

Hartwig UA (1998) The regulation of symbiotic $N_2$ fixation: a conceptual model of N feedback from the ecosystem to the gene expression level. Perspect Plant Ecol Evol Syst 1:92–120

Jackson LE, Martin Burger M, Cavagnaro TR (2008) Roots, nitrogen transformations, and ecosystem services. Annu Rev Plant Biol 59:341–363

Joshi S (1987) Effect of soil salinity on nitrogen metabolism in *Cajanus cajan* L. Indian J Plant Physiol 30:223–231

Läuchli A (1984) Salt exclusion: an adaptation of legumes for crops and pastures under saline conditions. In: Staples R, Toenniessen GH (eds) Salinity tolerance in plants: strategies for crop improvement. Wiley, New York, pp 171–187

Läuchli A, Wieneke J (1979) Studies on growth and distribution of $Na^+$, $K^+$ e $Cl^-$ in soybean varieties differing in salt tolerance. Z Pflanzenk Pflanzens 142:3–13

Lauter DJ, Munns DN (1986) Salt resistance of chickpea genotypes in solutions salinised with NaCl and $Na_2SO_4$. Plant Soil 95:271–279

Lee TD, Tjoelker MG, Reich PB, Russelle MP (2003) Contrasting growth response of an N2-fixing and nonfixing forb to elevated $CO_2$: dependence on soil N supply. Plant Soil 255:475–486

Leidi EO, Sliberbush M, Soares MIM, Lips SH (1992) Salinity and nitrogen nutrition studies on peanut and cotton plants. J Plant Nutr 15:591–604

Leigh RA, Wyn-Jones RG (1984) A hypothesis relating critical potassium concentration for growth to the distribution and functions of this ion in the plant cell. New Phytol 97:1–13

Lewis OAM, Leidi EO, Lips SH (1989) Effect of nitrogen source on growth response to salinity stress in maize and wheat. New Phytol 111:155–160

Lovell PH (1977) Correlative influences in seedling growth. In: Sutcliffe JF, Pate JS (eds) The physiology of the garden pea. Academic, London, pp 265–290

Maas EV, Hoffman GJ (1977) Crop salt tolerance – current assessment. Am Soc Civil Eng 103:115–134

Marschner H (1995) Mineral nutrition of higher plants. Academic, London

Martínez V, Cerdá A (1989) Influence of N source on rate of $Cl^-$, N, $Na^+$, and $K^+$ uptake by cucumber seedlings grown in saline conditions. J Plant Nutr 12:971–983

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681

Pate JS, Flinn AM (1977) Fruit and seed development. In: Sutcliffe JF, Pate JS (eds) The physiology of the garden pea. Academic, London, pp 431–468

Pereira SIA, Lima AIG, Figueira EMAP (2008) *Rhizobium leguminosarum* isolated from agricultural ecosystems subjected to different climatic influences: the relation between genetic diversity, salt tolerance and nodulation efficiency. In: Liu TX (ed) Soil ecology research development. Nova Science, New York, pp 247–263

Rengasamy P (2002) Transient salinity and subsoil constraints to dryland farming in Australian sodic soils: an overview. Aust J Exp Agric 42:351–361

Robson AD, Bottomley PJ (1991) Limitations in the use of legumes in agriculture and forestry. In: Dilworth MJ, Glenn AR (eds) Biology and biochemistry of nitrogen fixation. Elsevier, Amesterdam, pp 320–349

Rogers ME, Noble CL, Halloran GM, Nicolas ME (1997) Selecting for salt tolerance in white clover (*Tripolium repens*): chloride ion exclusion and its heritability. New Phytol 135:645–654

Salehi M, Salehi F, Poustini K, Heidari-Sharifabad H (2008) The effect of salinity on the nitrogen fixation in 4 cultivars of *Medicago sativa* L. in the seedling emergence stage. Res J Agric Biol Sci 4:413–415

Sanders D (1980) The mechanism of $Cl^-$ transport at the plasma-membrane of *Chara corallina*. I Cotransport with $H^+$. J Membr Biol 53:129–141

Serraj R, Roy G, Drevon JJ (1994) Salt stress induces a decrease in the oxygen uptake of soybean nodules and in their permeability to oxygen diffusion. Physiol Plantarum 91:161–168

Siddiqi MY, Malhotra B, Min XJ, Glass ADM (2002) Effects of ammonium and inorganic carbon enrichment on growth and yield of a hydroponic tomato crop. J Plant Nutr Soil Sci 165:191–197

Silberbush M, Lips SH (1991a) Potassium, nitrogen, ammonium/nitrate ratio, and sodium chloride effects on wheat growth. I. Shoot and root growth and mineral composition. J Plant Nutr 14:751–764

Silberbush M, Lips SH (1991b) Potassium, nitrogen, ammonium/nitrate ratio, and sodium chloride effects on wheat growth. II. Tillering and grain yield. J Plant Nutr 14:765–773

Singleton PW, Bholool BB (1983) Effect of salinity on functional components of the soybean-*Rhizobium japonicum* symbiosis. Crop Sci 23:451–460

Singleton PW, El Swaify SA, Bohlool BB (1982) Effect of salinity on *Rhizobium* growth and survival. Appl Environ Microbiol 44:884–890

Smart DR, Bloom AJ (1998) Investigations of ion absorption during $NH_4^+$ exposure. I. Relationship between $H^+$ efflux and $NO_3^-$ absorption. J Exp Bot 49:95–100

Sprent JI, Zahran HH (1988) Infection, development and functioning of nodules under drought and salinity. In: Beck DP, Materon LA (eds) Nitrogen fixation by legumes in Mediterranean agriculture. Martinus Nijhoff, Dordrecht, pp 145–151

Subbarao GV, Johansen C (1994) Strategies and scope for improving salinity tolerance in crop plants. In: Pessarakli M (ed) Handbook of plant and crop stress. Marcel Dekker, New York, pp 559–579

Subbarao GV, Johansen C, Jana MK, Kumar Rao JFDK (1990) Effects of sodium/ calcium ratio in modifying salinity response of pigeonpea (*Cajanus cajan*). J Plant Physiol 136:439–443

Tawfik KM (2008) Evaluating the use of rhizobacterin on cowpea plants grown under salt stress. Res J Agric Biol Sci 4:26–33

Tu JC (1981) Effect of salinity on *Rhizobium*-root-hair interaction, nodulation and growth of soybean. Can J Plant Sci 61:231–239

Tyerman SD (1992) Anion channels in plants. Annu Rev Plant Physiol Plant Mol Biol 43:351–373

Velagaleti RR, Schweitzer SM (1994) General effects of salt stress on growth and symbiotic nitrogen fixation in soybean. In: Pessarakli M (ed) Handbook of plant and crop stress. Marcel Dekker, New York, pp 461–471

Wahab AMA, Zahran HH (1981) Effects of salt stress in the nitrogenase activity and growth of four legumes. Biol Plant 23:16–23

Ward JM (1997) Patch-clamping and other molecular approaches for the study of plasma membrane transporters demystified. Plant Physiol 114:1151–1159

Williams LE, Miller AJ (2001) Transporters responsible for the uptake and partitioning of nitrogenous solutes. Annu Rev Plant Physiol Plant Mol Biol 52:659–688

Yeo AR (1998) Molecular biology of salt tolerance in the context of whole-plant physiology. J Exp Bot 49:915–926

Zahran HH (1991) Conditions for successful *Rhizobium*-legume symbiosis in saline environments. Biol Fertil Soils 12:73–80

Zahran HH, Rsanen LA, Karsisto M, Lindstrom K (1994) Alteration of lipopolysaccharide and protein profiles in SDS-PAGE of rhizobia by osmotic and heat stress. World J. Microbiol. Biotechnol. 10:100–105

Zhu JK (2002) Salt and drought signal transduction in plants. Annu Rev Plant Biol 53:247–273

# Chapter 14
# Plant Growth-Promoting Diazotrophs and Productivity of Wheat on the Canadian Prairies

**Anthony O. Anyia, Daniel J. Archambault, Carols J. Bécquer, and Jan J. Slaski**

**Abstract** Nitrogen is an essential plant nutrient, which is limiting for wheat production in Canada. To achieve higher yields, farmers often apply large amounts of nitrogen fertilizers at a considerable cost. The prospects of extending biological nitrogen fixation (BNF) to non legume crops, as a way to overcome the high cost and ecological issues of nitrogenous fertilizers, has remained a dream to date. Several studies have reported colonization and $N_2$ fixation by rhizobia strains in chemically-induced para-nodules (nodule-like structures). The extent to which nitrogen fixation in para-nodules and intercellular spaces benefits plants, especially under field conditions remains unclear. Exploiting the benefits of diazotrophs as plant growth-promoting rhizobacteria (PGPR) appears to be a more promising approach, assuming that issues of lack of consistency of growth stimulation can be resolved. This chapter describes experiences and progress made in Canada towards the application of *Azorhizobium caulinodans* and native rhizobia strains as inoculants for improving wheat production.

A.O. Anyia (✉)
Alberta Research Council, Bioresource Technologies, P.O. Bag 4000, Vegreville, Alberta, Canada, T9C 1T4
e-mail: Anthony.Anyia@arc.ab.ca

D.J. Archambault
Laurentian University, 935 Ramsey Lake Road, Sudbury, Ontario, Canada, P3E 2C6
e-mail: darchambault@laurentian.ca

C.J. Bécquer
Ministerio de la Agricultura, Instituto de Ganadería Tropical, Estación Experimental Sancti-Spiritus, Cuba
e-mail: becquer@pastos.yayabo.inf.cu

J.J. Slaski
Alberta Research Council, Bioresource Technologies, P.O. Bag 4000, Vegreville, Alberta, Canada, T9C 1T4
e-mail: Jan.Slaski@arc.ab.ca

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_14, 

## 14.1 Introduction

### *14.1.1 Wheat in Canada*

Wheat (common, *Triticum aestivum* and durum, *T. turgidum*) is the most important cereal crop in the world, which, together with rice and maize, account for about 73% of world cereal production. Canada is one of the world's largest producers and exporters of wheat. In 2007, Canada ranked as the eighth largest wheat-producing country in the world (Fig. 14.1). Over the last decade, the annual production of wheat in Canada has consistently exceeded 20 million tons, except in 2002 when production dropped to about 16 million tons due to severe drought in the Prairie Provinces. Wheat is Canada's largest crop both in terms of area seeded and production. All Canadian provinces, except Newfoundland and Labrador, grow wheat; Saskatchewan, Manitoba, Alberta, and Ontario are the leading wheat producers in Canada. The annual revenue derived from wheat export in Canada exceeds $5 billion, making wheat the highest earner of all exported agricultural products. Only one class of durum is grown in Canada, spring amber durum; however, there are several classes of common wheat grown in Canada, based on seed hardness and color, and on sowing time (autumn or spring).

### *14.1.2 Nitrogen Use in Wheat Production*

Canadian wheat yields are relatively low averaging 2.4 $tha^{-1}$ between 1998 and 2007 compared to the world average of 2.7 $tha^{-1}$ (FAOStart, Statistics Canada).

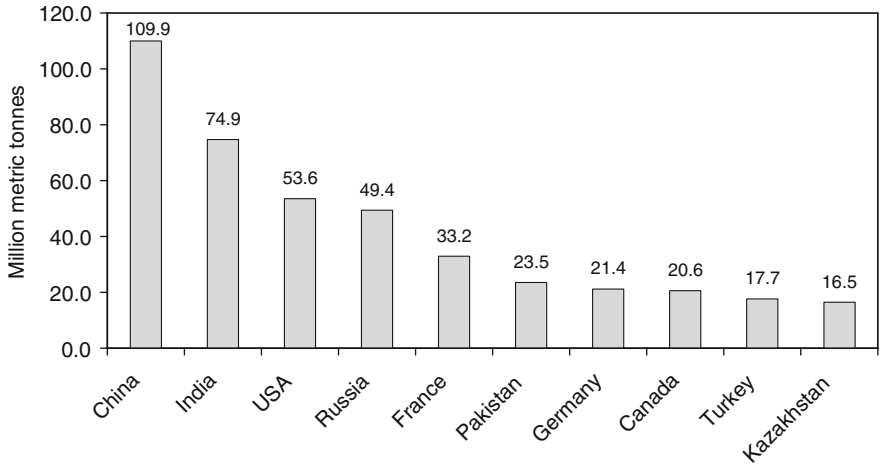



**Fig. 14.1** Wheat-producing top 10 countries of the world (FAOSTAT data, 2007)

Nitrogen is a very important plant nutrient whose limitation can affect yield considerably. The emergence of nitrogen-containing synthetic fertilizers is one of the factors that contributed to the "green revolution" in the middle of the twentieth century. The Haber-Bosch process allows for the production of ammonia from nitrogen and hydrogen, which are the main inputs used in the production of all nitrogen fertilizers. The hydrogen used in the Haber-Bosch process is derived from natural gas. Farmers in Canada as in many other countries are highly dependent on nitrogen fertilizers to achieve high yields. In terms of tonnage, nitrogen accounted for about 60% of the total sales of chemical fertilizers in Canada in 2004 (Canadian Fertilizer Institute). Production of chemical nitrogen fertilizers, besides being very cost intensive, also depletes nonrenewable resources and poses human and environmental hazards. To complement and eventually substitute mineral fertilizers with biologically fixed nitrogen would represent an economically beneficial and ecologically sound alternative (Glick et al. 1999).

Analysis of nitrogen use on the Canadian prairies over the last three decades shows a steady increase from approximately 125,000 metric tons per year in 1969 to more than 1.25 million tons per year since 1996. The Canadian Fertilizer Institute reported total sales of nitrogen-containing fertilizers of 2.57 million metric tons in western Canada in the year ending June 30, 2000. Data show that nitrogen applications represent 22.1 and 33%, respectively, of the operating costs of corn and wheat production. In the black soils zone of Alberta, a wheat farmer would spend approximately three times more on nitrogen fertilizer than his counterpart who grows feed peas (*Pisum sativum*) inoculated with rhizobia. This suggests that the introduction of nitrogen-fixing bacteria to wheat could significantly reduce the cost of nitrogen fertilizer input. Norman Borlaug, in his Nobel Peace Prize lecture in 1970, highlighted the need to extend the symbiotic nitrogen fixation of legumes with rhizobia to the world's major cereals, maize *(Zea mays*), wheat and rice (*Oryza sativa*) to sustain the green revolution (Borlaug 1970). Haber, who invented the famous "Haber-Bosch process" of ammonia production, in his 1920 Nobel Prize acceptance speech noted that "it may be that this solution is not the final one." Nitrogen bacteria teach us that nature, with her sophisticated forms of the chemistry of living matter, still understands and utilizes methods, which we do not as yet know how to imitate (Cocking 2005).

## 14.2 Nitrogen Fixation and Non legume Plants

Many researchers have long dreamt of the prospects of extension of biological nitrogen fixation (BNF) to non legume crops. The extension was thought to be able to overcome the high cost and ecological issues of nitrogenous fertilizers. When in 1969 Van Overbeek urged botanists to tackle the problem of nitrogen deficiencies with boldness (Quispel 1991), molecular biology was still in its infancy. Van Overbeek stated then that "we need to device more efficient ecosystems, put root nodules on cereal crops, and put nitrogen-fixing chloroplasts in their leaves." At

that time, many thought that, with increased knowledge and advances in molecular biology, nitrogen fixation would be easily achieved in non legume crops. Several decades later, nitrogen fixation by non legumes is still only a dream. The complexity of the genes of the nitrogenase enzyme complex involved in nitrogen fixation has so far prevented their successful transfer with function in plant cells. Quispel (1991) concluded that "in the light of our present knowledge the prospect of active nitrogenase in plant cells seems farther removed than it appeared in 1969." As sad as it may be, the same conclusion still holds today.

A 1987 patent (Publication No.: WO/1987/004182) by NIELSEN, Sven-Erik SO/RENSEN, Grete, Mo/rch, published at the World Intellectual Property Organization claims to have invented "Rhizobia transformants that nodulate and fix nitrogen in non legumes. The nodulated non legume plants can be grown without nitrogenous fertilizer and have at least the same or higher protein content, dry matter content and nitrogen content than their nonnodulated counterparts which are fertilized by the addition of nitrogenous fertilizer. The straw remaining after harvesting the nodulated non legumes is also high in protein content". There is no indication if these transformants were ever tested in field experiments and how well they performed. As was noted by Quispel (1991) "we are able to select and modify bacterial strains, host plants and bacteria–plant combinations, which are highly efficient under laboratory conditions. Successful field inoculation methods have been developed. Yet results in the field are mostly disappointing since the introduced selected bacteria have to compete with the indigenous *Rhizobium* populations in the soil."

## 14.3 Plant Growth-Promoting Effects of Diazotrophs

Bacteria are abundantly present in the rhizosphere and in close vicinity of the root. It has long been recognized that several genera of these rhizobacteria have the ability to promote plant growth, and these have been termed plant growth-promoting rhizobacteria or PGPR. Some of these rhizobacteria interact with the plant in different mutually beneficial ways and may thereby promote plant growth or yield by direct or indirect mechanisms. Direct growth promotion may be through biofertilization by synthesis of elements or compounds utilizable by the plant, or by aiding the plant in uptake of nutrients and/or water. Indirect growth promotion on the other hand may entail biocontrol of infection by phyto-pathogenic organisms. The bacteria that provide some benefit to plants are of two general types: those that form a symbiotic relationship, which involves formation of specialized structures or nodules on host plant roots, and those that are free-living in the soil; the latter are often found near, on, or even within the roots of plants (Kloepper et al. 1988). Examples of symbiotic diazotrophs include: (1) rhizobia (includes soil bacteria of the genera *Rhizobium*, *Azorhizobium* and *Bradyrhizobium*), which interact with leguminous plants (belonging to the Fabaceae family) to form $N_2$-fixing nodules; (2) *Frankia* – "actinorhizal" nitrogen fixers that form similar symbiosis to rhizobia, with a number of woody plant species; and (3) symbiotic cyanobacteria which,

unlike rhizobia and Frankia, do not form nodules. The host range of cyanobacteria varies widely from fungi, mosses, and liverworts to waterferns (*Azolla*), some Gymnosperms (e.g., *Cycas* and *Macrozamia*) and Angiosperms (*Gunnera*) (Quispel 1991). In these symbioses, the plant supplies energy materials to the diazotrophs, which in turn reduce atmospheric nitrogen to ammonia. This ammonia is transferred from the bacteria to the plant to meet the plant's nutritional nitrogen needs for the synthesis of proteins, enzymes, nucleic acids, and chlorophyll. Some examples of free-living growth-promoting rhizobacteria are *Azobacter* species, *Azospirillum* species, pseudomonads, bacilli, and *Burkholderia* species (Brown 1974; Elmerich 1984; Kloepper et al. 1989; Quispel 1991).

Associative nitrogen fixation in rice was reported to have supplied 20–25% N of the total need in a study at International Rice Research Institute, Philippines (Ladha et al. 1987). Rice seedlings inoculated with *Burkholderia* spp. previously isolated from rice plants contained a relatively higher level of N due to BNF (Baldani et al. 2000). Sevilla and Kennedy (2000) suggested that *Acetobacter diazotrophicus* could promote rice growth, which might be related to the transfer of biologically fixed N, although other factors such as auxin production could be involved. Inoculation of wheat with *Azospirillum brasilense* and *A. lipoferum* significantly increased the biomass and grain yield as well as branching of root hairs (Kennedy and Tchan 1992).

## 14.4 Beneficial Effects of Rhizobia in Non legume Crops

According to Saikia and Jain (2007) advances in agricultural sustainability will require an increase in the utilization of BNF as a major source of nitrogen for plants. The beneficial effects of legumes in crop rotation have long been known. Albrecht Thaer – the famous German pioneer of agricultural research – said this in 1856: "Latterly the practice of sowing white clover (*Trifolium repens*) with the last crop has become very general. Only a very few apathetic and indolent agriculturalists or men who are firmly wedded to their opinions and customs neglect this practice." Legumes have also been used for soil improvement for centuries because of their N and non-N rotational benefits to non legume crops (Lupwayi et al. 2005). Seeding a non legume crop after legumes in a rotation can reduce the amount of nitrogen required to maintain yield. Noel et al. (1996) suggested that *Rhizobium* not only interacts with the roots of legumes but may also interact beneficially with nonleguminous plants. These authors concluded that "as *Rhizobium* may be found as a free-living rhizobacterium and appears to promote growth of non legumes as well as host legume species, the term PGPR seems to apply."

Yanni et al. (2001) investigated the benefits of inoculating rice with *Rhizobium leguminorarum* bv *trifolii*. The authors exploited the long-term association between this *Rhizobium* and rice in the Egyptian Nile delta where rice has been rotated successfully with berseem clover (*Trifolium alexandrinum* L) for ages. Greenhouse and field inoculation results of these experiments show an increase in plant

performance and grain yield, which was associated more with rhizobial effects on rice root architecture for enhanced uptake of soil nutrients. The authors suggested that some rhizobia may have evolved an additional ecological niche enabling them to form a three-component life cycle including a free-living heterotrophic phase in soil, a $N_2$-fixing endosymbiont phase within legume root nodules, and beneficial growth promoting endocolonizer phase within cereal roots in the same crop rotation.

*Azorhizobium caulinodans* is a stem and root nodulating nitrogen-fixing bacteria, which was isolated from the stem nodules of *Sesbania rostrata* (Dreyfus et al. 1988). The nitrogenase enzyme of *A. caulinodans* is reported to be more tolerant of oxygen than other rhizobia studied so far (Dreyfus et al. 1983). Several studies have shown that this bacterium is able to colonize intercellular spaces of cortex, xylem and root meristems of several non legumes through crack entry of emerging lateral roots (Sabry et al. 1997; Reddy et al. 1997; Webster et al. 1997; Cocking 2001). In the studies of Sabry et al. (1997), inoculation of aseptically grown wheat with *A. caulinodans* resulted in stimulation of root development accompanied by increased yield and N content. Since inoculated plants showed some level of acetylene reduction activity (a measure of $N_2$ fixation), the increase in plant growth and N content were attributed to nitrogen fixation by the bacterium. In follow-up studies with *A. caulinodans* by Mathews et al. (2001) in a "temperate" controlled environment, inoculation of wheat again resulted in increased yield and plant N content. However, application of a non-$N_2$-fixing strain of *A. caulinodans* or a filter-sterilized supernatant of the bacterium produced similar growth stimulation effects as the $N_2$-fixing strain. Mathews et al. (2001) concluded that response of wheat to *A. caulinodans* was not due to $N_2$ fixation but probably related to plant growth substances produced by the bacteria in culture.

## 14.5 Trials with *A. caulinodans* in Alberta, Canada

In a preliminary greenhouse study with a genetically improved strain of *A. caulinodans* ORS 571 (Geelen et al. 1995) using soil beds to simulate field conditions, Archambault and Li (2002) reported that inoculation of wheat (variety CDC Teal ) with *A. caulinodans* increased biomass and grain yield by 49 and 34%, respectively. In subsequent growth chamber and greenhouse experiments, growth of wheat cv. CDC Teal was consistently enhanced by inoculation with *A. caulinodans* ORS 571 (Anyia et al. 2004). In one greenhouse experiment, leaf area, leaf area index, and biomass of inoculated plants were significantly higher in inoculated plants than in the uninoculated controls at 7 weeks after seeding (Table 14.1 and Fig. 14.2). Following successful and consistent positive results with *A. caulinodans* ORS 571 inoculation with wheat, it was thought that similar effects could be achieved under field conditions. During the summer of 2004, field trials were conducted at one location in Vegreville, Alberta, Canada, and in another location at Whitewater, Wisconsin, USA, using seeds of spring wheat pre-inoculated with *A. caulinodans* ORS 571. The trials were repeated in 2005 at four locations in Alberta, Canada,

**Table 14.1** Means and standard errors of growth parameters at 7 weeks of inoculated and uninoculated plants of wheat cv. CDC Teal grown in soil beds in a greenhouse

| Treatment | Height (cm) | Leaf area ($cm^2$) | Leaf area index | Tillers | Biomass ($g^{-5\ plants}$) |
|---|---|---|---|---|---|
| Control | 37.72±0.72 | 52.12±5.01 | 0.35±0.03 | 0.83±0.19 | 4.89±0.50 |
| Inoculated | 38.09±0.50 | 62.11±5.32 | 0.50±0.04 | 1.17±0.10 | 5.63±0.36 |
| Difference (%) | 1 | 19 | 41 | 40 | 15 |

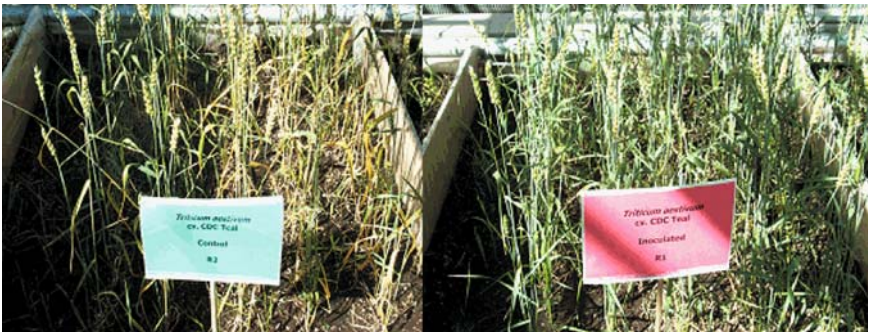



**Fig. 14.2** Wheat cv. CDC Teal grown in the presence (*right*) and absence (*left*) of *A. caulinodans*

representing different agro-ecological zones. One location in the southern part of the province was irrigated due to low precipitation. *A. caulinodans* inoculant was also tested in eight field locations including five winter wheat and three spring wheat plots in the USA. Results of the 2 years of field testing are presented in Table 14.2. In 2004, inoculated plots at the Vegreville location consistently showed greater biomass production and grain yields than control plots. Overall, across all four nitrogen levels used in the trial, grain yield of inoculated plots increased by 10% over controls (Table 14.1). The yield advantage of inoculated treatments over controls, although consistent at all nitrogen levels used, was however not statistically significant at $p=0.05$. In Whitewater, Wisconsin, inoculated plots showed significant positive effects. Grain yield increased by 6.4% (Table 14.2) while plant vigor increased by 10.6% (data not shown).

In the 2005 trial, grain yield of spring wheat at all locations in Alberta, Canada, except one showed positive effects of inoculation, which was again not statistically significant. Grain yield of inoculated plots were 4% (Vegreville), 8% (St. Vincent) and 14% (Taber) higher than the corresponding uninoculated control plots. In the USA, across all eight trials, inoculation showed an average increase of 1.3%. Considering the Wisconsin trials alone, inoculation with *A. caulinodans* caused an average increase of 6.5%. Half the trial locations (winter and spring wheat) did not respond significantly to inoculation. Although *A. caulinodans* inoculation showed very positive effects at some of the locations tested, the effects were not consistent. Compared with the greenhouse results, which were highly consistent and significant, those of the field trials were disappointing. Results of the

**Table 14.2** Field testing of wheat inoculated with *A. caulinodans* in Canada and USA

| Location | Year | Control | Inoculated | % increase |
|---|---|---|---|---|
| Alberta, Canada, trial spring wheat (extrapolated grain yield in kg $ha^{-1}$) | | | | |
| Vegreville | 2004 | 2,664 | 2,932 | 10.1 |
| Vegreville | 2005 | 2,975 | 3,095 | 4.0 |
| St Vincent | 2005 | 4,199 | 4,533 | 8.0 |
| Innisfail | 2005 | 2,712 | 2,637 | −2.8 |
| Taber | 2005 | 4,142 | 4,705 | 13.6 |
| Wisconsin and Idaho, USA, trial spring wheat (grain yield in kg/ha) | | | | |
| Whitewater, WI | 2004 | 1,197 | 1,273 | 6.4 |
| Genesee, ID | 2005 | 1,496 | 1,578 | 5.5 |
| Moscow, ID | 2005 | 1,768 | 1,768 | 0.0 |
| Nezperce, ID | 2005 | 1,605 | 1,632 | 1.7 |
| Wisconsin and Idaho, USA, trial winter wheat (grain yield in kg/ha) | | | | |
| Whitewater, WI | 2005 | 2,068 | 2,231 | 7.9 |
| Whitewater, WI | 2005 | 2,422 | 2,585 | 6.7 |
| Genesee, ID | 2005 | 2,286 | 2,258 | −1.2 |
| Lewiston, ID | 2005 | 2,912 | 2,857 | −1.9 |
| Nezperce, ID | 2005 | 2,830 | 2,830 | 0.0 |

*A. caulinodans* trial did not meet the minimum requirement of 95% significance required for registration of new inoculants or soil amendments in Canada.

Several reasons could be responsible for the difference observed between effects of inoculation in greenhouse and field studies. Two obvious ones in the experiments with *A. caulinodans* in Canada would be the methods of inoculation and pretreatment of plant growth medium. Firstly, in the greenhouse and growth chamber experiments, plants were grown in presteamed soil, which eliminated or minimized competition from indigenous soil microbes. Secondly, pre-inoculated seeds were used in field trials while in the greenhouse trials, seeds were pre-inoculated and multiple soil inoculations were performed subsequently. It is common knowledge that inoculation methods usually affect $N_2$ fixation in traditional rhizobium–legume symbioses. Soil inoculation (e.g., granular inoculants) is usually more effective than seed inoculation for initiating nodulation and $N_2$ fixation (Lupwayi et al. 2005). Multiple soil inoculations ensured that high number of cells of *A. caulinodans* was introduced to the soil, which perhaps aided this strain in its competition with indigenous soil microbes. In addition to the two obvious differences between the greenhouse and field experiments, it should be mentioned that *A. caulinodans* is adapted to conditions in tropical and subtropical environments. Early spring soil temperatures are usually very cold in the Canadian prairies. Lupwayi et al. (2005) suggested that the most adaptable rhizobia or legume genotypes are usually the ones isolated from similar environments. This might explain why attempts to re-isolate the microbes in inoculated plots during the growing season were not successful. Inconsistency of response is a perennial problem limiting the wide acceptance of PGPR as substitutes for chemical fertilizers in nonleguminous crops. Andrews et al. (2003) noted that the effects of *Azotobacter* and *Azospirillum* inoculants on growth and yield of graminaceous crops have been tested in many experiments in several

countries with inconsistent results obtained. Several reasons were suggested to account for the inconsistent results including unfavorable soil environment, competition with better adapted indigenous soil bacteria, or predation by protozoans (Dowling and Broughton 1986; Quispel 1991; Jjemba and Alexander 1999).

Although most of the effects of *A. caulinodans* inoculation on field grown wheat were positive, the trials were discontinued because the effects were not deemed to be large enough. Irrespective of the inconsistency of response observed in the field, it was obvious that the positive inoculation trends could not be attributed to chance alone. More fundamental research is needed to optimize field inoculation methods and to enhance survival and adaptation of *A. caulinodans* to temperate soil and temperature conditions.

### *14.5.1 Monitoring of Introduced Microbes in Field Soils*

All field locations used for testing of *A. caulinodans* were placed under strict monitoring requirements by the Canadian regulatory authorities. On each trial site, the experimental plots were spatially isolated from other field operations with a 12-m fallowed buffer zone. The buffer zone acted as inoculum trap providing effective trial confinement isolating treated crop and minimizing treatment drift to nontarget plants. The entire perimeter was fenced to restrict access to wildlife or unauthorized personnel. Wheat seeds were inoculated with liquid culture of *A. caulinodans* at a rate of 2.8 mL $kg^{-1}$. The inoculum was estimated to contain approximately $9.4 \times 10^8$ CFU per mL. Rhizosphere soil and root samples collected from the experimental sites at different time intervals during the growing season were analyzed for presence of *A. caulinodans* using BIOLOG® system and PCR technology. Soil and plant samples for PCR and BIOLOG® analysis were extracted and analyzed using methods described by Yeates et al. (1998) and Slaski et al. (2002), respectively. The substrate utilization pattern of the isolate was detected using an Emax BIOLOG® plate reader. The detected pattern was compared to a pattern produced from the original stock culture of *A. caulinodans*. The minimum detection limits of the PCR and BIOLOG® were estimated to be about 10 cells and 2,000 cells per gram of soil, respectively. None of the samples tested was positive for *A. caulinodans* suggesting that the microbe did not persist or spread in any of the locations tested. *A. caulinodans* does not produce spores, and thus low soil temperatures (below 0°C) may cause cell rupture and death of the organism. Low soil temperature was suggested as an important environmental factor drastically affecting survival of *A. caulinodans* in temperate climatic conditions of UK soils (Bullard 1999). The author reported that moderate temperatures 18/8°C reduced introduced populations of *A. caulinodans* by an order of magnitude in 35 days. Therefore, it appears that hostile fall and winter conditions of Alberta may have totally exterminated this strain from the environment. The depth of frost in prairie soils is typically greater than that in any other agricultural land in Canada, mostly due to the length of the winter season and the typically shallow snow depths. Frozen soils at depths

below 40 cm have been observed in April on some of the field sites tested, and therefore, for the organism isolated from tropical soils, environmental conditions were not favorable for it to thrive in the test locations.

## 14.6 Native Canadian Rhizobia and Wheat Production

Empirical data suggest that native rhizobia strains are usually more adapted than introduced strains. In a search for rhizosphere bacteria capable of promoting growth of wheat on the Canadian Prairies, several strains of rhizobia were isolated, identified, and characterized from rangeland ecosystems in the Castor region of central Alberta. This region is prone to drought due to low precipitation and high temperatures in the summer months. Sixteen native rhizobia strains identified as belonging to *Sinorhizobium meliloti* (Bécquer et al. 2008) were isolated from roots of *Melilotus officinalis* and *Medicago sativa*. The host legumes are adapted to the local pedological and climatic conditions prevalent in the region. Plant growth-promoting effects of the isolated rhizobia strains were evaluated in a greenhouse experiment using a Canadian variety of wheat, CDC Teal. The seeds disinfected with alcohol and 8% sodium hypochloride (Webster et al. 1997) were planted in pots containing standard soil mixture with a low N level, while fertilized control received 150 ppm N $kg^{-1}$ in a form of $NH_4NO_3$. Three milliliters per plant of inoculum containing approximately $10^8$ cells $mL^{-1}$ was applied 5 days after seeding. Two subsequent inoculations were performed 20 and 30 days after seeding. Wheat plants were grown to maturity under 29/25ºC (day/night) and 14 h of light. In general, inoculation with native rhizobia led to increase in root weight and plant height with no obvious effects on aerial dry matter (Bécquer et al. 2007). All but one of the isolated strains enhanced plant height while 12 strains stimulated root growth. Although changes in root morphology of wheat plants inoculated with rhizobia were not studied, literature data suggest that rhizobial inoculants could induce a higher volume of root hairs and lateral roots, which favors more efficient nutrient extraction (Biswas et al. 2000). Stimulation of root growth accompanied by increased yield and N content by *A. caulinodans* have also been reported (Sabry et al. 1997). Similarly, inoculation of barley (*Hordeum vulgare)* with *Mesorhizobium mediterraneum* (strain PECA21) considerably increased the dry matter yield and the content of macroelements in the plant (Peix et al. 2001). A well-developed root system is advantageous for the wheat plants cultivated in semiarid zones or for crops experiencing periodic soil moisture deficit. Thus, stimulation of root growth by inoculation with rhizobia may increase production of wheat in drought conditions through enhanced efficiencies of water and nutrient uptake/utilization.

The nine best performing native rhizobia strains identified in the greenhouse studies described above were evaluated for wheat growth promotion under field conditions at the Sancti-Spíritus Experimental Station in Cuba. In addition to the nine strains isolated from rangeland ecosystem of central Alberta, another strain (CAS2) isolated from legume grown in soil contaminated with petrochemical

sludge containing high concentration of salts and heavy metals, was tested. The well-described rhizobia strains (USDA 191 and ATCC 10004) were included in the field trial as reference strains. All strains were used to inoculate seeds of wheat (*T. aestivum*, L., var. Cuba-204) provided by the National Institute of Fundamental Research of Tropical Agriculture (INIFAT), La Habana, Cuba. Inoculation was performed by immersion of seeds in a bacterial inoculum containing approximately $10^8$ cells $mL^{-1}$ at room temperature for 24 h. Liquid inoculum was reapplied at the base of young seedlings 18 days after sowing to ensure adequate number of rhizobia cells in the rhizosphere. A fertilized and unfertilized control was included for comparison with *Rhizobium* inoculation effects. The fertilized treatment received 150 kg N $ha^{-1}$ $NH_4NO_3$. The experimental design was a randomized complete block with four replicates. Since the experiment was conducted on sandy loam soil with low mineral content ($P_2O_5$: 2.63 mg/100 g; $K_2O$: 10.00 mg/100 g; OM: 1.61%; pH: 5.4), a second application of fertilizer (NPK: 9–13–17) was made 21 days after sowing on the basis of 88 kg N $ha^{-1}$.

Plant growth-promoting and grain yield-enhancing effects of inoculation with nine Canadian native *Rhizobium* strains were observed (Fig. 14.3). Two strains, CAC2 and CAC 5, significantly improved plant agronomic parameters including grain yield, weight of 1,000 seeds, yield of total aboveground biomass, and stem height of the Cuban wheat cv. Cuba-204. Several of the native Canadian strains

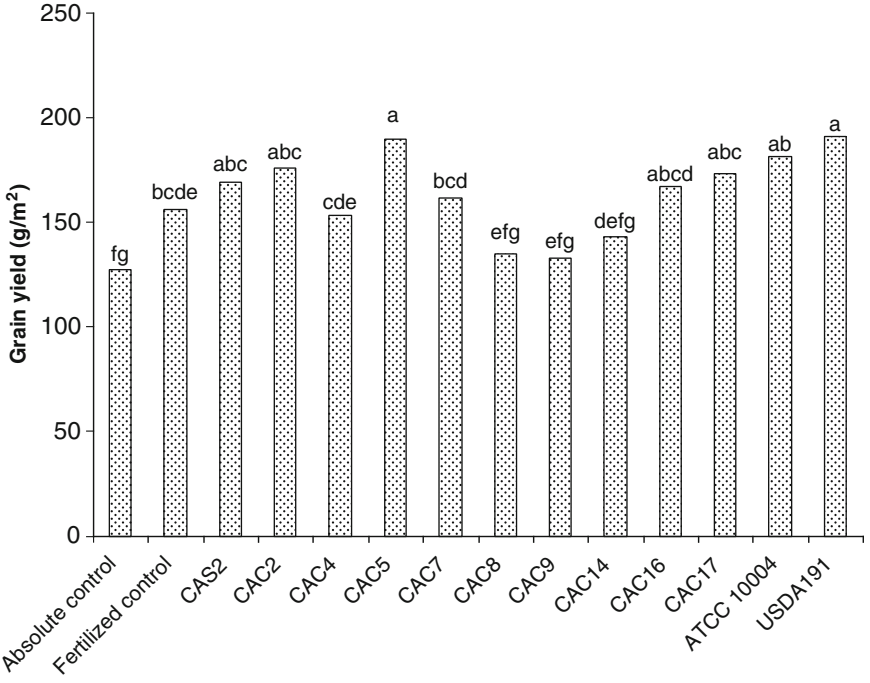



**Fig. 14.3** Effects of inoculation with *Rhizobium* strains isolated from forage legumes of Alberta, Canada on grain yield of wheat cv. Cuba 204 (Bécquer et al. 2007, modified)

tested clearly demonstrated plant growth-promoting effects on wheat grown in the field in Cuba. Positive growth-promoting effects of these strains were also observed on sorghum grown at the Sancti-Spiritus experimental station in Cuba (Bécquer, personal communication). The next steps would be to further characterize these strains and test their growth-promoting effects on wheat under field conditions in the Canadian prairies.

## 14.7 Conclusion

Although the process of BNF offers an economically attractive and ecologically sound means of reducing chemical nitrogen use in agriculture, $N_2$ fixation by non legumes remains a dream. Quispel (1991) suggested that only in endophytic systems are the prerequisites for effective nitrogen fixation likely to be fulfilled in non legume rhizobial interactions. Colonization and $N_2$ fixation by *A. caulinodans* in para-nodules (nodule-like) structures induced by plant growth hormones (2,4-D) treatment has been reported (Chen et al. 1993). Intercellular colonization of wheat by *A. caulinodans* has also been reported by several authors. While some levels of nitrogenase activity have been detected in wheat plants inoculated with *A. caulinodans* (Sabry et al. 1997), the extent to which the fixed nitrogen benefits the plant remains to be verified. In the absence of BNF by non legumes, most researchers have focused on the growth-promoting effects of diazotrophs in non legume systems. The theory proposed by Yanni et al. (2001), suggesting that some rhizobia can form a three-component life cycle including a free-living heterotrophic phase in soil, a $N_2$-fixing endosymbiont phase within legume root nodules, and beneficial growth-promoting endocolonizer phase within cereal roots in the same crop rotation, would need to be further verified. In addition to the known rotational benefits of legumes, optimization of conditions that promote endophytic colonization of cereal roots in rotation with legumes may improve crop performance while decreasing the use of chemical fertilizers required to maintain yield. Despite perennial inconsistencies in the positive effects of PGPR in Canada, the potential for the use of PGPR in non legume crops remains interesting, especially given the potential environmental benefits over the use of conventional agricultural methods. More fundamental research is needed to optimize field inoculation methods that will enhance survival and adaptation of PGPR, especially of *A. caulinodans*, in temperate conditions.

## References

Andrews M, James EK, Cummings SP, Zavalin AA, Vinogradova LV, McKenzie BA (2003) Use of nitrogen fixing bacteria inoculants as a substitute for nitrogen fertiliser for dryland graminaceous crops: Progress made, mechanisms of action and future potential. Symbiosis 35:209–229

Anyia AO, Archambault DJ, Slaski JJ (2004) Growth promoting effects of the diazotroph *Azorhizobium caulinodans* on Canadian wheat cultivars. Proceedings of the 4th International

Crop Science Congress, Brisbane, Australia, pp 201–202. www.cropscience.org.au/icsc2004/poster/2/5/6/863_anyiaa.htm#TopOfPage
Archambault DJ, Li X (2002) Potential benefit of endophytic nitrogen fixing bacteria to wheat (*Triticum aestivum*, L.) and barley (*Hordeum vulgare)*. Stage 1 Investment Project Final Report (unpublished internal report). Alberta Research Council, Vegreville, Alberta, Canada
Baldani VLD, Baldani JI, Dobereiner J (2000) Inoculation of rice plants with the endophytic diazotrophs *Herbasprillum seropedicae* and *Burkholderia* spp. Biol Fertil Soil 30:485–491
Bécquer CJ, Salas B, Archambault D, Slaski JJ, Anyia A (2007) Inoculation of wheat (*Triticum aestivum*, L.) with rhizobia adapted to livestock ecosystems of Alberta, Canada. Pastos Forrajes 30:133–140
Bécquer CJ, Salas B, Archambault D, Slaski J, Anyia A (2008) Selection of rhizobia adapted to livestock production ecosystems from Alberta, Canada, inoculated in corn (*Zea mays*, L.). Stage I: greenhouse. Pastos Forrajes 31:341–354
Biswas JC, Ladha JK, Dazzo FB (2000) Rhizobia inoculation improves nutrient uptake and growth of lowland rice. Soil Sci Soc Am J 64:1644–1650
Borlaug NE (1970) The green revolution: peace and humanity. Nobel peace price. CIMMYT, Mexico DF
Brown ME (1974) Seed and root bacterization. Annu Rev Phytopathol 12:181–197
Bullard M (1999) Further studies with *Azorhizobium caulinodans* as a novel diazotrophic symbiont in temperate cereals. Final Project Report (MAFF Project; Code CE0153), Ministry of Agriculture, Fisheries and Food, ADAS Arthur Rickwood, Mepal Ely, Cambridgeshire
Chen TW, Scherer S, Boger P (1993) Nitrogen fixation of *Azorhizobium* in artificially induced root para-nodules in wheat. In: Nester EW, Verma DPS (eds) Advances in molecular genetics of plant-microbe interactions. Kluwer, Netherlands, pp 593–606
Cocking EC (2001) Xylem colonization of tomato by *Azorhizobium caulinodans* ORS571. Acta Biol Hungarica 52:189–194
Cocking E (2005) OBPC Symposium: Maize 2004 and beyond – Intracellular colonization of cereals and other crop plants by nitrogen-fixing bacteria for reduced inputs of synthetic nitrogen fertilizers. In Vitro Cell Dev Biol Plant 41:369–373
Dowling DN, Broughton WJ (1986) Competition for nodulation of legumes. Annu Rev Microbiol 40:131–157
Dreyfus B, Elmerich C, Dommergues YR (1983) Free-living *Rhizobium* strains able to grow on $N_2$ as sole nitrogen source. Appl Environ Microbiol 45:711–713
Dreyfus B, Garcia JL, Gillis M (1988) Characterization of *Azorhizobium caulinodans* gen. nov., sp. nov., a stem nodulating nitrogen-fixing bacterium isolated from *Sesbania rostrata*. Int J Syst Bacteriol 38:89–98
Elmerich C (1984) Molecular biology and ecology of diazotrophs associated with nonleguminous plants. Biotechnology 2:967–978
Geelen D, Montagu MV, Holsters M (1995) Cloning of an *Azorhizobium caulinodans* endoglucanase gene and analysis of its role in symbiosis. Appl Environ Microbiol 61:3304–3310
Glick BR, Patten CL, Holguin G, Penrose DM (1999) Biochemical and genetic mechanisms used by plant growth promoting bacteria. Imperial College Press, London, p 267
Jjemba PK, Alexander M (1999) Possible determinants of rhizosphere competence of bacteria. Soil Biol Biochem 31:623–632
Kennedy IR, Tchan YT (1992) Biological nitrogen fixation in non-leguminous field crops: Recent advances. Plant Soil 141:93–118
Kloepper JW, Lifshitz R, Schroth MN (1988) Pseudomonas inoculants to benefit plant production. ISI Atlas Sci Anim Plant Sci 8:60–64
Kloepper JW, Lifshitz R, Zablotowicz RM (1989) Free-living bacterial inocula for enhancing crop productivity. Trends Biotechnol 7:39–43
Ladha JK, Tirol-Padre PGC, Watanabe I (1987) Nitrogen fixing ($C_2H_2$-reducing) activity and plant growth characters of 16 wetland rice varieties. Soil Sci Plant Nutr 33:187–200

Lupwayi NZ, Rice WA, Clayton GW (2005) Rhizobial inoculants for legume crops. J Crop Improv 15:289–321

Mathews SS, Sparkes DL, Bullard MJ (2001) The response of wheat to inoculation with the diazotroph *Azorhizobium caulinodans*. Aspects Appl Biol 63:35–42

Noel TC, Sheng C, Yost CK, Pharis RP, Hynes MF (1996) *Rhizobium leguminosarum* as a plant growth-promoting rhizobacterium: Direct growth promotion of canola and lettuce. Can J Microbiol 42:279–283

Peix A, Rivas-Boyero AA, Mateos PF, Rodriguez-Barrueco C, Martínez-Molina E, Velazquez E (2001) Growth promotion of chickpea and barley by a phosphate solubilizing strain of *Mesorhizobium mediterraneum* under growth chamber condition. Soil Biol Biochem 33:103–110

Quispel A (1991) A critical evaluation of the prospects for nitrogen fixation with non legumes. Plant Soil 137:1–11

Reddy PM, Ladha JK, So RB, Hernandez RJ, Ramos MC, Angeles OR, Dazzo FB, de Bruijn K (1997) Rhizobial communication with rice roots: induction of phenotypic changes, mode of invasion and extent of colonization. Plant Soil 194:81–98

Sabry SRS, Saleh SA, Batchelor CA, Jones J, Jotham J, Webster G, Kothari SL, Davey MR, Cocking EC (1997) Endophytic establishment of *Azorhizobium caulinodans* in wheat. Proc R Soc Lond B 264:341–346

Saikia SP, Jain V (2007) Biological nitrogen fixation with non legumes: An achievable target or a dogma? Curr Sci 92:317–322

Sevilla M, Kennedy C (2000) Colonization of rice and other cereals by *Acetobacter diazotrophicus*, an endophyte of sugarcane. In: Ladha JK, Reddy PM (eds) The quest for nitrogen fixation in rice. International Rice Research Institute, Manila, Philippines, pp 151–165

Slaski JJ, Archambault DJ, Li X, Macyk T (2002) BIOLOG® system as a tool for the evaluation of functional diversity of microbial communities in soils. 39th Alberta Soil Science Workshop, Nisku, AB, pp 204–209

Webster G, Gough C, Vasse J, Batchelor CA, O'Callaghan KJ, Kothari SL, Davey MR, Denarie J, Cocking EC (1997) Interactions of rhizobia with rice and wheat. Plant Soil 194:115–122

Yanni YG, Rizk RY, Abd El-Fattah FK, Squartini A, Corich V, Giacomini A, de Bruijn F, Rademaker J, Maya-Flores J, Ostrom P, Vega-Hernandez M, Hollingsworth RI, Martinez-Molina E, Mateos P, Velazquez E, Wopereis J, Triplett E, Umali-Garcia M, Anarna JA, Rolfe BG, Ladha JK, Hill J, Mujoo R, Ng PK, Dazzo FB (2001) The beneficial plant growth promoting association of *Rhizobium leguminosarum* bv. trifolii with rice roots. Aust J Plant Physiol 28:845–870

Yeates C, Gillings MR, Davison AD, Altavilla N, Veal DA (1998) Methods for microbial DNA extraction from soil for PCR amplification. Biol Proc Online 1:40–47

# Chapter 15
# Factors Affecting the Variation of Microbial Communities in Different Agro-Ecosystems

**Munees Ahemad, Almas Zaidi, Md Saghir Khan, and Mohammad Oves**

**Abstract** Soil microbial communities play an important role in supplying essential nutrients to plants by decomposing various organic matters. Composition, structure and functions of microbial communities in soil are, however, under the constant control of the environment including various agricultural management practices. Due to scarcity of convenient methods for exploration, our understanding of the different degrees and dynamics of microbial community variations are limited. An attempt will be made to understand such structural and functional variations employing molecular tools. Earlier it was believed that it is the plant community that exerts control over the microbial community, but recently, some findings have suggested that it is actually the microbial community that acts as a driver of plant community structure and dynamics. Attention will therefore be paid to highlight some of these issues, and the effect of various farm management practices on the composition and functions of microbial communities This is likely to lead to the development of best management practices for improving soil fertility and, consequently, agricultural productivity to improve the sustainability of agro-ecosystems.

## 15.1 Introduction

Microorganisms are a fundamentally important component of the soil habitat where they play key roles in ecosystem functioning through controlling nutrient cycling reactions essential for maintaining soil fertility, and also contribute to the genesis and maintenance of soil structure (Kirk et al. 2004). Despite their importance to the functioning of ecosystems, they are rarely explicitly considered in individual ecosystem or global process models. In addition to methodological limitations, a

Md. S. Khan(✉), M. Ahemad, A. Zaidi, and M. Oves
Faculty of Agricultural sciences, Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh, 202002, Uttar Pradesh, India
e-mail: khanms17@rediffmail.com

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_15, 

primary reason for this gap is their overwhelming diversity. Estimates of soil microbial diversity range from thousands to a million microbial species in a few grams of soil (Torsvik and Øvreås 2002; Gans et al. 2005), and how this diversity affects ecosystem processes is largely unknown (Torsvik et al. 2002; Crawford et al. 2005; Azam and Malfatti 2007). Furthermore, it is extremely difficult to assess, identify and track each individual microorganism in an ecosystem. Due in part to the lack of convenient and appropriate methods for exploration, our understanding of the different degrees and dynamics of microbial community variation as induced by soil type, plant type, or plant development is so far limited (Mahaffee and Klöpper 1997; Duineveld et al. 1998).

Despite all these problems, in recent times, attention has been focused on the impacts that microbial communities have on soil fertility and crop productivity in different agro-ecosystems. In soils, biologically mediated processes are known to exert profound influences on the status of soil health by: (1) degrading organic residues, (2) transformation of organic matter, (3) mineralization of nutrients, and (4) formation of soil aggregates (Haynes 1999). However, a great variety of abiotic and biotic factors shape soil- and plant-associated habitats and modify the compositions and activities of inhabiting microbial communities, which in turn bear upon the quality of their environment, the growth of plants, and the production of root exudates (Bever et al. 1997). For example, changes in physico-chemical variables such as pH and nutrient levels (Kennedy et al. 2004, 2005), floristic community change (Grayston et al. 1998; Grayston et al. 2004), changes in soil physical structure (Ibekwe et al. 2002; Sessitich et al. 2006), and the impacts of grazing animals have all been proposed as principal causes of shifts in microbial community structure, although it is still not fully understood which environmental factors influence microbial community change. Microbial communities in root-associated habitats respond with respect to density, composition, and activity to the abundance and great diversity of organic root exudates, eventually yielding plant species-specific microflora which may also vary during plant development stages (Lynch and Whipps 1990; Bowen and Rovira 1991; Mahaffee and Klöpper 1997; Weisskopf et al. 2006). Thus, interactions between plants and soil microbes are highly dynamic in nature and based on coevolutionary pressures (Reinhart and Callaway 2006). Such microbial communities are greatly influenced by plant species (Innes et al. 2004; Batten et al. 2006), genotypes within species (Kowalchuk et al. 2006), and diversity of carbon substrates and signaling compounds provided by the plants (Zak et al. 2003; Broeckling et al. 2008).

Agricultural improvement by applying chemical fertilizers has become a common practice in different parts of the world. However, the excessive and injudicious application of these fertilizers cause change in the soil chemical and physical properties by disturbing the soil microbial biomass and activities, an early indicator of soil fertility (Brookes 1995; Trasar-Cepeda et al. 1998). For instance, N fertilization has been shown to significantly affect soil microbial biomass and activities (Saliana-Garcia et al. 1997; Li et al. 2002), and long-term application of $NH_4^+$ or $NH_4^+$ forming fertilizers may lead to changes in the soil microbial communities in terms of promoting nitrifying populations, enhancing nitrification rates, and

increasing potential risks of groundwater contamination with $NO_3^-$–N (Tabatabai et al. 1992). Likewise, phosphate application affects soil microbial community structures in grassland ecosystems (Rooney and Clipson 2009). The understanding of interactions between fertilizers, plants and microbial communities in soils, in turn will lead to better understanding of the health of soils and the design of strategies for crop improvement in different agro-ecosystems.

## 15.2 Microbial Community Structure and Functional Analysis

It has long been recognized that the activity of soil microorganisms plays an intrinsic role in residue decomposition, nutrient cycling, and crop production. And, hence, the microbial diversity at the functional level plays a crucial role in the long-term stability of an ecosystem. Variations in the structure and function of microbial communities in soils have, however, been linked primarily to the quantities and qualities of soil organic materials (Saetre and Bååth 2000), which in turn are greatly affected by the availability and biochemical composition of the litter (Johansson 1995), vegetation (Priha et al. 2001), and root exudates (Grayston and Campbell 1996). A patchy distribution of organic litter may result in a high degree of biochemical compartmentalization (Bauhus et al. 1998; Priha and Smolander 1999; Côté et al. 2000; Priha et al. 2001) and may ultimately cause a spatial aggregation of the forest soil microbiota (Saetre and Bååth 2000). Thus a post-fire successional sequence from deciduous to coniferous species will have a significant impact on the morphological and physiological profiles of soil microbial communities (Bormann and Sidle 1990; Bending et al. 2002; Merilä et al. 2002). This may be due to differences in the structure and composition of leaf and needle litter, which alter nutrient availability in the soil (Priha and Smolander 1997) and cause shifts in microbial populations as the community adapts to new environmental conditions. Understanding microbial community structure shifts following implementation of various land use and management systems, therefore may lead to the development of best management practices for different agro-ecosystems.

Microbial diversity describes complexity and variability at different levels of biological organization. It includes genetic variations within taxons (species), and the number (richness) and relative abundance (evenness) of taxons and functional groups (guilds) in communities. Important aspects of diversity at the ecosystem level are the range of processes, complexity of interactions, and number of trophic levels. Although animal and plant ecologists can quantify and identify different species through (relatively) easily identifiable traits, it is extremely difficult to do this with microbial communities, because microbes vary greatly in their activities. Thus, measures of microbial diversity should include multiple methods integrating holistic measures at the total community-level and partial approaches targeting structural or functional subsets. However, the assessment of the composition and/or function of soil microbial communities still presents a number of challenges, due

to which less than 1% of bacterial species and an unknown percentage of fungi have been so far recovered (Rozak and Colwell 1978; Van Elsas et al. 1997). Unfortunately, changes in microbial community structure and diversity due to seasonal and temporal variations in nutrient or physical conditions are slow and gradual, making it difficult to further interpret the data and obtain conclusive results. Disturbances in microbial community equilibrium influenced by changes in environmental conditions and soil management practices have been reported (Sun et al. 2004). The challenge is how to quantify the changes and link them to the corresponding ecosystems.

In the premolecular biology era, microbial communities were determined by isolating and screening them onto commercially available standard laboratory media, and the total numbers and/or species of microbial communities in a particular soil were assessed using conventional methods, such as the most probable number (MPN) and plate-counting techniques. Such methods, however, have certain limitations (Torsvik et al. 1990), such as they address only the culturable populations present in the rhizosphere and provide only limited information about the functional diversity of the microbial communities. Similarly, microscopic techniques can be used to obtain information about numbers and, potentially, spatial distribution, but these approaches lack the discriminating ability to assess diversity and distinguish between multiple microbial populations. Recently, several modern methods including BIOLOG$^{TM}$ analysis, phospholipid fatty acid (PLFA) analysis and nucleic acid-based analysis have been developed to identify and characterize soil microbial diversity. Despite various limitations (Konopka et al. 1998; Garland 1999; Preston-Mafham et al. 2002), the Biolog system has been used to characterize microbial communities associated with boreal forest soils (Staddon et al. 1998b; Adkins et al. 2001), various crop types (Garland and Mills 1991; Garland 1996), grasslands (Zak et al. 1994), tree species (Grayston and Campbell 1996), plant rhizospheres (Grayston et al. 1998; Kent and Triplett 2002), and environmental samples. In addition, differences between soil microbial communities along a climatic gradient (Staddon et al. 1998a) and bacterial communities affected by soil types have also been analyzed (Winding 1994).

Garland and Mills (1991) compared the patterns of carbon source utilization at community-level for microbial communities of different habitats, such as samples from freshwater, saltwater, estuarine, and hydroponic solutions, from the rhizosphere of hydroponically grown wheat, and from soils, using commercially available microtiter plates that contain 95 carbon substrate. This and other studies suggest that, in addition to establishing ecologically relevant classifications of microbial communities, substrate utilization profiles might offer information with regard to community function, metabolic potential (Winding 1993), or functional diversity (Zak et al. 1994; Haack et al. 1995). When inoculum density was controlled, patterns of positive and negative responses exhibited by microbial communities to each of the carbon sources were reproducible. Even so, the rates and extents of substrate oxidation by the communities were reproducible but were not simply the sum of those exhibited by community members when tested separately. Replicates of the same model community clustered when analyzed by principal

components analysis (PCA), and model communities with different compositions were clearly separated on the first PCA axis, accounting for >60% of the dataset variation. However, the substrates were interpreted by PCA to be most significant in distinguishing the communities that changed with reading time, reflecting the nonlinearity of substrate oxidation rates. Although whole-community substrate utilization profiles were reproducible signatures for a given community, the extent of oxidation of specific substrates and the numbers or activities of microorganisms using those substrates in a given community were not correlated.

Compared to substrate utilization profiles, PLFA profiles have been shown to be more sensitive and can be used as biomarkers to identify and quantify microbial biomass as they are essential components of microorganisms (Priha and Smolander 1999; Waldrop et al., 2000; Grayston et al. 2003). The two community-based microbiological measurements, namely potential C source utilization patterns in Biolog microtiter plates and PLFA profiles, have been employed to examine metabolic fingerprints of soil microbial communities and changes in species composition of Palouse and Ritzville silt loams collected from the rhizosphere of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.), pea (*Pisum sativa* L.), jointed goatgrass (*Aegilops cylindrica* L.), and downy brome (*Bromus tectorum* L.) grown under field and greenhouse environments. PCA of PLFA profiles and C source utilization patterns were used to describe changes in microbial biomass and metabolic fingerprints from the two soil types. Biomass measurements from extractable PLFA profiles per g dry weight ranged from 28.8 nmol in wheat soil in the greenhouse to 71.4 nmol in pea soil in the field. In general, biomass was higher in all the field samples than in the greenhouse samples. PCA of the two soils with different plants in the field and greenhouse showed clear separation. PCA of C utilization patterns on the effects of environment on soil microbial community yielded similar results with PLFA measurements. However, higher variability observed among different plants with the Biolog data resulted in the low amount of variance for Biolog data explained by the first two dimensions of the PCA suggesting that PLFA may be more sensitive for community analysis than the Biolog technique (Ibekwe and Kennedy 1998).

Furthermore, the introduction of bio-molecular approaches like nucleotide sequence analysis of ribosomal RNA genes (the 16S rRNA genes in bacteria and 18S rRNA genes in fungi) into microbial ecology has allowed characterization of microbial communities without pure cultures. In such approaches, the total microbial community DNA can be directly extracted from soils; hence, the repeated subculturing of microbial communities could be avoided. Such DNA is used as a template for amplification of ribosomal genes from all the members of that community in the polymerase chain reaction (PCR), a technique that allows for high resolution analysis of the microbial community structure. The resulting individual gene products can then be cloned and sequenced to affirm the identity of the microbial species from which the original target sequence was amplified (Giovannoni et al. 1990; Hugenholtz et al. 1998). Furthermore, the heterogeneous mix of PCR products recovered from the environment by specific amplification of bacterial 16S rDNA sequences can be separated by a culture-independent method,

denaturing gradient gel electrophoresis (DGGE), which separates the DNA fragments based on their nucleotide content rather than size alone (Muyzer et al. 1993). The benefit of this approach is that a molecular fingerprint of the community structure is generated for each soil, such that each band in each lane of the gel theoretically represents a different bacterial species. Molecular approaches, in general, are rapid, specific and more reliable, compared to conventional culturing techniques, and have provided a further insight into the identification of the remaining 99% of microbial species.

Both conventional and molecular approaches have been employed to assess the impact of management practices, changes in seasons, and environment stress on microbial community structure and functions. For instance, de Lima et al. (1999) isolated 82 bacterial strains from the sugarcane (*Saccharum officinarum*) agroecosystem and grouped them in 16 different genera and 35 species of bacteria. The microbial diversity was found greater in wastewater collected from the stabilization ponds and in the soil sample collected from the sugarcane plantation burned before harvesting. Such variations in microbial communities supported the very concept of Odum's (1971) classical observation that communities with low energy cost for maintaining the entropy (high respiration: biomass ratio) divert their energy supply into diversity, which may be happening to the microbiota of the environments. A high respiration:biomass ratio observed in unproductive soil irrigated with vinasse in the tableland soil of the Usina Japungu (de Luna and Grisi 1996) also supported this theory. However, Atlas (1984) further pointed out that diversity changes in response to environmental stress may lead to an increase in diversity by selective toxicity causing elimination of dominant organism, or diversity may decrease by elimination of many species. To substantiate this further, Torsvik et al. (1997) observed a substantial reduction in diversity in perturbed soil due to agriculture, as compared to undisturbed environments. Also, molecular analysis using PCR amplification of small-subunit rRNA of microbial diversity in Amazonia soils, demonstrated microbial population shifts related to deforestation in the Amazonian forest, with predominance of *Bacillus* and high G+C Gram-positive-like sequences in pasture and predominance of *Clostridium* and unclassified bacteria in the forest (Borneman and Triplett 1997). Of these organisms, *Bacillus* seems to be a natural indicator of inhospitable environmental conditions whose endospore-forming ability certainly explains their occurrence in these situations.

## 15.3 Factors Causing Change in Microbial Community Structure and Function

Soil microbial communities are subjected to a range of factors that can be broadly classified as: (1) stress factors that constantly limits microbial growth and do not change markedly over time, and (2) disturbance factors that involves rapid changes and often causes destruction of organism biomass. Although both stress

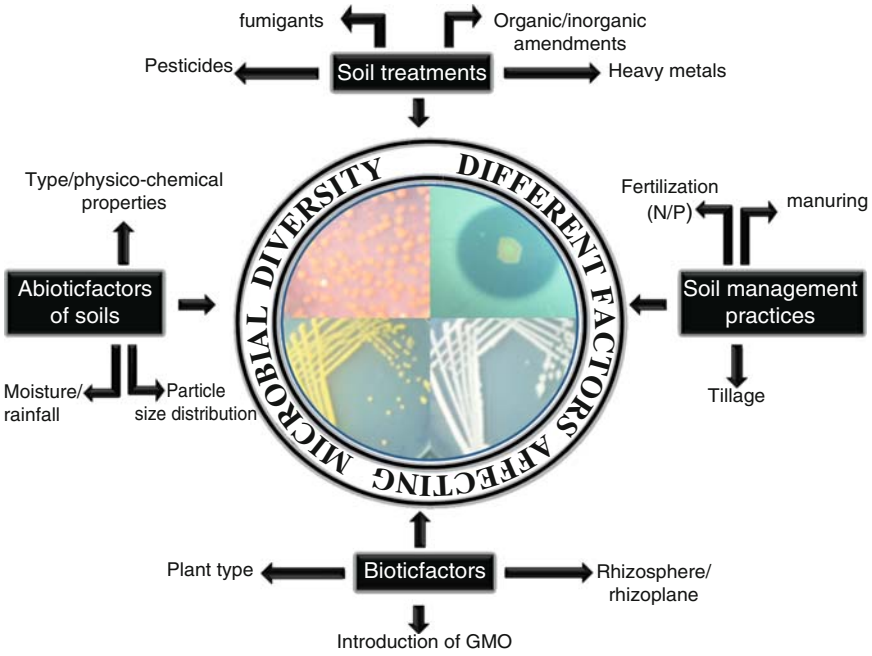



**Fig. 15.1** Environmental factors influencing the microbial structure and functions

and disturbance are strong drivers of the microbial community, they exert their effects independently from one another (Williamson and Wardle 2007). The factors causing changes in microbial community structure and functions (Fig. 15.1) are reviewed and discussed in the following section.

### *15.3.1 Rhizosphere, Root Exudates and Soil pH*

The plant rhizosphere, a term introduced by Hiltner to denote the region of the soil that is subjected to the influence of plant roots (Hiltner 1904), is a dynamic environment in which many factors affect the structure and composition of the microbial communities that colonize the roots. The rhizosphere communities vary spatially in a radial direction from the root surface, including the endorhizosphere, rhizoplane, and rhizosphere zones (Assmus et al. 1995; Bosse and Frenzel 1997; Gilbert and Frenzel 1998), as well as in specific root locations along the root axis (Gilbert and Frenzel 1998; De Leij et al. 1994). Microbial communities associated with the rhizosphere also vary depending on the plant species (Grayston et al. 1998), the soil type (Campbell et al. 1997), and cultural practices (Table 15.1) such as crop rotation or tillage (Lupwayi et al. 1998). Understanding the structure and

**Table 15.1** Effect of plant and soil types on microbial community structure

| System and factors studied | Methods used | Results and conclusion | References |
|---|---|---|---|
| Plant cultivar (maize) and soil-effect on a specific bacterial group *Paenibacillus* | PCR-TGGE | Soil type showed higher effect than plant cultivar on *Paenibacillus* communities | Da Silva et al. (2003) |
| Plant species (chickpea, rape, and Sudan grass); soil type (sandy, sandy loam, and clay); root zone location | PCR-DGGE | Bacterial community structure in the rhizosphere was affected by a complex interaction between soil type, plant species, and root zone location | Marschner et al. (2001) |
| Soil type, plant type (clover, bean, alfalfa), plant age | PCR-TGGE | The plant species type had the greatest effect on microbial community structure | Wieland et al. (2001) |
| Microbial community in the spermosphere as affected by soil type and seed type | Biolog-CLPP; FAME | Soil type affected microbial community structure more than seed type | Buyer et al. (1999) |
| Soil type, plant type (wheat, ryegrass, bentgrass, and clover) | Biolog-CLPP | Plant effect with significant difference in microbial communities from the different plant species | Grayston et al. (1998) |
| Plant (maize) development, cultivar, and soil-effect | Cultivation–plating enumeration | Between the factors studied, soil had the dominant effect on microbial diversity | Chiarini et al. (1998) |
| Plant type (canola, wheat); soil type | FAME | Effect of plant type stronger than that of soil type soil-effect stronger than plant effect | Germida et al. (1998) |
| Plant (flax, tomato) and soil type; effect on fluorescent pseudomonads | Cultivation; REP-PCR, RFLP | Soil-effect stronger than plant effect | Latour et al. (1996) |

Adapted from Garbeva et al. (2004)

species composition of these communities is fundamental to understanding how soil biological processes are influenced by such factors.

Generally, the microbes that inhabit the rhizosphere serve as an intermediary link between the plant, which requires soluble inorganic nutrients, and the soil, which contains the necessary nutrients but mostly in complex and inaccessible forms. However, the magnitude of the rhizosphere effect depends mainly on the nature and amount of root exudates, a complex mixture of chemicals and organic compounds secreted into the soil by the roots that affects microbial interactions (Bais et al. 2004). Root exudate concentrations are determined by many factors including species and nutritional status of the plant, plant age, and edaphic and climatic factors (Marschner 1995). About 5% to 60% (Marschner 1995) of the photosynthetic carbon fixed by the plant is released into rhizosphere by exudation through plant roots. Root exudates may consist of water soluble compounds such as amino acids, sugars, hormones, and vitamins that leak from the root surfaces, or actively secreted polymeric carbohydrates and enzymes. The products of the roots

may also include gases such as $CO_2$ and ethylene, lysates released when cells autolyse, solid materials including cell walls, sloughed cells and root border cells, and eventually parts as large as root hairs or roots themselves (Kang and Mills 2004) (Table 15.2). The effect of root exudates on the rhizosphere microbes is likely to be more intense, stimulatory or inhibitory. Depending on the composition of the exudates, plant may be able to: (1) enhance the possibilities and success of symbiotic relationships, (2) affect the soil microbial community, (3) alter the physical and chemical properties of the soil, and (4) inhibit the propagation or growth of another plant species. In fact, most rhizosphere bacteria and fungi are highly dependent on associations with plants that are clearly regulated by root exudates (Yang and Crowley 2000; Bais et al. 2004), and in the rhizosphere, density of microorganisms can reach $10^{10}$–$10^{12}$ organisms $g^{-1}$ soil. The influence of individual plants is reflected in the rhizosphere as the R:S (rhizosphere to nonrhizosphere ratio). For bacteria and fungi, values commonly range from 5 to 20. Actinomycetes, a somewhat less affected group of microorganisms of the rhizosphere, may reveal R:S ratios between 2 and 12.

Based on differences in root exudation and rhizodeposition in different root zones, rhizosphere microbial communities can vary in structure and composition in different root locations or in relation to soil type, plant species, nutritional status, age, stress, disease, and other environmental factors (Griffiths et al. 1999; Mahaffee and Klöpper 1997). As an example, Wieland et al. (2001)) observed while studying 32 microcosms in three habitats, namely soil, rhizosphere, and rhizoplane, that the type of plant species (clover, bean, or alfalfa) had the greatest effect in plant-associated habitats and also affected soil patterns through the variation of microbial communities as represented by the patterns of a sequence-specific separation of rRNA target sequences. Plant development demonstrated a minor habitat-dependent effect that was partly obscured by replicate variation. Ruiyu et al. (2007), while investigating the dynamics of microbial populations and their functional diversities in the seedling rhizospheres of rice (*Oryza sativa*) cultivars with varied allelopathic activities by employing agar plate bioassay, fumigation, and Biolog analysis, concluded that the rice cultivars significantly affected the microbial carbon content in their associated rhizospheric soil. Moreover, microbial carbon contents and the respiration rate of the soils varied with the type of cultivar. The microbial flora in the rhizospheric soil of different rice cultivars was dominated by bacteria (58.4–65.6%), followed by actinomycete (32.2–39.4%) and fungi (2.2–2.8%). Moreover, Biolog analysis showed that the value of average well color development (AWCD) differed significantly among rice cultivars. It was always the highest in the rhizospheric soil of the strongly allelopathic rice cv. PI312777 and the lowest in the rhizospheric soil of the poorly allelopathic rice cv. Lemont. The AWCD value reached the maximum in all the sampled soils after 144 h of incubation. The AWCD values from the rhizospheric soils of PI312777, IAC47, Iguape Cateto, and Lemont were 1.89, 1.79, 1.60, and 1.43 times higher than that of the control soil, respectively. PCA identified three principal component factors (PCF) in relation to carbon sources, accounting for 70, 11, and 7% of the variation, respectively, and 19 categories of carbon sources were positively correlated to the three principal

**Table 15.2** Components of root exudates, functions in the rhizosphere, and examples identified in root exudates of different plant species

| Exudate component | Rhizosphere functions | Specific compounds identified in root exudates |
|---|---|---|
| Organic acids | Nutrient source | Citric, glutaric |
| | Chemoattractant signals to microbes | Oxalic, malonic |
| | Chelators of poorly soluble mineral nutrients | Malic, aldonic |
| | Acidifiers of soil | Fumaric, erythronic |
| | Detoxifiers of Al | Succinic, ferulic |
| | *nod* gene inducers | Acetic, butanoic, butyric syringic, valeric, rosmarinic, Glycolic, *trans*-cinnamic, piscidic, vanillic, formic tetronic, aconitic, lactic, pyruvic |
| Amino acids | Nutrient source | α- and (β-alanine, proline |
| | Chelators of poorly soluble mineral nutrients | Asparagine, valine |
| | Chemoattractant signals to microbes | Aspartate, tryptophan, cystein, ornithine, cystine histidine, glutamate, arginine,glycine, homoserine, isoleucine, phenylalanine, leucine, aminobutyric acid, lysine, aminoadipic acid, methionine, serine, threonine |
| Sugars and vitamins | Promoters of plant and microbial growth | Glucose, deoxyribose |
| | Nutrient source | Fructose, oligosaccharides, galactose, biotin, maltose thiamine, ribose, niacin, xylose, pantothenate, rhamnose, rhiboflavin, arabinose, raffinose |
| Enzymes | Catalysts for P release from organic molecules | acid/alkaline phosphatase |
| | Biocatalysts for organic matter transformations | Invertase, amylase, protease |
| Purines | Nutrient source | Adenine, guanine, cytidine, uridine |
| Inorganic ions and gases | Chemoattractant signals to microbes | $HCO_3$, $OH^-$, $H^+$, $CO_2$, $H_2$ |
| Phenolics | Nutrient source | Liquiritigenin, luteolin |
| | Chemoattractant signals to microbes | Daidzein, 4′,7-dihydroxyflavanone |
| | Microbial growth promoters | Genistein, 4′,7-dihydroxyflavone |
| | *nod* gene inducers in rhizobia | Coumetrol, 4,4′-dihydroxy -2′-methoxychalcone |
| | *nod* gene inhibitors in rhizobia | Eriodictyol, 4′-7-dihydroxyflavone |
| Root border cells | Produce signals that control mitosis, produce signals controlling gene expression, stimulate microbial growth, release chemoattractant, synthesize defense molecules for the rhizosphere, act as decoys that keep root cap infection-free, release mucilage and proteins | |

Compiled from Dakora (2003), Dakora and Phillips (2002), and Bais et al. (2004)

components. In addition, the total microbial population in the rhizospheric soil was significantly positively correlated with AWCD, microbial biomass carbon, microbial respiration, and Shannon index. There was a significantly positive correlation between the total microbial population and the inhibition rate (IR) on the root length of lettuce owing to the different allelopathic activities of the rice cultivars. The results suggested that changes in microbial population, activity, and functional diversity in the rhizospheres are highly cultivar-dependent.

During the growth of new roots, exudates secreted in the zone of elongation behind the root tips support the growth of primary root colonizers that utilize easily degradable sugars and organic acids. In the older root zones, carbon is deposited primarily as sloughed cells and consists of more recalcitrant materials, including lignified cellulose and hemicellulose, so that fungi and bacteria in these zones are presumably adapted to crowded, oligotrophic conditions. Other nutritionally distinct sites include the sites of lateral root emergence and the secondary, nongrowing root tips, which are relatively nutrient-rich environments colonized by mature communities. Soil chemical changes related to the release of organic and inorganic compounds, and the respective products of their microbial metabolism, are important factors affecting microbial populations, availability of nutrients, solubility of toxic elements in the rhizosphere, and thereby the ability of plants to cope with adverse soil chemical conditions. Organic compounds in root exudates are continuously metabolized by root-associated microorganisms and in the rhizosphere. Mobilization of micronutrients or heavy metals in the rhizosphere has also been related to rhizosphere acidification and to complexities with organic acids in root exudates (Marschner 1995; Pinton et al. 2001; Waisel et al., 1991).

Soil pH is an important factor affecting the functioning of soil microbial communities in soils, and may influence rates of substrate utilization; the values of substrate utilization were significantly higher in soils of higher pH (Anderson and Joergensen 1997). And, hence, soil pH has been found as the most influential environmental factor responsible for discrimination among microbes (Grayston et al. 2003). For example, soil acidity has shown a considerable decrease in the availability of carbon to microbial communities (Anderson and Domsch 1993; Baath et al. 1995), and to slower bacterial growth rates (Baath 1998). However, studies have also shown optimum pH for growth of bacterial communities to be correlated with soil pH from which the communities are extracted, indicating that different bacterial communities are adapted to different pH values (Baath 1996; Andersson and Ingvar Nilsson 2001). For instance, bacterial communities associated with coniferous forest soils may contain larger proportions of Gram-positive bacteria adapted to the acidifying environment; in contrast, increases in soil pH may result in larger proportions of Gram-negative bacteria (Frostegard et al. 1993; Pennanen 2001). Additionally, numbers of bacteria have been shown to decrease in acidified soils (Baath et al. 1980), possibly because bacteria are less adapted to acidic conditions in soil compared to fungi (Matthies et al. 1997). However, microbial biomass C and inorganic N do not respond to changed soil moisture while $N_2O$ and $CO_2$ efflux and respiration increase after increasing moisture in the agricultural soils. Moreover, in the agricultural systems, reductions in both

the measures of microbial diversity and the resistance of the microbial community to change after a perturbation have been found to be associated with lower microbial responses to increased moisture availability (Steenwerth et al. 2005).

### *15.3.2 Soil Management Practices*

Agricultural management practices have strong impacts on soil microbes including both the indices related to biomass and activity as well as those related to community composition (Follett and Schimel 1989; Aon et al. 2001; Steenwerth et al. 2003; Potthoff et al. 2006). Therefore, in recent times, attention has been focused on the influence that agricultural management practices have on biological and biogeochemical properties of soils (Gregorich et al. 1994; Franzluebbers et al. 1995). Soil microbial biomass and activities are frequently used as an indicator of changes in soil chemical and physical properties resulting from soil management and environmental stress in agricultural ecosystems (Brookes 1995; Trasar-Cepeda et al. 1998). Understanding how management practices influence soil fertility and agricultural productivity is essential to improve the sustainability of agro-ecosystems (Wardle et al. 1999). The effect of different management regimes and perturbations on the soil microbial community (e.g., crop rotations, manure applications, and tillage) has been studied in a wide range of soil and management systems.

#### 15.3.2.1 Tillage

Cultivation and tillage practices profoundly disrupt the soil by: (1) breaking up soil aggregates, (2) increasing soil compaction, (3) exposing previously protected organic matter, and (4) mixing soil horizons (Beare 1997; Allison et al. 2005). In turn, such alterations lead to a significant decline in microbial biomass, reduce the abundance of fungi, aerobic microorganisms, and facultative anaerobes, while increasing the relative abundance of Gram-negative bacteria (Doran 1980; Beare 1997). Tillage practice in agricultural soils not only affects the microbial diversity but also influences species composition, N transformation processes mediated by microbes, and interactions between organisms and the soil pore network, and modulates the role of soil structure in mediating oxygen movement to sites of microbial activity in soil (Young and Ritz 2000). The agricultural soils which are tilled frequently and subjected to crop rotations have shown lower microbial diversity compared to those observed in soils which are either not tilled or have been infrequently tilled (Øvreås and Torsvik 1998). For example, by extracting microbial RNA, Buckley and Schmidt (2001) found that the abundance of groups comprising of Gram-positive bacteria (aerobes) and fungi were significantly lower in fields abandoned from agriculture 7 years earlier. In another study, Fraterrigo et al. (2006) reported that old farms had similar compositional patterns, despite

having experienced relatively little tillage and having been abandoned since 1930. The high bulk density of former farms (Fraterrigo et al. 2005) and the elevated abundance of Gram-negative bacterial markers suggest that these enduring microbial patterns may be partly due to a continued state of soil hypoxia; a common condition in highly managed soils. Moreover, tillage events contribute to decreased soil quality by increasing emissions of greenhouse gases (i.e., $CO_2$, NO, or $N_2O$), and increasing the potential for nitrate leaching to groundwater. Such negative aspects require proper attention before considering the benefits of tillage for increasing the health and productivity of crops (Jackson et al. 2003). In addition, research is also needed to assess potential effects of long-term agricultural management practices that may mask microbial responses to recent management change, as well as to identify conditions that lead to high microbial community resiliency in response to management so that communities remain similar under a given crop despite different preceding crops (Stromberger et al. 2007).

#### 15.3.2.2 Nutrient Management

Agricultural improvement through fertilizer application by the field practitioners is being practised around the world to achieve maximum plant productivity; but this practice is not only expensive, but the repeated and injudicious application of chemical fertilizers also leads to the loss of soil fertility (Gyaneshwar et al. 2002) by disturbing microbial diversity and catabolic activity (Zhang et al. 2007; Guanghua et al. 2008), and consequently reduces yield of crops. For instance, N fertilization has been shown to significantly affect soil microbial biomass and activities (Ladd et al. 1994; Saliana-Garcia et al. 1997). Long-term application of $NH_4^+$ or $NH_4^+$-forming fertilizers may lead to changes in the structure of soil microbial communities in terms of promoting nitrifying populations, enhancing nitrification rates, and increasing potential risks of groundwater contamination with $NO_3^-$–N (Tabatabai et al. 1992). However, such deleterious effects of N fertilizers could be reduced by controlled release and deep application (0–10 cm) of mineral fertilizers (Chu et al. 2005). Similarly, the long-term effect of different sources of phosphate fertilizers, like single superphosphate, North Carolina phosphate rock, partially acidulated North Carolina phosphate rock, and diammonium phosphate, on microbial activities, like basal respiration, substrate-induced respiration, inhibition of substrate-induced respiration by streptomycin sulfate (fungal activity) and actidione (bacterial activity), and microbial biomass C of pasture soils, has been reported (Bolan et al. 1996). The fertilizer addition caused an initial decrease in basal and substrate-induced respiration but had no effect on total microbial biomass. The initial decline in basal and substrate-induced respiration with the fertilizer addition was restored within 8 weeks after incubation. In the field experiment, the fertilizer addition had no significant effect on basal respiration but increased substrate-induced respiration and microbial biomass C (Bolan et al. 1996). Similarly, the application of triple superphosphate (at 94 kg $ha^{-1}$) has produced a substantial

reduction in microbial respiration and metabolic quotient ($qCO_2$) (Chandini and Dennis 2002). Recently, multidimensional scaling plots and canonical correspondence analysis revealed that phosphate ($K_2PO_4$ at 25 kg P $ha^{-1}$) addition and its interaction with upland grassland plant species (*Agrostis capillaries*, *Festuca ovina*, *Lolium perrene*) resulted in considerable changes in the fungal and bacterial communities of upland soil. Both fungal and bacterial community structures were significantly affected by phosphate suggesting that phosphate application may be an important contributor to microbial community structural change during agricultural management (Rooney and Clipson 2009). Similarly, Vineela et al. (2008) studied the long-term effect of fertilization on soil microbial communities and found that the bacterial counts were higher in treatments where combinations of organic and inorganic fertilizers were applied compared to control. Fungal population was higher in treatments under continuous inorganic fertilization whereas a high number of bacteria were found in integrated use of organic and inorganic fertilizers. At most of the locations, soil organic C and microbial biomass C showed a significant ($p \leq 0.05$) positive correlation with microbial populations. In contrast, Sun et al. (2004)) while examining 16S rRNA gene fingerprints of bacterial communities in six agro-ecosystems soils treated with manure for over a century or different fertilizers for over 70 years reported that the bacterial community structure and diversity in the manure-treated soil was more closely related to the structure in the untreated soil than that in soils treated with inorganic fertilizers. In addition, soils treated with P and N–P had bacterial community structures more closely related to each other than to those of soils given other treatments, suggesting that bacterial community structure was closely related to agro-ecosystem management practices conducted for over 70 years. In contrast, application of mineral fertilizers (e.g., N, P, K) in combination with farmyard manure (FYM) had greater numbers of soil microbes and more complex structure of the ammonium-oxidizing bacteria (AOB) community than those receiving mineral fertilizers alone, suggesting that mineral fertilizers along with FYM could be more effective for increasing the quantity of soil microbes, enriching the AOB community, and improving the soil bio-fertility (Guanghua et al. 2008).

### *15.3.3 Impact of Seasonal Variations*

Another important issue to elucidate is how seasonal variations influence qualitative variation in community composition. In this regard, the impact of seasonal variations on soil microbial communities of wheat grown under a field of an experimental farm in The Netherlands was assessed in different seasons over a 1-year period, using both cultivation-based and molecule-based methods (Smit et al. 2001). Fatty acid-based typing of bacterial cultures obtained via plating revealed a diverse community including predominantly Gram-positive bacteria, and only a few isolates represented *Proteobacteria* and green sulfur bacteria. Interestingly, *Micrococcus*, *Arthrobacter*, and *Corynebacterium* were detected throughout the year, while

*Bacillus* was found only during the month of July. The isolate diversity was lowest in July, and the most abundant bacteria, *Arthrobacter oxydans*, and members of the genus *Pseudomonas*, were found in reduced numbers in July. Moreover, molecular analysis suggested that diversity of cloned 16S ribosomal DNA (rDNA) sequences was greater than the diversity among cultured isolates. In addition, based on analysis of 16S rDNA sequences, there was a more even distribution among five main divisions, *Acidobacterium*, *Proteobacteria*, *Nitrospira*, cyanobacteria, and green sulfur bacteria, but no clones were found belonging to the Gram-positive bacteria, which dominated the cultured isolates. Cluster analysis of the patterns revealed that the bacterial community observed in July was clearly different from those observed in the other months. Thus, both molecular- and cultivation-based methods indicated that the community present in July had the largest difference from the communities of the other months. Based on the distribution of 16S rDNA sequences among the bacterial divisions found in this study and in literature, it was suggested that the ratio between the number of *Proteobacteria* and *Acidobacterium* organisms might be indicative of the trophic level of the soil. Effects of seasonal shifts on rhizosphere microbial populations of pea, wheat, and sugar beet (*Beta vulgaris* var. amythyst) have also been determined by culturing, rRNA gene density gradient gel electrophoresis, and Biolog. Culturable bacterial and fungal rhizosphere community densities were stable in pea and wheat rhizospheres, with dynamic shifts observed in the sugar beet rhizosphere. Successional shifts in bacterial and fungal diversity as plants mature demonstrated that different plants select and define their own functional rhizosphere communities. Assessment of metabolic activity and resource utilization by bacterial community-level physiological profiling demonstrated greater similarities between different plant species rhizosphere communities at the same rather than at different developmental stages. Marked temporal shifts in diversity and relative activity were observed in rhizosphere bacterial communities with the developmental stage for all plant species studied. Shifts in the diversity of fungal and bacterial communities were more pronounced in maturing pea and sugar beet plants. This extensive study demonstrates that plant species select for specialized microbial communities that change in response to plant growth and plant inputs (Houlden et al. 2008).

### *15.3.4 Pesticides*

A wide variety of pesticides including herbicides, fungicides and herbicides are used in agricultural practices in order to improve the yield of various crops. The use of such pesticides beyond recommended concentrations or their accumulation into soil following continuous application leads to changes in microbial populations agro-ecosystem. For instance, the application of endosulfan, profenophos with alphamethrin, and methamidophos has shown considerable inhibition in bacterial population in cotton (*Gossypium hirsutum*) agro-ecosystem while monocrotophos and bifenthrin with acetamiprid enhanced the bacterial population of soil. Fungal

population was depressed with endosulfan while monocrotophos, methamidophos, endosulfan with dimethoate, fenpropathrin, bifenthrin with acetamiprid, or with ethion or with a mixture of carbosulfan and chloropyrifos and profenophos alone or with ethion or cypermethrin or alphamethrin stimulated fungal counts (Iqbal et al. 2001). In another study, Ekundayo (2003) investigated the effect of 11 pesticides on the populations of bacteria, actinomycetes, fungi, and protozoa by treating a garden soil with their recommended rates. Phenylmercuric acetate (agrosan) at 50 μg $g^{-1}$ inhibited bacterial density severely. Pentachloronitrobenzene (PCNB) at 240,000 μg $g^{-1}$ reduced bacterial population from $4.6 \times 10^6$ to $2.1 \times 10^2$ cells $g^{-1}$, whereas tetramethylthiuram disulphide (thiram) at 100 μg $g^{-1}$ suppressed it by twofold. Soil application of 1-naphthylmethylcarbamate (Vetox 85) at 100 μg $g^{-1}$ and 1,2,3,4,5,6,-hexachlorocyclohexane (Gamalin 20) at 1,300 μg $g^{-1}$ repressed the bacterial numbers by two orders each. PCNB reduced the actinomycetes density from $3.4 \times 10^5$ to $3.2 \times 10^2$ cells $g^{-1}$ and completely eliminated all fungal and protozoan propagules from the soil. The Gammalin 20 completely wiped out all the fungi, whereas phenylmercuric acetate totally eliminated all the protozoa and reduced the fungal population from $3.4 \times 10^4$ to 60 cells $g^{-1}$. In general, protozoa and fungi were more susceptible to fungicides than bacteria and actinomycetes. PCNB, 1,2,3,4,5,6,-hexachlorocyclohexane and phenylmercuric acetate were toxic particularly to soil microorganisms, whereas the herbicides dacthal, preforan and dual were quite harmless in soil at application rates of 0.1, 0.06 and 0.02 μg $g^{-1}$, respectively. Wang et al. (2006) showed that both a low and a higher dose of methamidophos (0, *S*-dimethyl phosphoroamidothioate) in soil significantly decreased microbial biomass C ($C_{mic}$) by 41–83% compared to control. In contrast, the respiration activity of the applied soils was significantly higher than the control. Pesticide application also significantly increased the soil total of N and P. In addition, substrate richness, Shannon and Simpson indices of microbial communities under chemical stresses increased significantly. Moreover, the populations of methamidophos-metabolized bacteria also increased significantly. It was concluded that methamidophos reduces microbial biomass and enhances functional diversities of soil microbial communities; meanwhile, some species of bacteria may be enriched in soils under methamidophos stress. Similarly, Ratcliff et al. (2006) reported that commercial formulation herbicide (glyphosate) applied at the recommended field rate to a clay loam and a sandy loam forest soil has a benign affect on community structure and produced a nonspecific, short-term stimulation of bacteria at a high concentration.

## 15.4 Resilience of Microbial Communities

Although microbial communities are highly sensitive to both natural and artificial disturbance, the community might still be resilient and quickly return to its predisturbed composition. Microorganisms in general possess a number of features that help them to acquire resilience against several adverse environmental variables.

Such factors that protect them from disturbances include, firstly, the fast growth rates of microorganisms; if their abundance is suppressed by a disturbance, they have the potential to recover quickly. Secondly, their high degree of physiological flexibility; for example, purple nonsulfur bacteria, which act as a phototroph under anoxic conditions are a heterotroph under aerobic conditions suggesting that if the relative abundance of some microbes decreased initially, such organisms possess the ability to acclimatize rapidly to the new abiotic conditions over time and could return to their original abundance. And thirdly, their rapid evolution potential; if physiological adaptation is not possible, then the rapid evolution (through mutations or horizontal gene exchange) could allow microbes to adapt to new environmental conditions and recover from disturbance (Allison and Martiny 2008). Thus, there are three ways in which microbial composition might not matter substantially to ecosystem functioning even if there are any disturbances in the agro-ecosystems: (1) ability of microbial communities to resist changes; however, in the majority of cases microbial communities have been found sensitive to elevated $CO_2$, mineral fertilization, temperature changes, and C amendments (Allison and Martiny 2008), (2) microbial composition might be resilient and quickly return to its original state, and (3) even if microbial composition changes, the new community might be functionally similar to the original one. Although this hypothesis is currently difficult to test, recent studies suggest that many microbial communities are probably not functionally redundant and different communities are not functionally similar (Allison and Martiny 2008).

## 15.5 Conclusion

Soil microbial communities are integrally involved in biogeochemical cycles and their activities are crucial to maintaining soil fertility and productivity and improving the functioning of the soil ecosystem. A reasonable selection of sensitive and robust soil indicators is, however, required to distinguish the trends in improvement and deterioration of soil quality in various agro-ecosystems. Novel methods and approaches enable us to explore the variation in compositions and functional diversity of microbial communities. Studies of sequence information from organisms in soil microhabitats and their gene expression under different conditions will provide guidelines for designing new and improved culturing methods that resemble their natural niches. However, despite the importance of soil microorganisms, little is known about the distribution of microorganisms in the soil or the manner in which microbial community structure responds to changes in environmental conditions and farm management practices that have shown a considerable impact on soil biota, affecting nutrient cycling processes and ecosystem functioning. Understanding the mechanisms that regulate microbial community structure and activity following implementation of various management practices may lead to the

development of best management practices for improving soil fertility and, consequently, agricultural productivity to improve the sustainability of agro-ecosystems.

## References

Adkins A, Wright RB, Scott GAJ, Adkins A, Wright RB, Scott GAJ (2001) Microbial diversity and activity in a stressed boreal forest ecosystem (Manitoba, Canada), vol Abstract I-37. American Society for MicroBiology, Orlando, FL

Allison SD, Martiny JBH (2008) Resistance, resilience, and redundancy in microbial communities. Proc Natl Acad Sci 105:11512–11519

Allison VJ, Miller RM, Jastrow JD, Matamala R, Zak DR (2005) Changes in soil microbial community structure in a tallgrass prairie chronosequence. Soil Sci Soci Am J 69:1412–1421

Anderson TH, Domsch KH (1993) The metabolic quotient for CO2 (qCO2) as a specific activity parameter to assess the effects of environmental conditions, such as pH, on the microbial biomass of forest soils. Soil Biol Biochem 25:393–395

Anderson TH, Joergensen RG (1997) Relationship between SIR and FE estimates of microbial biomass C in deciduous forest soils at different pH. Soil Biol Biochem 29:1033–1042

Andersson S, Ingvar Nilsson S (2001) Influence of pH and temperature on microbial activity, substrate availability of soil-solution bacteria and leaching of dissolved organic carbon in a mor humus. Soil Biol Biochem 33:1181–1191

Aon MA, Cabello MN, Sarena DE, Colaneri AC, Franco MG, Burgos JL, Cortassa S (2001) I. Spatio-temporal patterns of soil microbial and enzymatic activities in an agricultural soil. Appl Soil Ecol 18:239–254

Assmus B, Hutzler P, Kirchhof G, Amann R, Lawrence JR, Hartmann A (1995) In situ localization of *Azospirillum brasilense* in the rhizosphere of wheat with fluorescently labeled, rRNA-targeted oligonucleotide probes and scanning confocal laser microscopy. Appl Environ Microbiol 61:1013–1019

Atlas RM (1984) Use of microbial diversity measurements to assess environmental stress. In: Klug MJ, Reddy CA (eds) Current perspectives in microbial ecology. ASM, Washington, pp 540–545

Azam F, Malfatti F (2007) Microbial structuring of marine ecosystems. Nat Rev Microbiol 5:782–791

Baath E (1996) Adaptation of soil bacterial communities to prevailing pH in different soils. FEMS Microbiol Ecol 19:227–237

Baath E (1998) Growth rates of bacterial communities in soils at varying pH: a comparison of the thymidine and leucine incorporation techniques. Microb Ecol 36:316–327

Baath E, Berg B, Lohm U, Lundgren B, Lundkvist H, Rosswall T, Soderstrom B, Wiren A (1980) Effects of experimental acidification and liming on soil organisms and decomposition in a Scots pine forest. Pedobiologia 20:85–100

Baath E, Frostegard A, Pennanen T, Fritze H (1995) Microbial community structure and pH response in relation to soil organic matter quality in wood–ash fertilized, clear-cut or burned coniferous forest soils. Soil Biol Biochem 27:229–240

Bais HP, Park S, Weir TL, Callaway RM, Vivanco JM (2004) How plants communicate using the underground information superhighway. Trends Plant Sci 9:26–32

Batten KM, Scow KM, Davies KF, Harrison SP (2006) Two invasive plants alter soil microbial community composition in serpentine grasslands. Biol Invasions 8:217–230

Bauhus J, Paré D, Côté L (1998) Effects of tree species, stand age and soil type on soil microbial biomass and its activity in a southern boreal forest. Soil Biol Biochem 30:1077–1089

Beare MH (1997) Fungal and bacterial pathways of organic matter decomposition and nitrogen mineralization in arable soils. In: Brussaard L, Ferrera-Cerrato R (eds) Soil ecology in sustainable agricultural systems. Lewis, Boca Raton, Florida, USA, pp 37–70

Bending GD, Turner MK, Jones JE (2002) Interactions between crop residue and soil organic matter quality and the functional diversity of soil microbial communities. Soil Biol Biochem 34:1073–1082

Bever JD, Westover KM, Antonovics J (1997) Incorporating the soil community into plant population dynamics: the utility of the feedback approach. J Ecol 85:561–573

Bolan NS, Currie LD, Baskaran S (1996) Assessment of the influence of phosphate fertilizers on the microbial activity of pasture soils. Biol Fertil Soils 21:284–292

Bormann BT, Sidle RC (1990) Changes in productivity and distribution of nutrients in a chronosequence at Glacier-Bay national park, Alaska. J Ecol 78:561–578

Borneman J, Triplett EW (1997) Molecular microbial diversity of soils from eastern Amazonia: evidence for unusual microorganisms and microbial population shifts associated with deforestation. Appl Environ Microbiol 63:2647–2653

Bosse U, Frenzel P (1997) Activity and distribution of methane-oxidizing bacteria in flooded rice soil microcosms and in rice plants (*Oryza sativa*). Appl Environ Microbiol 63:1199–1207

Bowen GD, Rovira AD (1991) The rhizosphere, the hidden half. In: Waisel Y, Eshel A, Kafkafi U (eds) Plant roots – the hidden half. Marcel Dekker, New York, pp 641–649

Broeckling CD, Broz AK, Bergelson J, Manter DK, Vivanco JM (2008) Root exudates regulate soil fungal community composition and diversity. Appl Environ Microbiol 74:738–744

Brookes PC (1995) The use of microbial parameters in monitoring soil pollution by heavy metals. Biol Fertil Soils 19:269–289

Buckley DH, Schmidt TM (2001) The structure of microbial communities in soil and the lasting impact of cultivation. Microb Ecol 42:11–21

Buyer JS, Roberts DP, Russek-Cohen E (1999) Microbial community structure and function in the spermosphere as affected by soil and seed type. Can J Microbiol 45:138–144

Campbell CD, Grayston SJ, Hirst DJ (1997) Use of rhizosphere carbon sources in soil carbon source tests to discriminate soil microbial communities. J Microbiol Methods 30:33–41

Chandini TM, Dennis P (2002) Microbial activity, nutrient dynamics and litter decomposition in a Canadian Rocky Mountain pine forest as affected by N and P fertilizers. For Ecol Manage 159:187–201

Chiarini L, Bevivino A, Dalmastri C, Nacamulli C, Tabacchioni S (1998) Influence of plant development, cultivar and soil type on microbial colonization of maize root. Appl Soil Ecol 8:11–18

Chu HY, Hosen Y, Yagi K, Okada K, Ito O (2005) Soil microbial biomass and activities in Japanese Andisol as affected by controlled release and application depth of urea. Biol Fertil Soils 42:89–96

CÔté L, Brown S, Paré D, Fyles J, Bauhus J (2000) Dynamics of carbon and nitrogen mineralization in relation to stand type, stand age and soil texture in the boreal mixed wood. Soil Biol Biochem 32:1079–1090

Crawford JW, Harris JA, Ritz K, Young IM (2005) Towards an evolutionary ecology of life in soil. Trends Ecol Evol 20:81–87

Da Silva KRA, Salles JF, Seldin L, van Elsas JD (2003) Application of a novel *Paenibacillus*-specific PCR-DGGE method and sequence analysis to assess the diversity of *Paenibacillus* spp. in the maize rhizosphere. J Microbiol Methods 54:213–231

Dakora FD (2003) Defining new roles for plant and rhizobial molecules in sole and mixed plant cultures involving symbiotic legumes. New Phytol 158:39–49

Dakora FD, Phillips DA (2002) Root exudates as mediators of mineral acquisition in low-nutrient environments. Plant Soil 245:35–47

De Leij FAAM, Whipps JM, Lynch JM (1994) The use of colony development for the characterization of bacterial communities in soil and on roots. Microb Ecol 27:81–97

de Lima TCS, Grisi BM, Bonato MCM (1999) Bacteria isolated from a sugarcane agroecosystem: their potential production of polyhydroxyalcanoates and resistance to antibiotics. Revista de Microbiologia 30:214–224

de Luna RG, Grisi BM (1996) Biomassa e atividade microbianas de solos cultivados com cana-de-açúcar, sob efeito da vinhaça. Rev Nordest Biol 11:15–29

Doran JW (1980) Soil microbial and biochemical changes associated with reduced tillage. Soil Sci Soc Am J 44:765–771

Duineveld BM, Rosado AS, van Elsas JD, van Veen JA (1998) Analysis of the dynamics of bacterial communities in the rhizosphere of the chrysanthemum via denaturing gradient gel electrophoresis and substrate utilization patterns. Appl Environ Microbiol 64:4950–4957

Ekundayo EO (2003) Effect of common pesticides used in the Niger Delta basin of southern Nigeria on soil microbial populations. Environ Monit Assess 89:35–41

Follett RF, Schimel DS (1989) Effect of tillage practices on microbial biomass dynamics. Soil Sci Soc Am J 53:1091–1096

Franzluebbers AJ, Zuberer DA, Hons FM (1995) Comparison of microbiological methods for evaluating quality and fertility of soil. Biol Fertil Soils 19:135–140

Fraterrigo JM, Turner MG, Pearson SM, Dixon P (2005) Effects of past land use on spatial heterogeneity of soil nutrients in southern Appalachian forests. Ecol Monogr 75:215–230

Fraterrigo JM, Balser TC, Turner MG (2006) Microbial community variation and its relationship with nitrogen mineralization in historically altered forests. Ecology 87:570–579

Frostegard A, Tunlid A, Baath E (1993) Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Appl Environ Microbiol 59:3605–3617

Gans J, Wolinsky M, Dunbar J (2005) Computational improvements reveal great bacterial diversity and high metal toxicity in soil. Science 309:1387–1390

Garbeva P, van Veen JA, van Elsas JD (2004) Microbial diversity in soil: selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annu Rev Phytopathol 42:243–270

Garland JL (1996) Patterns of potential C source utilization by rhizosphere communities. Soil Biol Biochem 28:223–230

Garland JL (1999) Potential and limitations of BIOLOG for microbial community analysis. In: Bell CR, Brylinsky M, Johnson-Green P (eds) Microbial biosystems: new frontiers. Proceedings of the 8th International Symposium on Microbial Ecology. Atlantic Canada Society for Microbial Ecology, Halifax, Canada, pp 1–7

Garland JL, Mills AL (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community level sole-carbon-source utilization. Appl Environ Microbiol 57:2351–2359

Germida JJ, Siciliano SD, Freitas JR, Seib AM (1998) Diversity of rootassociated bacteria associated with fieldgrown canola (*Brassica napus* L.) and wheat (*Triticum aestivum* L.). FEMS Microbiol Ecol 26:43–50

Gilbert B, Frenzel P (1998) Rice roots and $CH_4$ oxidation: the activity of bacteria, their distribution and the microenvironment. Soil Biol Biochem 30:1903–1916

Giovannoni SJ, Britschgi TB, Moyer C, Field KG (1990) Genetic diversity in Sargasso Sea bacterioplankton. Nature 345:60–63

Grayston SJ, Campbell CD (1996) Functional biodiversity of microbial communities in the rhizosphere of hybrid larch (*Larix eurolepis*) and Sitka spruce (*Picea sitchensis*). Tree Physiol 16:1031–1038

Grayston SJ, Wang S, Campbell C, Edwards A (1998) Selective influence of plant species on microbial diversity in the rhizosphere. Soil Biol Biochem 30:369–378

Grayston SJ, Campbell CD, Bardgett RD, Mawdsley JL, Mawdsley Clegg CD, Ritz K, Griffiths BS, Rodwell JS, Edwards SJ, Davies WJ, Elston DJ, Millard P (2003) Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques. Appl Soil Ecol 25:63–84

Grayston SJ, Campbell CD, Bardgett RD, Mawdsley JL, Clegg CD, Ritz K, Griffith BS, Rodwell JS, Edwards SJ, Davies WJ (2004) Assessing shifts in microbial community structure across a range of grasslands of differing management intensity using CLPP, PLFA and community DNA techniques. Appl Soil Ecol 25:63–84

Gregorich EG, Carter MR, Angers DA, Monreal CM, Ellert BH (1994) Towards a minimum set to assess soil organic matter quality in agricultural soils. Can J Soil Sci 76:367–385
Griffiths BS, Ritz K, Ebblewhite N, Dobson G (1999) Soil microbial community structure: effects of substrate loading rates. Soil Biol Biochem 31:145–153
Guanghua W, Junjie L, Qi Xiaoning Q, Jian J, Yang W, Xiaobing L (2008) Effects of fertilization on bacterial community structure and function in a black soil of Dehui region estimated by Biolog and PCR-DGGE methods. Acta Ecol Sin 28:220–226
Gyaneshwar P, Kumar GN, Parekh LJ, Poole PS (2002) Role of soil microorganisms in improving P nutrition of plants. Plant Soil 245:83–93
Haack SK, Garchow H, Klug MJ, Forney LJ (1995) Analysis of factors affecting the accuracy, reproducibility, and interpretation of microbial community carbon source utilization patterns. Appl Environ Microbiol 61:1458–1468
Haynes RJ (1999) Size and activity of the soil microbial biomass under grass and arable management. Biol Fertil Soils 30:210–216
Hiltner L (1904) Uber neuere Erfahrungen und Probleme auf dem Gebiet der Bodenbakteriologie und unter besonderer Berucksichtigung der Grundungung und Brache. [German]. Arb Dtsch Landwirtsch Ges 98:59–78
Houlden A, Timms-Wilson TM, Day MJ, Bailey MJ (2008) Influence of plant developmental stage on microbial community structure and activity in the rhizosphere of three field crops. FEMS Microbiol Ecol 65:193–201
Hugenholtz P, Goebel BM, Pace NR (1998) Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J Bacteriol 180:6793–6794
Ibekwe AM, Kennedy AC (1998) Phospholipid fatty acid profiles and carbon utilization patterns for analysis of microbial community structure under field and greenhouse conditions. FEMS Microbiol Ecol 26:151–163
Ibekwe AM, Kennedy AM, Frohne PS, Papiernik SK, Yang CH, Crowley DE (2002) Microbial diversity along a transect of agronomic zones. FEMS Microbiol Ecol 26:151–163
Innes L, Hobbs PJ, Bardgett RD (2004) The impacts of individual plant species on rhizosphere microbial communities in soils of different fertility. Biol Fertil Soils 40:7–13
Jackson LE, Calderon FJ, Steenwerth KL, Scow KM, Rolston DE (2003) Responses of soil microbial processes and community structure to tillage events and implications for soilquality. Geoderma 114:305–317
Johansson M (1995) The chemical composition of needle and leaf litter from Scots pine, Norway spruce and white birch in Scandinavian forests. Forestry 68:49–62
Kang S, Mills AL (2004) Soil bacterial community structure changes following disturbance of the overlying plant community. Soil Sci 169:55–65
Kennedy N, Connolly J, Clipson N (2004) Impact of lime and nitrogen and plant species on fungal community structure in grassland microcosms. Environ Microbiol 7:780–788
Kennedy NM, Gleeson DE, Connolly J, Clipson NJW (2005) Seasonal and management influences on bacterial community structure in an upland soil. FEMS Microbiol Ecol 53:329–337
Kent AD, Triplett EW (2002) Microbial communities and their interactions in soil and rhizosphere ecosystems. Annu Rev Microbiol 56:211–236
Kirk JL, Beaudette LA, Hart M, Moutoglis P, Klironomos JN, Lee H, Trevors JT (2004) Methods of studying soil microbial diversity. J Microbiol Methods 58:169–188
Konopka A, Oliver L, Turco JRF (1998) The use of carbon substrate utilization patterns in environmental and ecological microbiology. Microb Ecol 35:103–115
Kowalchuk GA, Hol WHG, van Veen JA (2006) Rhizosphere fungal communities are influenced by *Senecio jacobaea* pyrrolizidine alkaloid content and composition. Soil Biol Biochem 38:2852–2859
Ladd JN, Amato M, Li-Kui Z, Schultz JE (1994) Differential effects of rotation, plant residue and nitrogen fertilizer on microbial biomass and organic matter in the Australian alfisol. Biol Fertil Soils 26:821–831

Latour X, Corberand T, Laguerre G, Allard F, Lemanceau P (1996) The composition of fluorescent *Pseudomonas* population associated with roots is influenced by plant and soil type. Appl Environ Microbiol 62:2449–2456

Li X, Inubushi K, Sakamoto K (2002) Nitrous oxide concentrations in an Andisol profile and emissions to the atmosphere as influenced by the application of nitrogen fertilizres and manure. Biol Fertil Soil 35:108–113

Lupwayi NZ, Rice WA, Clayton GW (1998) Soil microbial diversity and community structure under wheat as influenced by tillage and crop rotation. Soil Biol Biochem 30:1733–1741

Lynch JM, Whipps JM (1990) Substrate flow in the rhizosphere. Plant Soil 129:1–10

Mahaffee WF, Klöpper JW (1997) Temporal changes in the bacterial communities of soil, rhizosphere, and endorhiza associated with field-grown cucumber (*Cucumis sativus* L.). Microb Ecol 34:210–223

Marschner H (1995) Mineral nutrition of higher plants. Academic, London

Marschner P, Yang CH, Lieberei R, Crowley DE (2001) Soil and plant specific effects on bacterial community composition in the rhizosphere. Soil Biol Biochem 33:1437–1445

Matthies C, Erhard HP, Drake HL (1997) Effects of pH on the comparative culturability of fungi and bacteria from acidic and less acidic forest soils. J Basic Microbiol 37:335–343

Merilä P, Strömmer R, Fritze H (2002) Soil microbial activity and community structure along a primary succession transect on the landuplift coast of western Finland. Soil Biol Biochem 34:1647–1654

Muyzer G, De Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes for 16S rRNA. Appl Environ Microbiol 59:695–700

Odum EP (1971) Fundamentals of ecology. W.B. Saunders, Philadelphia

Øvreås L, Torsvik V (1998) Microbial diversity and community structure in two different agricultural soil communities. Microb Ecol 36:303–315

Pennanen T (2001) Microbial communities in boreal coniferous forest humus exposed to heavy metals and changes in soil pH-a summary of the use of phospholipid fatty acids, Biologw and 3H thymidine incorporation methods in field studies. Geoderma 100:91–126

Pinton R, Varanini Z, Nannipieri P (2001) The rhizosphere. Biochemistry and organic substances at the soil-plant interface. Marcel Dekker, New York

Potthoff M, Steenwerth KL, Jackson LE, Drenovsky RE, Scow KM, Joergensen RG (2006) Soil microbial community composition as affected by restoration practices in California grassland. Soil Biol Biochem 38:1851–1860

Preston-Mafham J, Boddy L, Randerson PF (2002) Analysis of microbial community functional diversity using sole-carbon-source utilization profiles-a critique. FEMS Microbiol Ecol 42:1–14

Priha O, Smolander A (1997) Microbial biomass and activity in soil and litter under *Pinus sylvestris*, *Picea abies* and *Betula pendula* at originally similar field afforestation sites. Biol Fertil Soils 24:45–51

Priha O, Smolander A (1999) Nitrogen transformations in soil under *Pinus sylvestris* and *Betula pendula* at originally similar forest sites. Soil Biol Biochem 31:965–977

Priha O, Grayston SJ, Hiukka R, Pennanen T, Smolander A (2001) Microbial community structure and characteristics of the organic matter in soils under *Pinus sylvestris*, *Picea abies*, and *Betula pendula* at two forest sites. Biol Fertil Soils 33:17–24

Ratcliff AW, Busse MD, Shestak CJ (2006) Changes in microbial community structure following herbicide (glyphosate) additions to forest soils. Appl Soil Ecol 34:114–124

Reinhart KO, Callaway RM (2006) Soil biota and invasive plants. New Phytol 170:445–457

Rooney DC, Clipson NJW (2009) Phosphate addition and plant species alters microbial community structure in acidic upland grassland soil. Microb Ecol 57:4–13

Rozak DB, Colwell RR (1978) Survival strategies of bacteria in the natural environment. Microbiol Rev 51:365–379

Ruiyu L, Hong R, Junjian Z, Cuiping Y, Chenying Y, Liangsheng C, Wenxiong L (2007) Impact of allelopathic rice seedlings on rhizospheric microbial populations and their functional diversity. Acta Ecol Sin 27:3644–3654

Saetre P, Bååth E (2000) Spatial variation and patterns of soil microbial community structure in a mixed spruce-birch stand. Soil Biol Biochem 32:909–917

Saliana-Garcia JR, Hons FM, Matocha JE (1997) Long-term effects of tillage and fertilization on soil organic matter dynamics. Soil Sci Soc Am J. 61:152–159

Sessitich A, Weilharter A, Gerzabek MH, Kirchmann H, Kandeler E (2006) Microbial population structure in soil particle size fractions of a long-term fertilizer field experiment. Appl Environ Microbiol 67:4215–4224

Smit E, Leeflang P, Gommans S, Broek JVD, Mil SV, Wernars K (2001) Diversity and seasonal fluctuations of the dominant members of the bacterial soil community in a wheat field as determined by cultivation and molecular methods. Appl Environ Microbiol 67:2284–2291

Staddon WJ, Duchesne LC, Trevors JT (1998a) Impact of clearcutting and prescribed burning on microbial diversity and community structure in Jack pine (*Pinus banksiana* Lamb.) clear-cut using Biologe gram-negative microplates. World J Microbiol Biotechnol 14:119–123

Staddon WJ, Trevors JT, Duchesne LE, Colombo CA (1998b) Soil microbial diversity and community structure across a climatic gradient in western Canada. Biodivers Conserv 7:1081–1092

Steenwerth KL, Jackson LE, Calderón FJ, Stromberg MR, Scow KM (2003) Soil microbial community composition and land use history in cultivated and grassland ecosystems of coastal California. Soil Biol Biochem 35:489–500

Steenwerth KL, Jackson LE, Calderon FJ, Scow KM, Rolston DE (2005) Response of microbial community composition and activity in agricultural and grassland soils after a simulated rainfall. Soil Biol Biochem 37:2249–2262

Stromberger M, Shah Z, Westfall D (2007) Soil microbial communities of no-till dryland agroecosystems across an evapotranspiration gradient. Appl Soil Ecol 35:94–106

Sun HY, Deng SP, Raun WR (2004) Bacterial community structure and diversity in a century-old manure-treated agroecosystem. Appl Environ Microbiol 70:5868–5874

Tabatabai MA, Fu MH, Basta NT (1992) Effects of cropping systems on nitrification in soils. Commun Soil Sci Plant Anal 23:1885–1891

Torsvik V, Øvreås L (2002) Microbial diversity and function in soil: from genes to ecosystems. Curr Opin Microbiol 5:240–245

Torsvik V, Salte K, Sorheim R, Goksoyr J (1990) Comparison of phenotypic diversity and DNA heterogenity in a population of soil bacteria. Appl Environ Microbiol 56:776–781

Torsvik VL, Daae FL, Goksfyr J, Sfrheim R, Vreas L (1997) Diversity of bacteria in soil and marine environments. In: Martins MT, Sato MIZ, Tiedje JM, Hagler LCN, Döbereiner J, Sanchez PS (eds) Progress in microbial ecology. SBM–ICOME, São Paulo, pp 115–120

Torsvik V, Øvreås L, Thingstad TF (2002) Prokaryotic diversity-magnitude, dynamics, and controlling factors. Science 296:1064–1066

Trasar-Cepeda C, Leiros C, Gil-Sotres F, Seoane S (1998) Towards a biochemical quality index for soils: an expression relating several biological and biochemical properties. Biol Fertil Soils 26:100–106

Van Elsas JD, Trevors JT, Wellington EHM (1997) Modern soil microbiology. Marcel Dekker, New York, NY

Vineela C, Wani SP, Ch S, Padmaja B, Vittal KPR (2008) Microbial properties of soils as affected by cropping and nutrient management practices in several long-term manurial experiments in the semi-arid tropics of India. Appl Soil Ecol 40:165–173

Waisel Y, Eshel A, Kafkafi U (1991) Plant roots- the hidden half. Marcel Dekkar, New York

Waldrop MP, Balser TC, Firestone MK (2000) Linking microbial community composition to function in a tropical soil. Soil Biol Biochem 32:1837–1846

Wang M, Ming G, Zang H, Hua X, Yao J, Pang Y, Yang Y (2006) Effect of methamidophos and urea application on microbial communities in soils as determined by microbial biomass and community level physiological profiles. J Environ Sci Health B 41:399–413

Wardle DA, Yeates GW, Nicholson KS, Bonner KI, Watson RN (1999) Response of soil microbial dynamics, activity and plant litter decomposition to agricultural intensification over a seven-year period. Soil Biol Biochem 31:1707–1720

Weisskopf L, Tomasi N, Santelia D, Martinoia E, Langlade NB, Tabacchi R, Abou-Mansour E (2006) Isoflavonoid exudation from white lupin roots is influenced by phosphate supply, root type and cluster-root stage. New Phytol 171:657–668

Wieland G, Neumann R, Backhaus H (2001) Variation of microbial communities in soil, rhizosphere and rhizoplane in response to crop species, soil type, and crop development. Appl Environ Microbiol 67:5849–5854

Williamson WM, Wardle DA (2007) The soil microbial community response when plants are subjected to water stress and defoliation disturbance. Appl Soil Ecol 37:139–149

Winding A (1993) Fingerprinting bacterial soil communities using Biologw microtiter plates. In: Ritz K, Dighten J, Giller KE (eds) Beyond the biomass: compositional and functional analysis of soil microbial communities. Wiley, Chichester, United Kingdom, pp 85–94

Winding A (1994) Fingerprinting bacterial soil communities with BIOLOGE microtitre plates. In: Ritz K, Dighton J, Giller KE (eds) Beyond the biomass: compositional and functional analysis of soil microbial communities. Wiley, Chichester, pp 85–94

Yang C, Crowley DE (2000) Rhizosphere microbial community structure in relation to root location and plant iron nutritional status. Appl Environ Microbiol 66:345–351

Young IM, Ritz K (2000) Tillage, habitat space and function of soil microbes. Soil Tillage Res 53:201–213

Zak JC, Willig MR, Moorhead DL, Wildman HG (1994) Functional diversity of microbial communities: a quantitative approach. Soil Biol Biochem 26:1101–1108

Zak DR, Holmes WE, White DC, Peacock AD, Tilman D (2003) Plant diversity, soil microbial communities, and ecosystem function: are there any links? Ecology 84:2042–2050

Zhang P, Zheng J, Pan G, Zhang X, Li L, Tippkotter R (2007) Changes in microbial community structure and function within particle size fractions of a paddy soil under different long-term fertilization treatments from the Tai Lake region, China. Colloids Surf B Biointerf 58:264–270

# Chapter 16
# Strategies for Utilizing Arbuscular Mycorrhizal Fungi and Phosphate-Solubilizing Microorganisms for Enhanced Phosphate Uptake and Growth of Plants in the Soils of the Tropics

**Nelson Walter Osorio and Mitiku Habte**

**Abstract** One of the major constraints for plant productivity in tropical regions is low soil phosphate (Pi) availability. Phosphate ions are rendered unavailable for plant uptake due to adsorption onto the surface of soil minerals and precipitation by free aluminum and iron ions. In highly weathered soils, this is so intense that plant crops commonly exhibit Pi-deficiency. High rates of soluble Pi-fertilizers are employed to meet plant P demands. However, the large quantity of Pi required in order to offset the high Pi-retention capacity of the soils and the high cost associated with it makes it inaccessible to the vast majority of growers in the region. An alternative means of improving plant Pi-uptake from insoluble native and applied rock phosphate is the use of arbuscular mycorrhizal (AM) fungi. These fungi form a symbiotic association with most plants and improve the efficiency of associated plants to take up Pi from the soil solution. Other soil microorganisms commonly known as phosphate-solubilizing microorganisms (PSM) can replenish soil solution Pi by solubilizing complex phosphorus compounds found in soil or added to it, mostly through the release of organic acids. In this chapter, an attempt is made to highlight the interactions of these two distinct groups of soil microorganisms and the mechanisms by which they facilitate plant available Pi and enhance plant growth in the soils of the tropics.

N.W. Osorio(✉)
Soil Microbiology Research Group, Universidad Nacional de Colombia at Medellin, College of Sciences, Calle 59A No. 63-20, Medellin, Colombia, USA
e-mail: nwosorio@unal.edu.co

M. Habte
University of Hawai'i at Manoa, College of Tropical Agriculture and Human Resources, St. John Room 102, 3190 Maile Way, Honolulu, HI, USA, 96822

M.S. Khan et al. (eds.), *Microbial Strategies for Crop Improvement*,
DOI: 10.1007/978-3-642-01979-1_16, 

## 16.1 Introduction

Phosphate (Pi)-fixation is a serious problem in agricultural soils, particularly in highly weathered soils and those formed from volcanic ash (Trolove et al. 2003; Sanchez and Uehara 1980). It is estimated that the soils that exhibit high Pi-fixation capacity occupy 1,018 million ha in the tropics (Sanchez and Logan 1992). In tropical America, there are 659 million ha affected by high Pi-fixation, 210 in Africa, and 199 in Asia. The term Pi-fixation is used to describe reactions that remove bioavailable Pi from the soil solution into the soil solid phase (Barber 1995). There are two types of reactions: (1) Pi-sorption onto the surface of soil minerals, and (2) Pi-precipitation by cations such as $Al^{3+}$ and $Fe^{3+}$ in the soil solution (Havlin et al. 1999). Of these, phosphate sorption is particularly strong on iron and aluminum hydrous-oxides (crystalline or noncrystalline) that predominate in the highly weathered soils of humid regions and acid savannas (Mattingly 1975). Jones (1981) characterized the Pi-sorption by 11 Puerto Rican soils and found that the surface area of Goethite was a primary factor accounting for Pi-sorption, with Gibbsite and Hematite contributing little to Pi-sorption. A similar result for Hawaiian soils has been reported (Jackman et al. 1997). Thus, soil Pi-sorption was satisfactorily predicted by soil mineralogical composition. In soils derived from volcanic parent materials, humus-Al/Fe complexes, Allophanes, Ferrihydrite, and Goethite are the soil minerals responsible for the strong Pi-sorption (Jackman et al. 1997; Shoji et al. 1993; Schwertmann and Herbillon 1992; Parfitt 1989). On the other hand, in calcareous soils, Pi is sorbed on the surface of calcium carbonate (Mattingly 1975).

In acidic soils, Pi-precipitation occurs with active forms of aluminum [$Al^{3+}$, $Al(OH)^{2+}$, $Al(OH)_2^{+}$) and iron ($Fe^{3+}$)], while in neutral and alkaline soils, it occurs mostly with calcium ($Ca^{2+}$) (Bohn et al. 1985). The extent of dominance of these cations depends mainly on the degree of soil weathering and soil pH. Phosphate ions precipitate to form initially amorphous (noncrystalline) compounds, becoming much more stable as crystalline forms are formed over time (Brady and Weil 1999). Amorphous minerals are slightly more soluble than their crystalline forms because they have smaller particle size, and consequently greater surface area. For instance, the crystalline mineral variscite ($AlPO_4.2H_2O$) has a surface area of 1.54 $m^2 g^{-1}$ (Taylor and Gurney 1964) whose solubility product ($K_{sp}$) is $10^{-30.5}$ (Bache 1963). In contrast, its amorphous aluminum-phosphate counterpart has a surface area of 10.5 $m^2 g^{-1}$ (Juo and Ellis 1968) and its $K_{sp}$ is $10^{-28.1}$ (Veith and Sposito 1977). In alkaline soils, Pi-compounds are similarly transformed to more insoluble forms. Initially Pi-ions precipitate to calcium-monohydrogen-phosphate ($K_{sp}=10^{-6.6}$) (Stumm and Morgan 1995), which is then converted to calcium-orthophosphate ($K_{sp}=10^{-24}$), and finally to apatite ($K_{sp}=10^{-55.9}$) (Snoeyink and Jenkins 1980). Fox and Kamprath (1970) showed that the degree of P fixation varies among soils, with Andisols, Oxisols, and Ultisols (USDA soil taxonomy) having a high Pi-fixation capacity (Buol et al. 1997). These soils are usually acidic and generally have higher amounts of exchangeable Al and clay minerals that can sorb high

**Table 16.1** Categories of soil Pi-sorption as measured by Pi-sorption isotherms following the method of Fox and Kamprath (1970) and usual mineralogy in each category

| Category | $P_{0.2}$ (P: mg $kg^{-1}$)[a] | Usual mineralogy |
|---|---|---|
| Very low (VL) | <10 | Quartz, organic materials |
| Low (L) | 10–100 | 2:1 clays, quartz, and 1:1 clays |
| Medium (M) | 100–500 | 1:1 clays with oxides |
| High (H) | 500–1,000 | Oxides, moderately weathered ash |
| Very high (VH) | >1,000 | Desilicated amorphous materials |

[a]Amount of P required to achieve a soil solution P of 0.2 mg $L^{-1}$

amounts of Pi (Sanchez and Uehara 1980). Juo and Fox (1977) proposed several categories for soil Pi-sorption capacity in tropical soils as measured by Pi-sorption isotherms and the usual mineralogy of each category (Table 16.1).

The immediate Pi-source for plants is the soil solution, which usually contains a low Pi-concentration (P: 0.001–0.01 mg $L^{-1}$) (Barber 1995; Fox 1979). When Pi is removed from the soil solution by roots and/or by Pi-fixation reactions, a gradient of Pi-concentration is created between the solid phase and the soil solution around the roots. Sorbed Pi on the soil solid surfaces must desorb in order to replenish Pi in the soil solution (Do Carmo Harta and Torrent 2007). Hence, Pi diffuses from the solid phase, where it is more concentrated, to the soil solution around the root surface where its concentration is continuously being depleted. However, the rate of Pi-diffusion is quite slow ($10^{-12}$–$10^{-15}$ $m^2 s^{-1}$) (Schachtman et al. 1998) which limits the Pi-supply and creates a depletion zone of Pi around the roots of approximately of 1–2 mm (Barber 1995). Phosphate ions beyond the zone of depletion cannot be accessed by the root surface (Barber 1995). Phosphate ions that slowly diffuse into the soil solution originate mainly from Pi weakly sorbed on soil colloids and from those freshly precipitated (Lindsay 2001; Stevenson 1986). Soil Pi-supply depends on the Pi-buffering capacity of soils (Pypers et al. 2006), which can be estimated from the relationship between the concentration of Pi in soil solution (intensity factor, I) and the quantity of labile Pi in the solid phase (quantity factor, Q) (Holdford 1997; Barber 1995).

## 16.2 Phosphate Fertilizer Management

Sanchez and Uehara (1980) discussed different strategies to increase soil Pi-availability of acidic tropical soils with high Pi-fixation capacity. One strategy consists of applying a high dose of soluble Pi-fertilizers (500–1,000 mg $kg^{-1}$), followed by small amounts of annual application. Although a great part of the added Pi is fixed, it may be released over several years, thus generating a residual effect. These Pi-fertilization rates are, however, not added by most farmers in developing countries due to its high cost. The proportion of the added Pi taken up by the first crop is quite low, ranging from 5 to 10%, suggesting that 90–95% of the added phosphatic fertilizer could be fixed in the soils in chemical forms that slowly release Pi for uptake by plants (Engelstad and Terman 1980).

Rock phosphates (RP) are highly recommended for acid soils with a high Pi-fixation capacity because other more soluble Pi forms are quickly fixed and are more expensive (Yusdar et al. 2007; Randhawa et al. 2006; Msolla et al. 2005). However, the greater the reactivity of a RP, the greater is its desirability, because less reactive minerals are very insoluble (Shrivastava et al. 2007; Ojo et al. 2007; Hammond and Leon 1992). There is an increasing interest in tropical countries to use organic amendments and RP concurrently. In this context, several studies conducted across the globe have shown that the effectiveness of RP to increase plant growth and crop yields could be enhanced by mixing it with farmyard manures, compost, and green manures (Msolla et al. 2007; Yusdar et al. 2007; Shrivastava et al. 2007). Moreover, added manures can also facilitate desorption of sorbed Pi from soil particles (Redding et al. 2006). Some treatments on RP such as "fine grinding," thermal alteration, and fusion with silica, sodium or magnesium carbonate have been satisfactorily used (Sanchez and Uehara 1980). These treatments are oriented to increase solubility (due to a lower particle size) and/or reduce Pi-sorption by including silicates that compete for the Pis soprtion sites. On the other hand, since RP are more soluble in acidic conditions, their acidulation with strong acids has been employed to produce more soluble fertilizers, such as superphosphates (Young and Davies 1980). Partial acidulation has also been applied; however, it increases the cost of production (Havlin et al. 1999). The direct application of nonacidulated RP is recommended for acid soils but not for neutral and alkaline soils. However, several authors have used RP successfully in alkaline soils with simultaneous inoculation of P-solubilizing microorganisms, which can release Pi rapidly and, in turn, increases plant Pi-uptake (Khan et al. 2007; Whitelaw 2000; Kucey et al. 1989). For instance, Bar-Yosef et al. (1999) tested *Pseudomonas cepacia* for P-solubilizing activity on a RP and recorded that it produced superphosphate. These researchers found that this bacterium produced gluconic acid and 2-ketogluconic acid using glucose as the sole carbonaceous substrate. Once these acids were dissociated in solution, the protons reacted with the RP and released Pi into the solution that was then reprecipitated with $Ca^{2+}$ to form superphosphate fertilizers.

The use of mycorrhizal fungi to increase the efficiency of Pi-uptake by plant is also reported (Habte and Osorio 2001). For instance, Manjunath et al. (1989) evaluated the effectiveness of AM-fungus (*Glomus aggregatum)* to enhance plant Pi-uptake of *Leucaena leucocephala* grown in an Oxisol fertilized with RP (P: 340–5,440 mg $kg^{-1}$). Although plant dry weight and shoot P concentration did not increase significantly in uninoculated soils, when soil was inoculated with *G. aggregatum,* a significant increase in plant dry weight and tissue P concentration was observed. However, in order to obtain adequate growth of mycorrhizal plants, it was necessary to apply a high (at least 2,720 mg $kg^{-1}$) P level. Despite the benefits of mycorrhizal inoculation, it is clear that it is necessary to apply a high rate of RP. This imposes economic limitations on use of the mycorrhizal association as a strategy to manage Pi-deficient soils. It is clear that AM fungi absorb only Pi from the soil solution, as plant roots do, and there is no evidence of their ability to solubilize insoluble soil P minerals (Bolan 1991). The use of

Pi-solubilizing microorganisms may increase the amount of available Pi in soil solution and, consequently, enhance the effectiveness of AM fungi to increase plant Pi-uptake.

## 16.3 Arbuscular Mycorrhizal Pi-Uptake

Some plants adopt different strategies to grow in P deficient soils: (1) changes in root morphology such as the production of an elongated root system with fine roots and abundance of root hairs, (2) release of phosphatase enzymes that release Pi from organic compounds, and (3) production and release of organic acids that solubilize Pi-compounds (Radersma and Grierson 2004; McCully 1999; Hetrick 1991). Plants with less root plasticity need to form symbiotic associations with mycorrhizal fungi that colonize the cortical tissue of roots (Smith et al. 2003; Smith 2002; Sylvia 1999) if they are to grow normally in Pi-deficient soils. During plant–AM fungal interactions, the plant supplies carbonaceous compounds to the fungus, while the fungus provides nutrients, particularly diffusion-limited ones such as Pi, $Cu^{2+}$, and $Zn^{2+}$ (Lynch and Ho 2005; Hamel 2004; Marschner 1995; Barber 1995; Habte and Manjunath 1991). It is clear that AM fungi can only take up soluble Pi from the same Pi-pool that is available for uptake by roots (Cardoso et al. 2006; Bolan 1991). In turn, roots can absorb available Pi from distances not exceeding a few millimeters from their surface while mycorrhizal hyphae can extend to several centimeters from the root surface, exploring a greater volume of soil (Jeffries et al. 2003; Habte and Osorio 2001; Miyasaka and Habte 2001). For example, 47 days after mycorrhizal inoculation of *Trifolium subterraneum*, Jacobsen et al. (1992) found mycorrihzal hyphae spreading from the root surface to 11 cm with the proportion between mycorrhizal hyphae and root length of 1–10 $m\,cm^{-1}$ of infected root while Barber (1995), however, reported a lower value of 0.8 $m\,cm^{-1}$ of root.

Mycorrhizal hyphae have a higher affinity for absorbing Pi than roots. Schachtman et al. (1998) reported that the hyphae of *Gigaspora margarita* had an affinity constant for Pi ($K_m$) of 2.5 μM (P: 0.077 mg $L^{-1}$), while many plants usually exhibited a $K_m$ of 6–44 μM (P: 0.19–1.36 mg $L^{-1}$), particularly those highly dependent on the mycorrhizal association (Barber 1995; Nye and Tinker 1977). In addition, Barber (1995) affirmed that due to the small radius of the mycorrhizal hypha (1–3 μm) there is no Pi-depletion zone around the hypha which allows the mycorrhizal hypha to take up Pi more effectively due to a higher and more constant Pi concentration. In comparison, Li et al. (1991) found a very narrow Pi-depletion zone around the mycorrhizal hypha. Roots with a greater radius (150 μm), however, generate a zone of depletion of at least 1 mm, resulting in low Pi concentration around the root surface. Smith and Read (1997) reported P influx in mycorrhizal roots of 3- to 5-fold higher than nonmycorrhizal roots ($10^{-11}$ mol $m^{-1}s^{-1}$).

Plant species exhibit different degrees of mycorrhizal dependency (MD) to produce maximum growth at a given level of soil fertility (Plenchette et al.

1983). Habte and Manjunath (1991) found that the MD of several plant species was determined by root characteristics, such as root length, root density, root surface area, and incidence and length of hair roots of the host species, which help them to explore and absorb Pi from the soil solution. Since MD was significantly affected by soil solution Pi-concentration, they proposed that MD should be estimated at different levels of available Pi, particularly at the soil solution P of 0.02 mg $L^{-1}$.

## 16.4 Phosphate-Solubilizing Microorganisms

Many soil microorganisms can solubilize inorganic soil P compounds, reversing the process of Pi-fixation (Khan et al. 2007; Gyaneshwar et al. 2002; Rao 1992). Soil bacteria of the genus *Pseudomonas, Enterobacter*, and *Bacillus* are particularly active as Pi-solubilizers (Canbolat et al. 2006; Pandey et al. 2006; Xavier and Germida 2003; Kim et al. 1998a, 1998b). Soil fungi especially those of the genus *Penicillium* and *Aspergillus* have also been demonstrated to be effective phosphate-solubilizing microorganisms (PSM) (Reddy et al. 2002; Whitelaw 2000). Kucey et al. (1989) found in Mollisols of Canada that 0.5 and 0.1% of the total population of bacteria and fungi, respectively, exhibited the ability to solubilize insoluble Pi-compounds. Although Pi-solubilizing bacteria have received greater attention, Whitelaw (2000) and Kucey (1983) indicated that Pi-solubilizing fungi are more effective in solubilizing P compounds. Moreover, bacteria on repeated subculturing can lose their ability to solubilize P, while subcultures of Pi-solubilizing fungi maintain this ability (Rashid et al. 2004; Whitelaw 2000). Apparently, there are some bacterial genes that could be repressed by high levels of Pi that control the production of some organic acids (e.g., gluconic acid); however, in spite of the progress made in the genetic control of Pi-solubilizing bacteria (Rodriguez et al. 2006), this is not completely understood and requires more study.

During the 1950s and 1960s, inoculation with *Bacillus megaterium* var. *phosphaticum* (phosphobacterin) in Russian soils (mainly Mollisols) was the best-known use of PSM (Kucey et al. 1989; Stevenson 1986). The mechanisms of Pi-solubilization were not fully understood, but the mineralization of organic P was proposed as the major mechanism. Trials carried out in many locations demonstrated little consistency in plant response; apparently other factors such as liming and/or organic material addition affected the effectiveness of phosphobacterin. The lack of response to phosphobacterin in many locations pointed to a possible intensified organic matter decomposition, and the poor understanding of the mechanisms of P solubilization carried out by this microorganism discouraged its use. Since then, the focus on microbial solubilization of P has been directed towards understanding the mechanisms of the dissolution of inorganic P compounds (Kucey et al. 1989).

Inoculation with PSM has produced positive results on growth, yield, and Pi-uptake in several plant species (Wani et al. 2007; Khan and Zaidi 2007; El-Azouni

**Table 16.2** Effect of PSM inoculation on plant Pi-uptake of mycorrhiza-free and mycorrhized plants grown in temperate soils

| Soil type/plant | P added | PSM | Increase of plant P uptake due to PSM inoculation (%) | | Reference |
|---|---|---|---|---|---|
| | | | − AMF | + AMF | |
| Mollisol pH 7.7 | None | Penicillium bilaji | 73 | | Kucey |
| Plant: wheat | RP | | 47 | | (1988) |
| Mollisol pH 8.0 | None | Penicillium bilaji | 25 | | Asea et al. (1988) |
| Mollisol pH >7.0 | None | Penicillum bilaji | 62 | | Kucey et al. |
| Plant: wheat | RP | | 36 | | (1989) |
| | MAP | | 19 | | |
| Calcareous | RP | Penicillium sp. | 17 | | Salih et al. |
| TypicTorrifluvent | RP | *Aspergillus foetidus* | 19 | | (1989) |
| pH 8.2 | TSP | Penicillium sp. | 8 | | |
| | TSP | *Aspergillus foetidus* | 4 | | |
| Mollisol pH 7.6 | None | Penicillium bilaji | 39 | | Gleddie |
| | TSP | | 18 | | (1993) |
| | TSP | | 18 | | |
| | TSP | | 7 | | |
| Hapludoll pH 6.2 | None | Aspergillus awamori | 58 (grain) | | Singh and Singh (1993) |
| | RP | | 13 (grain) | | |
| Calcareous mixed with sand, pH 6.7 Plant: kudzu | RP | *Azospirillum* | 33 | 0–33 | Toro et al. (1996) |
| | | *Penicillium* | 33 | 0 | |
| | | Unidentified | 33 | 22 | |
| | | Pseudomonas | 33 | 33 | |
| Calcareous, pH 7.5 Plant: wheat | None | *Aspergillus* | 30 | 39 | Omar (1998) |
| | | *Penicillum* | 24 | 26 | |
| | | *Aspergillus* + *Penicillium* | 78 | 49 | |
| | RP | *Aspergillus* | 116 | 24 | |
| | | *Penicillum* | 118 | 11 | |
| | | *Aspergillus* + *Penicillium* | 151 | 57 | |
| Vertic Epiaqualf mixed with sand and vermiculite, pH 5.9 Plant: Tomato | RP | Enterobacter agglomerans | 54 (35 days)[a] | 124 | Kim et al. (1998a) |
| | | | 27 (55 days) | 27 | |
| | | | 8 (75 days) | 11 | |
| Sandy soil pH 7.6 Plant: wheat | None | *Bacillus* | 26 | 52 | Singh and Kapoor (1999) |
| | | *Cladosporium* | 47 | 69 | |
| | | *Bacillus* + *Cladosporium* | 73 | 98 | |
| | RP | *Bacillus* | – | 248 | |
| | | *Cladosporium* | – | 301 | |
| | | *Bacillus* + *Cladosporium* | 51 | 344 | |
| Rhodic Haplustox Plant: leucaena | None | *Mortierella* sp. | 9 | 13 | Osorio and Habte (2001) |
| | RP | | 14 | 73 | |

(*continued*)

**Table 16.2** (continued)

| Soil type/plant | P added | PSM | Increase of plant P uptake due to PSM inoculation (%) | | Reference |
|---|---|---|---|---|---|
| | | | − AMF | + AMF | |
| Alluvial sandy loam pH 7.2 Plant: wheat | | *Azotobacter chroococcum* | 39 | 99.6 | Khan and Zaidi (2007) |
| | | *Bacillus* sp. | 77 | 120 | |
| | | *Penicillium variable* | 94 | – | |
| Typic Haplustox Plant: leucaena | None | *Mortierella* sp. | 0 | 0 | Osorio (2008) |
| | RP [b] | (150 mg P $kg^{-1}$) | 0 | 40 | |
| | RP [c] | (300 mg P $kg^{-1}$) | 0 | 66 | |
| Typic Haplustoll Plant: leucaena | None | *Mortierella* sp. | 155 | 26 | Osorio (2008) |

[a]Days after planting; [b, c]150 and 300 mg of P $kg^{-1}$ of soil, respectively

2008) (Table 16.2). Some effective Pi-solubilizing fungi that have shown a substantial increase in plant growth and P uptake by different plants including *Aspergillus niger* (Omar 1998), *Aspergillus flavus* (Rashid et al. 2004), *Penicillium bilaji* (Kucey et al. 1989), *Penicillium italicum* (El-Azouni 2008), *Penicillium radicum* (Whitelaw 2000), and *Mortierella* sp. (Osorio 2008). Salih et al. (1989) inoculated a calcareous soil (Typic Torrifluvent, pH 8.2) with two PSM, *Penicillium* sp. and *Aspergillus* sp. They observed that, when the soil was fertilized with RP, the PSM inoculation increased sorghum (*Sorghum bicolor*) Pi-uptake by 17 and 19%, respectively, compared to the sole application of RP. Also, Kucey (1988) inoculated a Mollisol (pH 7.7) of Canada with *P. bilaji*, the soil was either fertilized or unfertilized with RP (Table 16.2). Wheat (*Triticum aestivum*) Pi-uptake increased only by 4% with RP alone, 14% with the PSM inoculation alone, and 12% with a combination of RP and *P. bilaji*. In a similar experiment on a Mollisol (pH 8), Asea et al. (1988) found that the addition of RP increased wheat plant Pi-uptake by only 2%; *P. bilaji* inoculation alone significantly increased it by 26%, and the combination of both (RP and *P. bilaji*) by 28% (Table 16.2).

Several authors have reported that soil microorganisms can increase soil P availability (Table 16.3). Although in some soils this increase does not have-practical implications, it has been important in other soils (Marschner et al. 2006). For instance, the increases in soil P availability reported by Goenadi (1995) and Goenadi et al. (1995) in two acidic Ultisols fertilized with RP were significant. The effectiveness of PSM to enhance plant Pi-uptake has been questioned by some authors (Bolan 1991, Tinker 1980) due to several reasons: (1) organic substances required for these microorganisms are scarce in nonrhizospheric sites, (2) antagonism and competition by other microorganisms in the rhizosphere can reduce the effectiveness of PSM, and (3) low translocation of solubilized Pi through the soil because it can be refixed by soil components. Of these, the latter point is more important in soils with a high Pi-fixation capacity as discussed earlier.

**Table 16.3** Enhancement of the soil Pi-available by phosphate-solubilizing microorganisms

| PSM | Soil | P source | SAP increase (mg $kg^{-1}$) | Reference |
|---|---|---|---|---|
| Aspergillus niger | Fluvaquent, pH 5.4 SAP: 9 mg $kg^{-1}$ | Nil P fertilizer | 2 | Banik and Dey (1981b) |
| A. fumigates | Fluvaquent, pH 7.4 SAP: 7 mg $kg^{-1}$ | RP and farmyard manure | 3 | Banik and Dey (1982) |
| *Penicillium bilaji* | Mollisol, pH 7.7 SAP: 4 mg $kg^{-1}$ | RP | 2 | Kucey (1988) |
| *Penicillium* sp. | Torrifluvent, pH 8.2 SAP: 4 mg $kg^{-1}$ | RP | 2 | Salih et al. (1989) |
| | | TSP | 9 | |
| *A. foetidus* | | RP | 1 | |
| | | TSP | 5 | |
| *A. awamori* | Hapludoll, pH 6.2 SAP: 27 mg $kg^{-1}$ | Nil P fertilizer | 8 | Singh and Singh (1993) |
| | | RP | 3 | |
| *Aspergillus* sp. | Ultisol, pH 3.9 SAP: 37 mg $kg^{-1}$ | RP | 24 | Goenadi et al. (1995) |
| *Aspergillus* sp. | Ultisol SAP: 0.5 mg $kg^{-1}$ | Nil P fertilizer | 14 | Goenadi (1995) |
| | | RP | 38 | |

*Source*: Whitelaw (2000). SAP Soil available P, *TSP* triple superphosphate

### *16.4.1 Mechanisms of Microbial Pi-Solubilization*

Several mechanisms have been proposed to explain the microbial solubilization of P compounds. The mechanisms include: -(1) release of organic acids produced during organic residue decomposition (Hameeda et al. 2006; Bar-Yosef et al. 1999), (2) excretion of protons due to $NH_4^+$ assimilation by microorganisms (Whitelaw 2000; Illmer and Schinner 1995; Abd-Alla 1994; Asea et al. 1988; Roos and Luckner 1984; Kucey 1983), (3) formation of complexes between organic acids/anions with cations ($Al^{3+}$, $Fe^{3+}$, $Ca^{2+}$) (Welch et al. 2002), and (4) desorption of Pi-sorbed onto soil clay and/or oxides (Osorio 2008). Nitric and sulfuric acid produced by *Nitrosomonas* and *Thiobacillus* species, respectively, have also been reported to dissolve Pi-compounds (Azam and Memon 1996). Equally, P compounds may be solubilized by carbonic acid formed as a result of organic matter decomposition (Memon 1996). An increase in soil P availability may be caused by several reactions involving microorganisms that produce organic acids and humic substances (Stevenson 1986). Presumably, these substances can replace or compete with Pi-ions for sorption sites.

Kim et al. (1997) found that the production of acidity was a major mechanism in the solubilization of hydroxyapatite by *Enterobacter agglomerans* under in vitro conditions. For comparison, Kim et al. (1997) employed citric acid, oxalic acid, lactic acid, and HCl at the same pH produced by *E. agglomerans*. They found that at pH 4.0–4.1 (and a shaking time of 48–50 h), there were no significant differences among P solubilization produced by this bacterium and that produced by the application of citric acid, oxalic, and HCl. However, lactic acid exhibited a

significantly ($P \leq 0.05$) lower capacity for solubilizing hydroxyapatite. IIlmer et al. (1995) reached the same conclusion while studying $AlPO_4$ solubilization by several PSM. For instance, *A. niger* produced organic acids but other PSM species did not produce detectable amounts of the organic acids. Under in vitro conditions, the pH of the growth medium decreased as a result of acid production by PSM. Osorio and Habte (2001) found an inverse relation between culture medium pH and Pi released from RP by PSM (*Mortierella* sp.) isolated from Hawaiian soils.

Some of the organic acids (or their respective anions) commonly associated with microbial solubilization of Pi are gluconic acid (Bar-Yosef et al. 1999; Di-Simine et al. 1998), oxalic acid, citric acid (Kim et al. 1997, Kucey et al. 1989), lactic acid, tartaric acid, and aspartic acid (Venkateswarlu et al. 1984). These acids are products of microbial metabolism, in some cases by oxidative respiration or by fermentation of carbonaceous substrates (e.g., glucose) (Trolove et al. 2003; Jones et al. 2003; Gyaneshwar et al. 2002; Prescott et al. 1999; Atlas and Bartha 1997). The reactions of P solubilization are believed to occur in the rhizosphere where carbonaceous compounds are released and where solubilized Pi may be taken up by the root or mycorrhizal system. Amos and Walters (2006) estimated that for maize (*Zea mays*) up to 29% of the total carbon fixed by photosynthesis could be excreted into the rhizosphere. According to Nguyen (2003), on average, 17% of the net C fixed by photosynthesis is lost by roots and recovered as rhizosphere respiration (12%) and soil residues (5%). Many rhizosphere microorganisms are heterotrophs and might use these carbonaceous substrates to produce organic acids. Recently, Hameeda et al. (2006) found that the type of carbon source affected the effectiveness of RP-solubilizing bacteria and, for *Serratia marcescens* and *Pseudomonas* sp., the more favorable carbon source for RP solubilization followed the order, glucose > galactose > xylose > mannose = maltose > cellobiose > arabinose. However, no solubilization of RP was detected with the last carbon source of this series. In addition, *Serratia marcescens* and *Pseudomonas* sp. were capable of solubilizing RP using different kinds of composted crop residues including rice (*Oryza sativa*), pigeon pea (*Cajanus cajans*), and a grass. Furthermore, Reyes et al. (2006) also compared the effect of the carbon source on RP solubilization and found that *Penicillium* sp. and *Azotobacter* sp. were more effective when the medium contained sucrose rather than dextrose. When PSM were inoculated in neutral or alkaline soils, the production of acids decreased rhizosphere pH, favoring the solubility of soil native calcium-phosphate and added RP (Kim et al. 1998a). These results have commonly been found in temperate-zone soils of Europe and North America (Kucey et al. 1989; Kucey 1983, 1987, 1988) and other countries, like Egypt (Omar 1998) where calcareous soils are abundant. The following reactions (1)–(3) suggest that the increase in $H^+$ activity leads to a substantial increase in solubilization of calcium-phosphates. Moreover, if $Ca^{2+}$ is chelated by organic anions, the dissolution of both solids is favored. For example, Welch et al. (2002) found that organic acid/anions produced by microorganisms were capable of dissolving apatite by forming a complex with Ca either in solution and/or directly at the mineral surface.

$$\text{(Dicalcium phosphate)}\ CaHPO_4 + H^+ \leftrightarrow H_2PO_4^- + Ca^{2+} \qquad K = 10^{0.3}, \quad (1)$$

$$\text{(Hydroxyapatite) } Ca_5(PO_4)_3OH + 7H^+ \leftrightarrow 3H_2PO_4{}^{2-} + 5Ca^{2+} + H_2O \quad K = 10^{14.5}, \quad (2)$$

$$\text{(Fluoroapatite) } Ca_5(PO_4)_3F + 6H^+ \leftrightarrow 3H_2PO_4{}^2 + 5Ca^{2+} + F^- \quad K = 10^{-0.2}. \quad (3)$$

On the other hand, in highly weathered acidic soils, P solubility is controlled by other compounds, mainly variscite ($AlPO_4.2H_2O$) and strengite ($FePO_4 \cdot 2H_2O$) (Bohn et al. 1985). In this case, the decrease in soil pH may not increase the dissolution of strengite and variscite (Lindsay 2001) and, hence, Pi is not released. This happens because gibbsite [Al $(OH)_3$] and kaolinite $[(Al_2Si_2O_5OH)_4]$ can control the solubility of Al in these soils, while goethite (FeOOH), hematite ($Fe_2O_3$) and soil-$Fe(OH)_3$ control the solubility of Fe. Reductions in the soil pH would release more Al and Fe ions, which would precipitate Pi. Thus, if soil pH decreases, Pi-solubility is likely to decrease, as shown in reactions (4)–(8) (Lindsay 2001).

$$AlPO_4 \cdot 2H_2O + H_2O \leftrightarrow H_2PO_4{}^- + Al(OH)_3 + H^+ \quad K = 10^{-10.5}, \quad (4)$$

$$AlPO_4 \cdot 2H_2O + SiO_2(\text{quartz}) + 0.5H_2O \leftrightarrow H_2PO_4{}^- + 0.5Al_2Si_2O_5(OH)_4 + H^+ \quad K = 10^{-9.2}, \quad (5)$$

$$FePO_4 \cdot 2H_2O + H_2O \leftrightarrow H_2PO_4{}^- + Fe(OH)_3 + H^+ \quad K = 10^{-9.6}, \quad (6)$$

$$FePO_4 \cdot 2H_2O \leftrightarrow H_2PO_4{}^- + FeOOH + H^+ \quad K = 10^{-6.8}, \quad (7)$$

$$FePO_4 \cdot 2H_2O \leftrightarrow H_2PO_4{}^- + 0.5H_2O + 0.5Fe_2O_3 + H^+ \quad K = 10^{-6.9}. \quad (8)$$

The microbial solubilization of soil P seems to be associated with the presence of calcium-phosphates. In fact, most of the research on microbial solubilization has been done with solubilizers of RP (mixture of hydroxy- and fluor-apatites) (Osorio and Habte 2001; Kim et al. 1998b) or tricalcium phosphate ($Ca_3PO_4$) (Vyas et al. 2007; Wani et al. 2007; Banik and Dey 1981a, 1981b, 1981c; Paul and Rao 1971; Agnihorti 1970; Louw and Webley 1959; Sperber 1957, 1958; Pikovskaia 1948). However, researchers on PSM no longer accept isolation of PSM using culture medium with $Ca_3PO_4$ because it can supply free Pi. Some authors reported that in vitro microbial solubilization not only occurred with calcium-phosphate but also with Al and Fe phosphates. However, the solubilization was higher with calcium phosphates (IIlmer et al. 1995; Banik and Dey 1983; Rose 1957).

Solubilization of Al and Fe phosphates could be easily observed under in vitro conditions where no soil minerals interfere with Pi-solubility (9) and (10). IIlmer

et al. (1995) found that *A. niger, Penicillium simplicissium, Pseudomonas aurantiogriseum*, and *Pseudomonas* sp. were effective in solubilizing $AlPO_4$ under in vitro conditions via organic acid production or proton excretion due to $NH_4^+$ assimilation. Aluminum and Fe ions in solution may be chelated by organic anions, such as oxalate and citrate (Bolan et al. 1994), favoring the dissolution of Al and Fe phosphates. However, the role of organic acids released by PSM in solubilizing Pi from Al- and Fe-phosphate under acidic soil conditions must be thoroughly investigated.

$$AlPO_4 \cdot 2H_2O + 2H^+ \leftrightarrow Al^{3+} + H_2PO_4^- + 2H_2O \quad K = 10^{-2.5}, \tag{9}$$

$$FePO_4 \cdot 2H_2O + 2H^+ \leftrightarrow Fe^{3+} + H_2PO_4^- + 2H_2O \quad K = 10^{-6.8}. \tag{10}$$

On the other hand, organic anions produced by PSM can also compete with phosphate for Pi-sorption sites onto the surface of soil minerals. In this regard, He and Zhu (1997, 1998) suggested that Pi-sorbed onto the surfaces of some minerals was displaced when a culture medium was inoculated with soil samples containing microorganisms (unidentified) that presumably excreted organic acids. Similarly, Osorio (2008) found that *Mortierella* sp., a PSF, was capable of desorbing Pi-sorbed from soil by releasing oxalic acid. The effectiveness of this fungus to desorb Pi was controlled by the type of soil and followed the order: mollisol > oxisol > ultisol > andisol. Moreover, the effectiveness of *Mortierella* sp. to desorb Pi-sorbed from soil minerals decreased in the order: montmorillonite > kaolinite > goethite > allophane.

### *16.4.2 Role of Organic Acids in Pi-Solubilization*

Many organic acids have been found effective in solubilizing soil P compounds (Hue 1991) and other soil minerals (Calvaruso et al. 2006; Welch et al. 2002). These acids or anions are produced by roots (Corrales et al. 2007; Radersma and Grierson 2004; Kirk et al. 1999; Marschner 1995) and by microbial activity during decomposition of organic matter or induced by Pi-deficiency stress (Jones et al. 2003; Fransson et al. 2004; Bohn et al. 1985). In accordance with these, Bolan et al. (1994) found organic acids or anions in high concentrations in poultry manure, lesser amounts in the rhizosphere soil, very little in the bulk soil, and trace amounts in leaf litter. Also, Le Bayon et al. (2006) reported a relatively higher production of organic anions (citrate, fumarate, and malate) in the rhizosphere of *Lupinus albus* under Pi-starvation. Furthermore, to substantiate the role of such organic anions in P solubilization, Bolan et al. (1994) assessed the influence of monocarboxylic (acetic, formic, and lactic), dicarboxylic (malic, tartaric, and oxalic), and tricarboxylic (citric) acids/anions in the solubilization and sorption of Pi on an andisol (Hydric Dystrandept) and an alfisol (Typic Fragiaqualf) of New Zealand. The

**Table 16.4** Stability constant of organic anions with aluminum (Log $K_{Al}$) and calcium (Log $K_{Ca}$) and their effects on soil Pi-sorption and solubilization of two Pi-fertilizers

| Organic acid | Log $K_{Al}$[a] | Log $K_{Ca}$[b] | Sorbed P (mmol $kg^{-1}$ soil) | Dissolved MCP[e] (%) | Dissolved NCPR[f] (%) |
|---|---|---|---|---|---|
| Water (control) | – | – | 52 | 2.35 | 1.28 |
| Formic | 1.36 | 1.43[b] | 47 | 12.27 | 11.86 |
| Acetic | 1.60 | 1.18[b] | 45 | 10.35 | 12.57 |
| Lactic | 2.41 | 1.63[c] | 46 | 8.71 | 13.92 |
| Malic | 5.40 | 2.25[c] | 37 | 32.65 | 31.98 |
| Tartaric | 5.62 | 2.80[b] | 35 | 30.49 | 32.31 |
| Oxalic | 6.16 | 3.44[d] | 32 | 36.44 | 34.02 |
| Citric | 7.98 | 4.87[b] | 18 | 83.78 | 86.41 |

Source: Bolan et al. (1994)
[a]Hue et al. (1986); [b]MINTEQA2 (a model for equilibrium speciacion in geochemical enviroments, accesed at www.epa.gov on March 15, 2008); [c]Bazin et al. (1995); [d]Finlayson et al. (1972); [e]Monohydrogen calcium phosphate; [f]*NCRP* North Carolina rock phosphate

addition of these organic acids significantly decreased Pi-sorption on allophane surface. The effectiveness of such acids followed the order: tricarboxylic > dicarboxylic > monocarboxylic. This was explained by the formation of complexes between Al with the conjugated anion of each organic acid and by their respective stability constant (Log $K_{Al}$) (Hue et al. 1986), as depicted in Table 16.4. The addition of organic acids or anions also favored the solubilization of North Carolina rock phosphate (NCRP) and monohydrogen calcium phosphate (MCP), increased dry matter yield of ryegrass (*Lolium rigidium*), and enhanced plant Pi-uptake (Bolan et al. 1994).

Hue (1991) obtained similar results on the availability of soil Pi when organic acids/anions were added to two andisols, an oxisol, an ultisol, and a vertisol of Hawaii. The effectiveness of the acids to reduce Pi-sorption from a soluble source ($KH_2PO_4$) was higher with malic acid (monohydroxy dicarboxylic), followed by protocatechuic acid (dihydroxy monocarboxylic), and acetic acid (monocarboxylic). The effect was higher when the acid was applied first and Pi last. The dry weight of lettuce (*Lactuca sativa*) was significantly higher when the soils received organic acids or anions plus Pi compared to those that received only Pi. The magnitude of this effect was much higher in the two andisols and in the oxisol whereby plant dry weight was 5- to 15-fold higher than control plants, whereas in the vertisol the increase was only 1.3- to 1.72-fold. Such variations in effects were suggested to be due to differences in clay mineralogy that determines the soil Pi-sorption capacity. From this study, it was concluded that the efficiency of Pi-fertilizers might be enhanced if they are added with organic acids or anions or, more practically, with green manures or animal wastes. Results of recent studies have also shown that RP could be more effective if it is applied with manures, composts, and crop litters (Reddy 2007; Bah et al. 2006; Singh et al. 2006).

The phenomenon of Pi-desorption by organic anions is widely accepted by soil scientists. Recently, Sato and Comerford (2006) used organic anions to model the Pi-desorption from a Brazilian soil. They concluded that the organic anions can increase soluble Pi by two process: (1) the desorption of P sorbed onto soil surface (ligand exchange), and (2) the dissolution of soil P compounds (ligand dissolution)

(e.g., calcium phosphates). Since PSM can release the same organic acids as reported by Hue (1991) and Bolan et al. (1994), these microorganisms can presumably reduce the activity of Al ions in the rhizosphere, decrease Pi-sorption, and enhance plant Pi-uptake. Miyasaka et al. (1991) found that the Al tolerance of plants is associated with the ability of roots to release organic acids/anions (citrate and oxalate, in particular) into the rhizosphere. Since some soil microorganisms are able to produce organic acids, it is possible that such microorganisms could help plants to grow in soils with toxic levels of $Al^{3+}$. In a series of experiments, De la Fuente and Herrera (1999) isolated a gene that encodes for citrate synthetase overproduction in the TCA cycle of a phosphate solubilizing strain of *Pseudomonas aeruginosa*. This gene was then transferred to tobacco (*Nicotiana tabacum*) cells of Al-intolerant plants. Transgenic plants were able to produce high amounts of citric acid and citrate, its conjugated anion, and grew in solutions with high concentration of Al. The process was successfully replicated with papaya (*Carica papaya*) plants. Although these experiments were aimed at enhancing Al tolerance of these plants, they also tested mechanisms proposed for the microbial solubilization of soil Pi. However, Delhaize et al. (2001) reported that they were not able to repeat the results obtained by De la Fuente and Herrera (1999) and, hence, suggested that the expression of *P. aeruginosa* genes are either sensitive to environmental conditions or that the observed Al tolerance and improved Pi-nutrition were due to other factors. Generally, the roles of organic acid/anion production in the rhizosphere on Pi-desorption and Pi-solubilization has been accepted. However, experimental results indicate that its efficiency in increasing plant Pi-uptake depends on plant species, age, physiological state, and soil mineralogy (Trolove et al. 2003). The concentration of these organic compounds is relatively low in most soils because they can be precipitated with free ions (e.g., $Al^{3+}$, $Fe^{3+}$, $Ca^{2+}$) or sorbed on soil clay reactive surfaces. Also, their persistence in rhizosphere soil is very low because they can be used as carbon sources by soil microorganisms (Jones et al. 2003). Thus, competent rhizosphere microorganisms capable of producing organic acids or anions can play an important role in the management of Pi-deficient and high Pi-sorbing soils.

## 16.5 Interactive Effect of Arbuscular Mycorrhizal Fungi and Phosphate-Solubilizing Microbes on Plant Pi-Uptake

The dual inoculation of PSM and AM fungi may overcome the limitations on the effectiveness of PSM to enhance plant Pi-uptake in soils with high Pi-fixation capacity. During this interaction, mycorrhizal plants release higher amounts of carbonaceous substances into the rhizosphere (Rambelli 1973; Linderman 1988) which is used as a carbon source by PSM (Azcon and Barea 1996). Moreover, the extensive mycorrhizal hyphae network formed around roots can efficiently take up Pi released by PSM, thus minimizing its refixation. As long as PSM grow in the rhizosphere (or mycorrhizosphere), there is a great opportunity to satisfy their

**Table 16.5** Effects of *E. agglomerans* (PSM) and *G. etunicatum* (AMF) inoculation on growth and Pi-uptake in tomato plants 75 days after inoculation

| Treatment | Shoot dry weight (g per plant) | Root dry weight (g per plant) | Shoot P content (mg per plant) |
|---|---|---|---|
| Control | 42.2 (100)[a] | 4.3 (100) | 116.6 (100) |
| PSM | 48.5 (115) | 5.1 (118) | 125.3 (107) |
| AMF | 47.6 (113) | 5.6 (130) | 120.9 (104) |
| PSM+AMF | 54.6 (129) | 6.8 (158) | 134.4 (115) |
| LSD ($P \leq 0.05$) | 1.96 | 0.5 | 9.8 |

*Source*: Kim et al. (1998a)
[a]Values in parenthesis indicates percentage increase over control.

carbon requirement and deliver Pi into the soil solution. Synergistic effects have been found in sunflower (*Helianthus annus*) with the triple inoculation of two PSM (*Azotobacter chroococcum* and *Penicillium glaucum*) and the AM fungus *G. fasciculatum* (Gururaj and Mallikarjunaiah 1995). Similar effects were found in cotton *(Gossypium hirsutum* L.*)* with the inoculation of *Pseudomonas striata* and *Azospirillum* sp. (PSM) and the arbuscular mycorrhizal fungi (AMF) *G. fasciculatum* (Prathibha et al. 1995). In rice (*O. sativa*), favorable effects were also reported with *P. striata* (PSM) and *Bacillus polymyxa* (PSM) and the AM fungus *G. fasciculatum* (Mohod et al. 1991). In chili (*Capsicum annuum*), synergistic effects werre reported with two AMF, *G. fasciculatum* or *G. macrocarpum,* and a PSM *P. striata* (Sreenivasa and Krishnaraj 1992). In tomato (*Lycopersicon lycopersicum*), beneficial results were found with *E. agglomerans* and *G. etunicatum* (Kim et al. 1998a) (Table 16.5). Moreover, positive results have been obtained in wheat with multiple combinations that included *P. striata* (PSM) and *G. fasciculatum* (AMF), *P. putida, P. aeruginosa* and *P. fluorescens* (PSM) with *G. clarum* (AMF), *P. striata* and *Agrobacterium radiobacter* (PSM) combined with two AMF*, G. fasciculatum* and *G. margarita* (Gaur et al. 1990).

Kopler et al. (1988) indicated that more legume nodulation was obtained with concurrent inoculation of *Rhizobium* and *Pseudomonas* spp. (PSM). Sturz et al. (1997) found that nodulation by *Rhizobium leguminosarum* b.v. *trifolii* in red clover (*Trifolium pratense*) was promoted when it was coinoculated with the PSM *Bacillus insolitus*, *B. brevis* or *Agrobacterium rhizogenes*. Similar results were obtained with the inoculation of *G. mosseae* (AMF) and *Azorhizobium caulinodans* (PSM) for *Sesbania rostrata* (Rahman and Parsons 1997). In soybean (*Glycine max*), the combination of *Bradyrhizobium japonicum* ($N_2$ fixer) with *P. fluorescens* (PSM) and *G. mosseae* (AMF) showed equally good results (Shabayey et al. 1996). Lately, El-Azouni (2008) also found that the dual inoculation of *A. niger* and *P. italicum* significantly increased the plant dry weight and yield of soybean. Such results are likely to be due to a higher plant Pi-uptake promoted by the combined action of PSM and AMF, which may satisfy the high Pi requirements of the $N_2$-fixing process (Azcon and Barea 1996; Young et al. 1990). More recently, Khan and Zaidi (2007) found synergistic effects with the triple inoculation of two plant growth-promoting rhizobacteria *A. chroococcum* and *Bacillus* sp. and *Glomus fasciculatrum* on plant growth, yield and nutrient uptake of wheat plants uinder field conditions.

Peix et al. (2001) found that the $N_2$-fixing bacterium *Mesorhizobium mediterraneum* was able to solubilize $Ca_3(PO_4)_2$ under in vitro and soil conditions. Inoculation with *M. mediterraneum* of seeds of chickpea (*Cicer arietinum*) and barley (*Hordeum vulgare*) planted in a Calcic Rhodoxeralf significantly increased plant growth and total N- and Pi-content in both plants, which were increased even further when *M. mediterraneum* and $Ca_3PO_4$ were applied simultaneously. Such enhancement in the overall performance of the tested crops was not only due to the symbiotic $N_2$ fixation but also to the availability of soluble P to the plants. Apparently, there is a certain degree of specificity among PSM, AMF, and P source. Toro et al. (1996) studied the combined effect of AMF (*Glomus* spp.) and eight PSM (bacteria) on plant growth and Pi-nutrition of a tropical legume, kudzu (*Pueraria phaseoloides*). The PSM were isolated from an oxisol and were characterized by their ability to solubilize RP, Al- and Fe-P compounds. In general, the combined inoculation of PSM, *Rhizobium* and AMF increased growth, yield, and nutritional properties of kudzu plants. However, such response was not observed for all combinations of AMF and PSM. For instance, the three PSM namely, *Azospirillum* sp., *Bacillus* sp., and *Enterobacter* sp., had a greater effect when they were coinoculated with *G. mosseae*. In contrast, *Pseudomonas* sp. and an unidentified isolate demonstrated a better performance when they were combined with *G. fasciculatum*. On the other hand, Fe-P solubilizers were more effective if they were used alone, while Al-P and RP solubilizers performed better when they were concurrently inoculated with AMF. Reasons for these differences may be due to the interactions found between the microorganisms. For instance, some of these PSM could be more effective in stimulating a rapid mycorrhizal colonization, and enhancing the length, distribution, and/or survival of external fungal mycelium. Mycorrhizal fungi might differ in the amount and type of hyphal exudates released into the mycorrhizosphere. In addition, a high capacity to solubilize Pi might stimulate plant growth and favor mycorrhizal activity. Kucey (1987) inoculated a mollisol (pH 7.2) of Canada with *P. bilaji*, in which either mycorrhizal or nonmycorrhizal wheat or beans were grown. In the case of wheat, mycorrhizal inoculation alone increased Pi-uptake significantly by 30%, while *P. bilaji* alone did not do so. However, *P. bilaji* increased Pi-uptake of mycorrhizal wheat by 10% in the unfertilized soil, but not in the soil fertilized with RP. In the case of bean, mycorrhizal inoculation alone did not increase plant Pi-uptake, but *P. bilaji* alone was able to increase it significantly by 31%. Dual inoculation, however, did not increase Pi-uptake beyond the level obtained with *P. bilaji*. In other words, no synergism was established between the inoculated microorganisms and, hence, Pi-uptake by bean plants grown in unfertilized and RP-fertilized soil did not increase. Mollisols usually exhibit a low Pi-fixation capacity. For this reason, it is not surprising that inoculation with this PSM alone increased bean Pi-uptake. However, the differences in the effect on wheat and bean plants could also be due to the release of different types and amounts of root exudates that otherwise stimulate acid production in the rhizosphere by PSM. The interactive effect of both PSM and AMF on plant P-uptake is explained in Fig. 16.1 (Osorio 2008). Once the plant releases

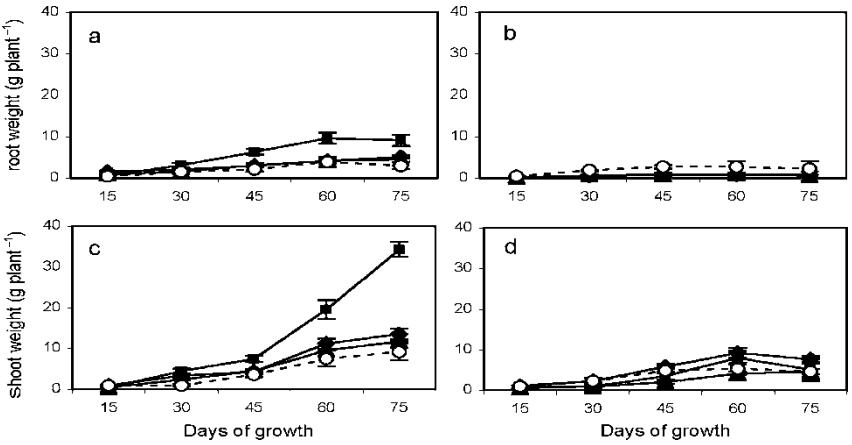



**Fig. 16.1** Diagram showing the microbial solubilization of soil and added P by *Mortierella* sp. and the mycorrhizal Pi-uptake (Osorio, original drawing)

organic compounds into its rhizosphere, the PSM produces organic acids that dissolve insoluble calcium phosphate compounds (added o native). Also, such organic acids can desorb Pi sorbed from soil minerals, which is controlled by the soil Pi-sorbing capacity. The Pi-release can be efficiently taken up by the mycorrhizal hyphae favoring in this way the plant growth.

## 16.6 Microbial Pi-Solubilization in Temperate and Tropical Soils

Currently, *P. bilaji* is commercially available in North America under the name of Provide$^{TM}$, which has been successfully tested to enhance plant Pi-uptake of some crop plants (Whitelaw 2000). However, the tests have been conducted in mollisols of low Pi-fixation capacity. In contrast, little research on PSM has been conducted in highly weathered and volcanic ash soils of the tropics with high Pi-fixation capacity. Toro et al. (1996) isolated various effective PSM from an oxisol of Venezuela. However, they were not tested in this soil from which they were originated but in a calcareous soil of Spain in which they were able to improve plant growth and Pi-uptake of tropical kudzu (*Pueraria phaseoloides*). Whitelaw et al. (1997) inoculated an acidic Pi-deficient soil of Australia (pH 4.6) with *P. radicum* in combination with several levels of $KH_2PO_4$ (P: 0–20 kg ha$^{-1}$). The inoculation with this PSF increased wheat Pi-uptake by 8% in the unfertilized soil. When the fungus was coinoculated with Pi-fertilizer, plant Pi increased between 2 and 28%; increase was highest when the rate of added P was 15 kg ha$^{-1}$. Young

**Table 16.6** Effect of AM fungus, phosphate-solubilizing microbes and rock phosphate on peanut yield (kg $ha^{-1}$) in two soils of Taiwan

| Treatment | Hualain soil (pH 4.2) | Yuanchang soil (pH 5.4) |
|---|---|---|
| Control | 1,875 b (100) | 3,667 c (100) |
| RP (660 kg $ha^{-1}$) | 2,250 a (120) | 6,167 a (169) |
| AMF | 2,350 a (125) | 6,208 a (140) |
| PSM | 2,259 a (120) | 6,333 a (173) |
| AMF+RP | 2,367 a (126) | 5,125 b (140) |
| PSM+RP | 2,275 a (121) | 6,083 ab (166) |

Letters indicate mean separation by Duncan's multiple range test ($P \leq 0.05$). Values in parenthesis indicate percentage increase over control.
*Source*: Young et al. (1990)

**Table 16.7** Effect of AM fungus and phosphate solubilizing microbes on growth (g per pot) of leucaena grown in three soils of Taiwan

| Treatment | Hinshe (pH 5.0; P: 2 mg $kg^{-1}$) | Wunfun (pH 5.5; P: 3 mg $kg^{-1}$) | Taitung (pH 7.8; P: 95 mg $kg^{-1}$) |
|---|---|---|---|
| Control | 8.4 c (100) | 13.7 b (100) | 22.0 b (100) |
| PSM | 10.4 b (124) | 12.9 b (94) | 30.8 a (140) |
| AMF | 13.1 a (156) | 27.3 a (199) | 26.8 b (122) |
| PSM+AMF | 12.8 a (152) | 26.0 a (190) | 23.6 b (107) |

Letters indicate mean separation by Duncan's multiple range test ($P \leq 0.05$). Values in parentheses indicate percentage increase over control.
*Source*: Young et al. (1990)

et al. (1990) found that inoculation with either PSM or AMF significantly increased peanut (*Arachis hypogea*) production in two subtropical–tropical acidic soils of Taiwan. Inoculation with either AMF or PSM in unfertilized soils was as effective as the addition of RP alone (Table 16.6). Inoculation with AMF or PSM of RP-fertilized soils did not increase peanut yield above that obtained with AMF or PSM inoculation in unfertilized soils. Unfortunately, dual inoculation of AMF and PSM was not evaluated. Interestingly, PSM inoculation alone increased peanut yield by 73% in the less acidic soil (Yuanchang soil), but the increase was only 20% in the strongly acidic soil (Hualain soil). In addition, Young et al. (1990) found that the responses to single or mixed inoculations with PSM and/or AMF had variable effects on plant growth of *Leucaena* grown in three soils of Taiwan. Inoculation with PSM was not as effective as AMF inoculation in enhancing plant growth in the soil with the lowest available Pi-level (Hinshe soil) (Table 16.7). In the Wunfun soil (also with a low soil available Pi-level), PSM inoculation was ineffective in increasing plant growth unlike AMF. In the alkaline soil containing the highest soil available Pi (presumably rich in calcium-phosphates), PSM inoculation alone significantly increased plant growth (40%) above the AMF inoculation effect, which did not increase growth.

Effectiveness of PSM inoculation alone to enhance plant Pi-uptake in subtropical and tropical acidic soils is relatively low and variable. The increases recorded were 8% (Whitelaw et al. 1997), 13% (Osorio and Habte 2001), and 24–25% (Young et al. 1990) compared with those reported in less weathered soils (mostly

Mollisols) of the temperate zone, where soil Pi-fixation capacity is low. By contrast, effectiveness of PSM inoculation to enhance plant Pi-uptake of mycorrhizal plants grown in tropical or subtropical soils can be relatively higher compared to data reported in temperate soils (Table 16.7). Mycorrhizal colonization in combination with PSM is often needed to obtain improvements in plant P uptake in highly weathered soils, in contrast to results obtained in less weathered soils. In these less weathered soils that normally exhibit low soil P sorption, the inoculation of PSM alone has been found effective to increase plant P-uptake of nonmycorrhizal plants (Peix et al. 2001; Omar 1998; Kucey 1983, 1987, 1988; Asea et al. 1988; Kucey et al. 1989; Gleddie 1993). Most of the soils used by these authors were mollisols, calcareous soils, or sandy soils, which are characterized by a low P-sorption capacity and relatively high soil Ca–Pi content (Cross and Schlesinger 1995). Therefore, the freshly released Pi by PSM can remain longer in the soil solution until its absorption by the roots. For instance, Toro et al. (1998) found that the PSM *Enterobacter* sp. alone was as effective as the mycorrhizal fungus *G. mosseae* when used alone, and increased the P uptake of alfalfa grown in a calcareous soil of Spain by twofold. Duponnois et al. (2006) found that single inoculation of fungus *Arthrobotrys oligospora* increased the P uptake and shoot dry weight of *Acacia holoserica* grown in a sandy soil of Senegal by 56 and 46%, respectively. The increase in plant P uptake and growth were even higher when RP was added with the PSM (74 and 103%, respectively). The application of RP alone, however, did not significantly increase growth and plant P uptake. Similar results were observed by Wakelin et al. (2004a, 2004b) for *Penicillum radicum*- inoculated wheat grown in sandy soils in Australia with neutral to alkaline soil reactivity. In these soils, Wakelin and coworkers observed increases in plant growth between 34 and 76%. Furthermore, *Penicillium thomii* has shown a threefold higher increase in plant P uptake of mint (*Mentha piperita*), grown in a soil-less medium (vermiculite–perlite) fertilized with RP compared to those observed for uninoculated plants and unfertilized control (Cabello et al. 2005). The RP alone was, however, ineffective. The impressive increase in plant P uptake is understandable given the very low P sorption on this kind of substrates.

The results obtained recently by Osorio (2008) indicate that the effectiveness of *Mortierella* sp., a phosphate-solubilizing fungus (PSF), in enhancing plant Pi-uptake and growth was controlled by the type of soil, particularly by the Pi-sorption capacity of the soil (Table 16.8). In a mollisol (low Pi-sorption capacity) *Mortierella* sp. alone was capable of increasing shoot dry weight of *Leucaena*. However, the effect was significantly higher in the presence of the mycorrhizal fungus *G. fasciculatum*. On the other hand, in two oxisols (medium Pi-sorption capacity), the PSF stimulated dry matter accumulation in shoots only in the presence of the mycorrhizal fungus. In contrast, the PSF was ineffective in increasing plant Pi-uptake in an andisol (very high P sorption capacity) even in the presence of the mycorrhizal association. The $P_{0.2}$ value (Tables 16.1 and 16.8) seems to be a good predictor of the effectiveness of PSM to increase plant Pi-uptake via Pi-desorption, RP solubilization, and Pi-uptake by mycorrhizal roots.

**Table 16.8** Effect of AM fungus (AMF) and phosphate- solubilizing fungus (PSF) on growth (g per pot) of leucaena grown in three tropical soils of Colombia

| Treatment | Mollisol ($P_{0.2} = 45$ mg kg$^{-1}$) | Oxisol ($P_{0.2} = 417$ mg kg$^{-1}$) | Andisol ($P_{0.2} = 2{,}222$ mg kg$^{-1}$) |
|---|---|---|---|
| Control | 0.73 | 0.32 | 0.26 |
| PSF | 1.18 | 0.30 | 0.28 |
| AMF | 1.36 | 0.84 | 0.28 |
| PSF + AMF | 1.48 | 0.97 | 0.26 |
| LSD | 0.09 | 0.12 | 0.09 |

Vertical comparisons (LSD test, $P \leq 0.05$).
Adapted from Osorio (2008)

## 16.7 Conclusion

One of the major limiting factors for plant productivity in the tropics is low soil Pi-availability. Phosphorus is rendered unavailable for plant uptake due to adsorption onto the surface of soil minerals and precipitation by free $Al^{3+}$ and $Fe^{3+}$ ions. As a result, the efficiency of Pi-fertilization becomes quite low and hence, require application of high rates of soluble P fertilizer. A viable alternative to overcome dependance on chemical phosphatic fertilizer is the use of phosphate rocks (RP), which are locally available and are cheaper. The use of rhizosphere PSM has received greater attention to increase the agronomic effectiveness of RP. The most accepted mechanism is the production and release of organic acids (e.g., citric acid, oxalic acid, gluconic acid, among others). The released protons decrease the rhizosphere pH, while the organic anions can form complex with calcium favoring thus the dissolution of RP. In addition, it has been demonstrated that organic anions produced by PSM can also desorb Pi from soil minerals. However, given the high Pi-sorption capacity in highly weathered soils of the tropics the effectiveness of PSM to increase plant Pi-uptake and growth can be low. Synergistic effects of PSM and AMF on plant nutrition, growth, and yields have been reported. This dual inoculation seems to be more relevant in the case of soil exhibiting high Pi-sorption capacity (e.g., oxisols, ultisols, andisols) that are abundant in the tropics. Further studies are, however, required in tropical soils in order to establish an effective use of PSM inoculants in a more predictable and sustainable manner.

## References

Abd-Alla MH (1994) Use of organic phosphorus by *Rhizobium leguminosarum* biovar. *viceae* phosphatases. Biol Fertil Soils 18:216–218

Agnihorti VP (1970) Solubilization of insoluble phosphates by some soil fungi isolated from nursery seedbeds. Can J Microbiol 16:877–880

Amos B, Walters DT (2006) Maize root biomass and net rhizodeposited carbon: an analysis of the literature. Soil Sci Soc Am J 70:1489–1503

Asea PEA, Kucey RMN, Stewart JWB (1988) Inorganic phosphate solubilisation by 2 *Penicillium* species in solution culture and soil. Soil Biol Biochem 20:459–464

Atlas R, Bartha R (1997) Microbial ecology. Addison Wesley, New York
Azam F, Memon GH (1996) Soil organisms. In: Bashir E, Bantel R (eds) Soil science. National Book Foundation, Islamabad, pp 200–232
Azcon C, Barea JM (1996) Interactions of arbuscular mycorrhiza with rhizosphere microorganisms. In: Guerrero E (ed) Mycorrhiza. Biological Soil Resource. FEN, Bogota, Colombia, pp 47–68
Bache BW (1963) Aluminum and iron phosphates studies relating to soils. I. Solution and hydrolysis of variscite and strengite. J Soil Sci 14:113–123
Bah A, Zaharah AR, Hussin A (2006) Phosphorus uptake from green manures and phosphate fertilizers applied in a acid tropical soil. Commun Soil Sci Plant Anal 37:2077–2093
Banik S, Dey BK (1981a) Phosphate solubilizing microorganisms of a lateritic soil. I. Solubilization of inorganic and production of organic acids by microorganisms isolated in sucrose calcium phosphate agar plates. Zentralblatt Bakteriol Parasitenkunde infectionskarankheiten, hygiene. 2. Naturwiss Miikrobiology Landwirtsch 136:476–486
Banik S, Dey BK (1981b) Phosphate solubilizing microorganisms of a lateritic soil. II. Effect of some tricalcium phosphate-solubilizing microorganisms on available phosphorus content of the soil. Zentralblatt Bakteriol. Parasitenkunde infectionskarankheiten, hygiene. 2. Naturwiss Miikrobiology Landwirtsch 136:487–492
Banik S, Dey BK (1981c) Phosphate solubilizing microorganisms of a lateritic soil. III. Effect of inoculation of some tricalcium phosphate-solubilizing microorganisms on available phosphorus content of rhizosphere soils of rice (*Oryza sativa* L. cv. IR-20). Zentralblatt Bakteriol. Parasitenkunde infectionskarankheiten, hygiene. 2. Naturwiss Miikrobiology Landwirtsch 136:493–501
Banik S, Dey BK (1982) Available phosphate content of an alluvial soil as influenced by inoculation of some isolated phosphate solubilizing microorganisms. Plant Soil 69:353–364
Banik S, Dey BK (1983) Phosphate solubilizing potentiality of the microorganisms capable of utilizing aluminium phosphate as a sole phosphate source. Zentralbl Mikrobiol 138:17–23
Barber SA (1995) Soil nutrient bioavailability: a mechanistic approach. Wiley, New York
Bar-Yosef B, Rogers RD, Wolfram JH, Richman E (1999) *Pseudomonas cepacia*-mediated rock phosphate solubilization in kaolinite and montmorillonite suspensions. Soil Sci Soc Am J 63:1703–1708
Bazin H, Bouchu A, Descotes G, Petit-Ramel M (1995) Comparison of calcium complexation of some carboxylic acids derived from D-glucose and D-fructuose. Can J Chem 73:1338–1347
Bohn H, McNeal BL, O'connor G (1985) Soil chemistry. Wiley, New York
Bolan NS (1991) A critical review on the role of mycorrhizal fungi in the uptake of phosphorus by plants. Plant Soil 134:189–207
Bolan NS, Naidu R, Mahimairaja S, Baskaran S (1994) Influence of low-molecular-weight organic acids on the solubilization of phosphates. Biol Fertil Soils 18:311–319
Brady NC, Weil RR (1999) The nature and properties of soils. Prentice Hall, Upper Saddle River, New Jersey
Buol S, Hole FD, McCraken RJ, Southard RJ (1997) Soil genesis and classification. Iowa State University Press, Ames
Cabello M, Irrazabal G, Bucsinszky AM, Saparrat M, Schalamuk S (2005) Effect of an arbuscular mycorrhizal fungus, *Glomus mosseae*, and a rock-phosphate-solubilizing fungus, *Penicillium thomii*, on *Mentha piperita* growth in a soilless medium. J Basic Microbiol 45:182–289
Calvaruso C, Turpault MP, Frey-Klett P (2006) Root-associated bacteria contribute to mineral weathering and to mineral nutrition in trees: a budgeting analysis. Appl Environ Microbiol 72:1258–1266
Canbolat MC, Bilen S, Cakmakci R, Sahin F, Aydin A (2006) Effect of plant growth-promoting bacteria and soil compaction on barley seedling growth, nutrient uptake, soil properties, and rhizosphere microflora. Biol Fertil Soils 42:350–357
Cardoso I, Boddington CL, Janssen BH, Oenema O, Kuyper T (2006) Differential acces to phosphorus pool of an Oxisol by mycorrhizal and non-mycorrhizal maize. Commun Soil Sci Plant Anal 37:1537–1552

Corrales I, Amenos M, Poschenrieder C, Barcelo J (2007) Phosphorus efficiency and root exhudates in two contrasting tropical maize varieties. J Plant Nutr 30:887–900

Cross AF, Schlesinger WH (1995) A literature review and evaluation of the Hedley fractionation: application to the biogeochemical cycle of soil phosphorus in natural ecosystems. Geoderma 64:197–214

De la Fuente JM, Herrera L (1999) Advances in the understanding of aluminum toxicity and the development of aluminum tolerant transgenic plants. Adv Agron 66:103–121

Delhaize E, Hebb DM, Ryan PR (2001) Expression of a *Pseudomonas aerugionosa* citrate synthase gene in tobacco is not associated with either enhanced citrate accumulation or efflux. Plant Physiol 125:2059–2067

Di-Simine CD, Sayer JA, Gadd GM (1998) Solubilization of zinc phosphate by a strain of *Pseudomonas fluorescens* isolated from a forest soil. Biol Fertil Soils 28:87–94

Do Carmo Harta M, Torrent J (2007) Phosphorus desorption kinetics in relation to phosphorus forms and sorption properties of Portuguese acid soils. Soil Sci 172:631–638

Duponnois R, Kisa M, Plenchette C (2006) Phosphate-solubilizing potential of the nematophagous fungus *Arthrobotrys oligospora*. J Plant Nutr Soil Sci 169:280–282

El-Azouni IM (2008) Effect of phosphate solubilizing fungi on growth and nutrient uptake of soybean (*Glycine max* L.) plants. J Appl Sci Res 4(6):592–598

Engelstad OP, Terman GL (1980) Agronomic effectiveness of phosphate fertilizers. In: Khasawneh FE, Sample E, Kamprath E (eds) The role of phosphorus in agriculture. Soil Science Society of America, Madison, WI, pp 311–332

Finlayson G, Roth R, Bubois L (1972) Calcium oxalate solubility studies in urinary calculi. In: International Symposium on Renal Stone Research Madrid, Spain, pp 1–7

Fox RL (1979) Comparative responses of field grown crops to phosphate concentrations in soil solutions. In: Munsell H, Staples R (eds) Stress physiology in crop plants. Wiley, New York, pp 81–106

Fox RL, Kamprath E (1970) Phosphate sorption isotherms for evaluating phosphorus requirements of soils. Soil Sci Soc Am Proc 34:902–907

Fransson AM, Valeur I, Wallander H (2004) The wood-decaying fungus *Hygrophoropsis aurantica* increases P availability in acid forest humus soil, while N addition hampers this effect. Soil Biol Biochem 36:1699–1705

Gaur A, Rana J, Jalali B, Chand H (1990) Role of VA mycorrhizae, phosphate solubilizing bacteria and their interactions on growth and uptake of nutrients by wheat crops. In: Trends in mycorrhizal research. Proceedings of the National Conference on Mycorrhizae, Hisar, India, pp 105–106

Gleddie SC (1993) Response of pea and lentil to inoculation with the phosphate-solubilizing fungus *Penicillium bilaii* (Provide). In: Proceedings of the Soils and Crops Workshops. Saskatoon, Saskatchewan, pp 47–52

Goenadi DH (1995) Suitability of selected mixtures of clay minerals with humic substances as carrier of phosphates-solubilizing microbes. Menara Perkebunan 63:102–113

Goenadi DH, Saraswati R, Nganro NN (1995) Nutrient-solubilizing and aggregate stabilizing microbes isolates from selected humic tropical soils. Menara Perkebunan 63:60–66

Gururaj R, Mallikarjunaiah R (1995) Interactions among *Azotobacter chroococcum, Penicillium glaucum* and *Glomus fasciculatum* and their effect on the growth and yield of sunflower. Helia 18(23):73–84

Gyaneshwar P, Kumar GN, Parekh LJ, Poole PS (2002) Role of soil microorganisms in improving P nutrition of plants. Plant Soil 245:83–93

Habte M, Manjunath A (1991) Categories of vesicular-arbuscular mycorrhizal dependency of host species. Mycorrhiza 1:3–12

Habte M, Osorio NW (2001) Arbuscular mycorrhizas: producing and applying arbuscular mycorrhizal inoculum. University of Hawaii, Honolulu

Hameeda B, Kumar YH, Rupela OP, Kumar GN, Reddy G (2006) Effect of carbon substrates on rock phosphate solubilization by bacteria from compost and macrofauna. Curr Microbiol 53:298–302

Hamel C (2004) Impact of arbuscular mycorrhizal fungi on N and P cycling in the root zone. Can J Soil Sci 84:383–395
Hammond L, Leon L (1992) Evaluation of the North Carolina natural phosphate as a phosphoric fertilizer. Suelos Ecuat 22:143–150
Havlin J, Beaton J, Tisdale SL, Nelson W (1999) Soil fertility and fertilizers: an introduction to nutrient management. Prentice Hall, Upper Saddle River, New Jersey
He ZL, Zhu J (1997) Transformation and bioavailability of specifically sorbed phosphate on variable-charge mineral soils. Biol Fertil Soils 25:175–181
He ZL, Zhu J (1998) Microbial utilization and transformation of phosphate adsorbed by variable charge minerals. Soil Biol Biochem 30:917–923
Hetrick BAD (1991) Mycorrhizas and root architecture. Experentia 47:355–362
Holdford ICR (1997) Soil phosphorus: its measurement, and its uptake by plants. Aust J Soil Res 35:227–239
Hue NV (1991) Effects of organic acids/anions on P sorption and phytoavailability in soils with different mineralogies. Soil Sci 152:463–471
Hue NV, Craddock GR, Adams F (1986) Effects of organic acids on aluminum toxicity in subsoils. Soil Sci Soc Am J 50:28–34
IIlmer P, Barbato A, Schinner F (1995) Solubilization of hardly-soluble $AlPO_4$ with P-solubilizing microorganisms. Soil Biol Biochem 27:265–270
Illmer P, Schinner F (1995) Solubilization of inorganic calcium phosphates-solubilization mechanisms. Soil Biol Biochem 27:257–263
Jackman JM, Jones RC, Yost RS, Babcock CJ (1997) Rietveld estimates of mineral percentages to predict phosphate sorption by selected Hawaiian soils. Soil Sci Soc Am J 61:618–625
Jacobsen I, Abbott LK, Robson AD (1992) External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. New Phytol 120:371–380
Jeffries P, Gianinazzi S, Perotto S, Turnau K, Barea JM (2003) The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. Biol Fertil Soils 37:1–16
Jones RC (1981) X-ray diffraction line profile analysis vs. phosphorus sorption by 11 Puerto Rican soils. Soil Sci Soc Am J 45:818–825
Jones DL, Dennis PG, Owen AG, Van Hees PAW (2003) Organic acid behavior in soils–misconceptions and knowledge gaps. Plant Soil 248:31–41
Juo A, Ellis B (1968) Chemical and physical properties of iron and aluminum phosphate and their relation to phosphorus availability. Soil Sci Soc Am Proc 32:216–221
Juo ASR, Fox RL (1977) Phosphate sorption characteristics of some bench-mark soils of West Africa. Soil Sci 124:370–376
Khan MS, Zaidi A (2007) Synergistic effects of the inoculation with plant growth-promoting rhizobacteria and a arbuscular mycorrhizal fungus on the perfomance of wheat. Turk J Agr For 31:355–362
Khan MS, Zaidi A, Wani PA (2007) Role of phosphate-solubilizing microorganisms in sustainable agriculture – a review. Agron Sustain Dev 27:29–43
Kim KY, McDonald GA, Jordan D (1997) Solubilization of hydroxyapatite by *Enterobacter agglomerans* and cloned *Escherichia coli* in culture medium. Biol Fertil Soils 24:347–352
Kim KY, McDonald GA, Jordan D (1998a) Effect of phosphate solubilizing bacteria and vesicular-arbuscular mycorrhizae on tomato growth and soil microbial activity. Biol Fertil Soils 26:79–87
Kim KY, Jordan D, McDonald GA (1998b) *Enterobacter agglomerans*, phosphate solubilizing bacteria and microbal activity in soil. Effect of carbon sources. Soil Biol Biochem 30:995–1003
Kirk GJD, Santos EE, Findenegg GR (1999) Phosphate solubilization by organic anion excretion from rice (*Orytza sativa* L.) growing in aerobic soil. Plant Soil 211:11–18
Kopler J, Lifshitz R, Schroth M (1988) *Pseudomonas* inoculants to benefit plant production. ISI Atlas Sci Anim Plant Sci 1:60–64

Kucey RMN (1983) Phosphate solubilising bacteria and fungi in various cultivated and virgin Alberta soils. Can J Soil Sci 63:671–678

Kucey RMN (1987) Increased phosphorus uptake by wheat and field beans inoculated with a phosphorus solubilising *Penicillium bilaii* strain and with vesicular-asbuscular mycorrhizal fungi. Appl Environ Microbiol 53:2699–2703

Kucey RMN (1988) Effect of *Penicillium bilaii* on the solubility and uptake of P and micronutrients from soil by wheat. Can J Soil Sci 68:261–270

Kucey RMN, Janzen HH, Leggett ME (1989) Microbial mediated increases in plant available phosphorus. Adv Agron 42:199–228

Le Bayon RC, Weisskopf L, Martinoia E, Jansa J, Frossard E, Keller F, Follmi KB, Gobat JM (2006) Soil phosphorus uptake by continuously cropped *Lupinus albus*: a new microcosm design. Plant Soil 283:309–321

Li X, Ecckhard G, Marschner H (1991) Phosphorus depletion and pH decrease at the root-soil and hypha-soil interfaces of VA mycorrhizal white clover fertilized with ammonium. New Phytol 119:397–404

Linderman RG (1988) Mycorrhizal interaction with the rhizosphere microflora: The mycorhizosphere effect. Phytopathology 78:366–371

Lindsay WL (2001) Chemical equilibria in soils. Blackburn, Caldwell, New Jersey

Louw HA, Webley DM (1959) The solubilization of insoluble phosphates. V. The action of some organic acids on iron and aluminium phosphates. NZ J Sci 2:215–218

Lynch JP, Ho MD (2005) Rhizoeconomics: carbon costs of phosphorus acquisition. Plant Soil 269:45–56

Manjunath A, Hue NV, Habte M (1989) Response of *Leucaena leucocephala* to vesicular-arbuscular mycorrhizal colonization and rock phosphate fertilization in an Oxisol. Plant Soil 114:127–133

Marschner H (1995) Mineral nutrition of higher plants. Academic, London

Marschner P, Solaiman Z, Rengel Z (2006) Rhizosphere properties of *Poacea* genotypes under P-limiting conditions. Plant Soil 283:11–24

Mattingly GEG (1975) Labile phosphate in soils. Soil Sci 119:369–375

McCully M (1999) Roots in soil: unearthing the complexities of roots and their rhizospheres. Annu Rev Plant Physiol Plant Mol Biol 50:695–718

Memon KS (1996) Soil and fertilizer phosphorus. In: Bashir E, Bantel R (eds) Soil science. National Book Foundation, Islamabad, pp 291–314

Miyasaka SC, Habte M (2001) Plant mechanisms and mycorrhizal symbioses to increase phosphorus uptake efficiency. Commun Soil Sci Plant Anal 32:1101–1147

Miyasaka S, Buta JG, Howell RK, Foy CD (1991) Mechanisms of aluminum tolerance in snapbeans. Plant Physiol 96:737–743

Mohod S, Gupta DN, Chavan AS (1991) Effects of P solubilizing organims on yield and N uptake by rice. J Maharashtra Agric Univ 16:229–231

Msolla MM, Semoka JMR, Borggaard OK (2005) Hard Minjingu phosphate rock. An alternative P source for maize production on acid soils in Tanzania. Nutr Cyc Agroecosyst 72:299–308

Msolla MM, Semoka JMR, Szilas C, Borggaard OK (2007) Crop (maize) response to direct application of local phosphate rock on selected acidic soils of Tanzania. Commun Soil Sci Plant Anal 38:93–106

Nguyen C (2003) Rhizodeposition of organic C by plants: mechanisms and controls. Agronomie 23:375–396

Nye PH, Tinker PB (1977) Solute movement in the soil-root system. Blackwell, Oxford

Ojo OD, Kintomo AA, Akinride EA, Akoroda MO (2007) Comparative effect of phosphorus sources for grain amaranth production. Commun Soil Sci Plant Anal 38:35–55

Omar SA (1998) The role of rock-phosphate-solubilizing fungi and vesicular-arbuscular-mycorrhiza (VAM) in growth of wheat plants fertilized with rock phosphate. World J Microbiol Biotechnol 14:211–218

Osorio NW (2008) Effectiveness of microbial solubilization of phosphate in enhancing plant phosphate uptake in tropical soils and assessment of the mechanisms of solubilization. Ph.D. dissertation, University of Hawaii, Honolulu

Osorio NW, Habte M (2001) Synergistic influence of an arbuscular mycorrhizal fungus and P solubilizing fungus on growth and plant P uptake of *Leucaena leucocephala* in an Oxisol. Arid Land Res Manage 15:263–274

Pandey A, Trivedi P, Kumar B, Palni LMS (2006) Characterization of a phosphate solubilizing microorganism and antagonistic strain of *Pseudomonas putida* (B0) isolated from a sub-apline location in the Indian Central Himalaya. Curr Microbiol 53:102–107

Parfitt RL (1989) Phosphate reactions with natural allophane, ferrihydrite and goethite. J Soil Sci 40:359–369

Paul NB, Rao WVBS (1971) Phosphate-dissolving bacteria in the rhizosphere of some cultivated legumes. Plant Soil 35:127–132

Peix A, Rivas-Boyero AA, Mateos PF, Rodriguez-Barrueco C, Martinez-Molina E, Velasquez E (2001) Growth promotion of chickpea and barley by a phosphate solubilizing strain of *Mesorrhizobium mediterraneum* under growth chamber conditions. Soil Biol Biochem 33:103–110

Pikovskaia RI (1948) Mobilization of phosphates in soil in conection with the vital activities of some microbial species. Mikrobiologie 17:362–370

Plenchette C, Fortin JA, Furlan V (1983) Growth response of several plant species to mycorrhiza in a soil of moderate P fertility. I. Mycorrhizal dependency under field conditions. Plant Soil 70:191–209

Prathibha CK, Alagawadi A, Sreenivasa M (1995) Establishment of inoculated organisms in rhizosphere and their influence on nutrient uptake and yield of cotton. J Agric Sci 8:22–27

Prescott L, Harley J, Klein DA (1999) Microbiology. McGraw-Hill, Boston

Pypers P, Delrue J, Diels J, Smolders E, Merckx R (2006) Phosphorus intensity determines short term P uptake by pigeon pea (*Cajanus cajan* L.) grown in soils with different P buffereing capacity. Plant Soil 284:217–227

Radersma S, Grierson P (2004) Phosphorus mobilization in agroforestry: organic anions, phosphatase activity and phosphorus fractions in the rhizophere. Plant Soil 259:209–219

Rahman MK, Parsons JW (1997) Effects of inoculation with *Glomus mosseae, Azorhizobium caulinodans* and rock phosphate on the growth of and nitrogen and phosphorus accumulation in *Sesbania rostrata*. Biol Fertil Soils 25:47–52

Rambelli A (1973) The rhizosphere of mycorrhyzae. In: Marks GC, Kozlowski TT (eds) Ectomycorrhyzae. Their ecology and physiology. Academic, London, pp 299–343

Randhawa P, Condron LM, Di HJ, Sinaj S, McLenaghen RD (2006) Phosphorus availability in soils amended with different phosphate fertilizers. Commun Soil Sci Plant Anal 37:25–39

Rao S (1992) Biofertilizers in agriculture. A. A. Balkema, Rotterdam

Rashid M, Khalil S, Ayub N, Alam S, Latif F (2004) Organic acids production and phosphate solubilization by phosphate solubilizing microorganisms (PSM) under *in vitro* conditions. Pak J Biol Sci 7(2):187–196

Redding MR, Shatte T, Bell K (2006) Soil-sorption-desorption of phosphorus from piggery effluent compared with inorganic sources. Eur J Soil Sci 57:134–146

Reddy DD (2007) Phosphorus solubilization from low-grade rock phosphates in the presence of decomposing soybean leaf litter. Commun Soil Sci Plant Anal 38:283–291

Reddy MS, Kumar S, Babita K, Reddy MS (2002) Biosolubilization of poorly soluble rock phosphates by *Aspergillus tubigensis* and *Aspergillus niger*. Bioresour Technol 84:187–189

Reyes I, Valery A, Valduz Z (2006) Phosphate-solubilizing microrganisms isolated from rhizospheric and bulk soils of colonizer plants at an abandoned rock phosphate mine. Plant Soil 287:69–75

Rodriguez H, Fraga R, Gonzalez T, Bashan Y (2006) Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. Plant Soil 287:15–21

Roos W, Luckner M (1984) Relationships between proton extrusion and fluxes of ammonium ions and organic acids in *Penicillium cyclopium*. J Gen Microbiol 130:1007–1014

Rose RE (1957) Techniques of determining the effect of microorganisms on insoluble inorganic phosphates. NZ J Sci Technol 38:773–780
Salih HM, Yahya AI, Abdul-Rahem AM, Munam BH (1989) Availability of phosphorus in a calcareous soil treated with rock phosphate or superphosphate as affected by phosphate-dissolving fungi. Plant Soil 120:181–185
Sanchez P, Logan T (1992) Myths and science about the chemistry and fertility of soils in the tropics. In: Lal R, Sanchez P (eds) Myths and science of soils of the tropics. Soil Science Society of America, Madison, WI, pp 35–46
Sanchez P, Uehara G (1980) Management considerations for acid soils with high phosphorus fixation capacity. In: Khasawneh FE (ed) The role of phosphorus in agriculture. Soil Science Society of America, Madison, WI, pp 471–514
Sato S, Comerford N (2006) Organic anions and phosphorus desorption and bioavailability in a humid Brazilian Ultisol. Soil Sci 171:695–705
Schachtman DP, Reid R, Ayling SM (1998) Phosphorus uptake by plants: from soil to cell. Plant Physiol 116:447–453
Schwertmann U, Herbillon AJ (1992) Some aspects of fertility associated with the mineralogy of highly weathered tropical soils. In: Lal R, Sanchez P (eds) Myths and science of soils of the Tropics. Soil Science Society of America, Madison, WI, pp 47–60
Shabayey VP, Smolin VY, Mudrik VA (1996) Nitrogen fixation and $CO_2$ exchange in soybeans inoculated with mixed cultures of different microorganisms. Biol Fertil Soils 23:425–430
Shoji S, Nanzyo M, Dahlgren RA (1993) Volcanic ash soils-genesis, properties, and utilization. Elsevier, Amsterdam
Shrivastava M, Bhujbal BM, D'Souza SF (2007) Agronomic efficiency of Indian rock phosphate in acidic soils employing radiotracer A-value technique. Commun Soil Sci Plant Anal 38:461–471
Singh S, Kapoor KK (1999) Inoculation with phosphate-solubilizing microorganisms and a vesicular-arbuscular mycorrhizal fungus improves dry matter yield and nutrient uptake by wheat grown in a sandy soil. Biol Fertil Soils 28:139–144
Singh HP, Singh TA (1993) The interaction of rock phosphate, *Bradyrhizobium*, vesicular arbuscular mycorrhizae, and phosphate-solubilising microbes on soybean grown in a sub-Himalayan Mollisol. Mycorrhiza 4:37–43
Singh V, Dhillon NS, Brar BS (2006) Effect of incorporation of crop residues and organic manures on adsorption/desorption and bioavailability of phosphate. Nutr Cyc Agroecosyst 76:95–108
Smith FW (2002) The phosphate uptake mechanism. Plant Soil 245:105–114
Smith SE, Read DJ (1997) Mycorrhizal symbiosis. Academic, San Diego
Smith FW, Mudge SR, Rae AL, Glassop D (2003) Phosphate transport in plants. Plant Soil 248:71–83
Snoeyink VL, Jenkins D (1980) Water chemistry. Wiley, New York
Sperber JI (1957) Solution of mineral phosphates by soil bacteria. Nature 180:994
Sperber JI (1958) Solution of apatite by soil microorganisms producing organic acids. Aust J Agric Res 9:782–787
Sreenivasa M, Krishnaraj M (1992) Synergistic interaction between VA mycorrhizal fungi and a phosphate solubilizing bacterium in chili. Zentralbl Mikrobiol 147:126–130
Stevenson FJ (1986) Cycles of soil. Wiley, New York
Stumm W, Morgan JJ (1995) Aquatic chemistry: chemical equilibria and rates in natural waters. Wiley, New York
Sturz AV, Christie BR, Matheson BG, Nowak J (1997) Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. Biol Fertil Soils 25:13–19
Sylvia D (1999) Mycorrhizal symbioses. In: Sylvia DJ, Fuhrmann J, Hartel P, Zuberer D (eds) Principles and applications of soil microbiology. Prentice Hall, Upper Saddle River, NJ, pp 408–426
Taylor AW, Gurney EL (1964) Solubility of variscite. Soil Sci 98:9–13

Tinker PB (1980) Role of rhizosphere microorganisms in phosphorus uptake by plants. In: Khasawneh FE, Sample EC, Kamprath EJ (eds) The role of phosphorus in agriculture. Soil Science Society of America, Madison, WI, pp 617–654

Toro M, Azcon R, Herrera R (1996) Effects on yield and nutrition of mycorrhizal and nodulated *Pueraria phaseolides* exerted by P-solubilizing rhizobacteria. Biol Fertil Soils 21:23–29

Toro M, Azcon R, Barea JM (1998) The use of isotopic dilution techniques to evaluate the interactive effects of rhizobium genotypes, mycorrhizal fungi, phosphate-solubilizing rhizobacteria and rock phosphate on nitrogen and phosphorus acquisition by *Medicago sativa*. New Phytol 138:265–273

Trolove SN, Hedley MJ, Kirk GJD, Bolan NS, Loganathan P (2003) Progress in selected areas of rhizosphere research on P acquisition. Aust J Soil Res 41:471–499

Veith JA, Sposito G (1977) Reactions of aluminosilicates, aluminum hydrous oxides, and aluminum oxide with o-phosphate: the formation of x-ray amorphous analog of variscite and montebrasite. Soil Sci Soc Am J 41:870–876

Venkateswarlu B, Rao AV, Raina P (1984) Evaluation of phosphorus solubilization by microorganisms isolated from Aridisols. J Indian Soc Soil Sci 32:273–277

Vyas P, Rahi P, Chauhan A, Gulati A (2007) Phosphate solubilization potential and stress tolerance of *Eupenicilium parvum* from tea soil. Mycol Res 111:931–938

Wakelin SA, Warren RA, Harvey PR, Ryder MH (2004a) Phosphate solubilization by *Penicillium* spp. closely associated with wheat roots. Biol Fertil Soils 40:36–43

Wakelin SA, Warren RA, Ryder MH (2004b) Effect of soil properties on growth promotion of wheat by *Penicillium radicum*. Aust J Soil Res 42:897–904

Wani PA, Khan MS, Zaidi A (2007) Synergistic effects of the inoculation with nitrogen fixing and phosphate-solubilizing rhizobacteria on the perfomance of field grwon chickpea. J Plant Nutr 27:599–610

Welch S, Taunton AE, Banfiled JF (2002) Effect of microorganisms and microbial metabolites on apatite dissolution. Geomicrobiol J 19:343–367

Whitelaw MA (2000) Growth promotion of plants inoculated with phosphate-solubilizing fungi. Adv Agron 69:99–151

Whitelaw MA, Harden TJ, Bender GL (1997) Plant growth promotion of wheat inoculated with *Penicillium radicum* sp. nov. Aust J Soil Res 35:291–300

Xavier LJC, Germida JJ (2003) Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. Biol Fertil Soils 35:471–478

Young R, Davies C (1980) Phosphate fertilizers and process technology. In: Khasawneh FE, Sample E, Kamprath E (eds) The role of phosphorus in agriculture. Soil Science Society of America, Madison, WI, pp 195–226

Young CC, Chen CL, Chao CC (1990) Effect of *Rhizobium*, vesicular-arbuscular mycorrhiza, and phosphate solubilizing bacteria on yield and mineral phosphorus uptake of crops in subtropical-tropical. In: 14th International Congress of Soil Science Transactions, vol. 3. International Society of Soil Science, Kyoto, Japan, pp 55–60

Yusdar H, Anuar AR, Hanafi MM, Azifah H (2007) Analysis of phosphate rock dissolution determining factors using principal component analysis in some acid Indonesian soils. Commun Soil Sci Plant Anal 38:273–282

# Index


A
Abiotic, 134, 135, 147, 148
*Acacia,* 229, 230, 236, 237, 239
ACC. *See* 1-Aminocyclopropane-1-carboxylate
ACC deaminase, 31, 36, 38, 39, 63, 65, 69–77
Acetylene, 245, 247
Actinorhizal nitrogen fixers, 290
Agriculture
  conventional, 207, 210, 213, 217
  high-input, 209, 210
  low-input, 210
  organic, 206, 208, 210, 212, 213, 218
  sustainable, 205–219
*Agrobacterium,* 236, 237
Agro-ecosystem(s), 134, 139, 152, 207, 209–215
*Albizia,* 236, 237
Alfalfa, 65, 68, 70, 73, 77
Alkalinity/salinity, 25
Allelochemicals, 194
Alphaproteobacteria, 229, 248
Aluminum, 326, 336, 337
Amino acids, 69, 71
1-Aminocyclopropane-1-carboxylate (ACC), 139, 140, 147, 151
Ammonia, 63, 65, 66, 71
Andisol, 326, 336, 337, 343
Antibiotics, 106, 108, 109, 120–122
Arbuscular mycorrhiza (AM), 256–260, 262
Arid land, 229
Arsenic, 87
*Aspergillus,* 330, 332
ATPase, 90
*Azorhizobium,* 229, 237
  *A. caulinodans,* 292–296, 298
*Azospirrlum,* 243, 245, 246

B
*Bacillus,* 94, 95, 98
*Bacillus thuringiensis,* 186–189, 192, 196, 197, 199, 201
Bacteria, 186, 187–189, 192, 195, 198
Bacteroids, 66, 72, 73, 75, 76
Barley, 340
Beneficial biofilms, 52–55
Bioaccumulation, 91, 94–95
Biochemical pesticides, 194–195
Bio-control, 28, 31–34, 38, 41, 147
Biocontroling agents, 53
Biodiversity, 135
Biofertilizer(s), 135, 138, 140, 151–153, 206, 208, 211, 213, 218, 228
Biofilm, 95
Biofilmed biofertilizers, 56–60
Biofilm formation, 52
BIOLOG, 295
Biological nitrogen fixation (BNF), 289, 291, 298
Biopesticides, 185–202
Bioremediation, 90–98, 100, 151
Biosorption, 91–94
Biotechnology, 134, 152, 153
Biotic stress, 148–150

BNF. *See* Biological nitrogen fixation
Botanicals, 193–194
*Bradyrhizobium*, 65, 69

C

Cadmium (Cd), 86–88, 90, 94, 96–99
Carbon
  drain, 212, 213
  sequestration, 206
Carolina phosphate rock, 313
Chelators, 117
Chickpea, 340
Chili, 339
Chitinase(s), 135, 140
Chrome Azurol S (CAS), 116
Chromosomal insertion, 34
Citric acid, 333, 334, 336
*Citrobacter,* 92
Clover, 291
Coinoculation, 150
Community level physiological profiles (CLPP), 107
Consistency, 138, 141, 152
Conventional biofertilizers, 56
Cotton, 339
Crop rotation(s), 210–213
Cropping system, 207, 210, 211, 218
  carbon sequestration, 171
  microbial community, 178
  rhizodeposition, 177
  soil health, 161–180
  tailoring, 178
Current agricultural problems
  agrochemical(s), 173
  farmers awareness, 174
  heavy vehicle traffic, 173–174
  inappropriate cropping system, 172–173
  intensification, 161, 162, 165, 172–173
  sewage and sludge, 173–174
  soil testing protocol, 174
Cytokinin(s), 66, 138, 139

D

Decontamination, 91
Denaturing gradient gel electrophoresis (DGGE), 306
Desertification, 219
DGGE. *See* Denaturing gradient gel electrophoresis
Diazotroph(s), 287–298
Diazotrophic, 137
Diazotrophic bacteria, 56, 59
Diversification
  cropping system, 177–178
  framing system, 178
  soil health, 177–178
DNA, 231, 235–237, 239, 240, 242, 245–247
Drought stress, 242

E

Ecosystem(s), 301–318
Efflux, 90, 94
Endophytes, 245
Endosymbiont, 292, 298
Engineering biocontrol agents, 198
Engineering crop plants, 186, 199
*Ensifer,* 229, 236
*Enterobacter,* 330, 333, 340, 343
Entomopathogenic fungi, 190–192
Entomopathogenic protozoa and microsporida, 193
Essential nutrients, 134
Ethylene, 63–77, 138, 139, 147
Exopolysaccharide (EPS), 29
Extracellular polymeric substances (EPS), 52, 55
Exudates, 138

F

Farming
  organic, 210, 218
  practices, 210
  system, 206, 212, 213, 218
Farm yard manure (FYM), 314
Fatty acid methyl ester (FAME), 108
Fertilizer
  biofertilizer(s), 206, 208, 211, 213, 218
  chemical, 206, 207, 211
  inorganic, 210
Flavonoids, 66, 230
Formulation, 135, 151–152
Four-microbe FRB, 58

Functional diversity, 105–124
Fungal–bacterial biofilm (FBB), 53–58
Fungal–rhizobial biofilm (FRB), 53–58

G
Gene density, 315
Gibberellins, 138, 139
*Gigaspora margarita,* 329
*Glomus aggregatum,* 328
*Glomus fasciculatum,* 339
Gluconic acid, 334
Glucose dehydrogenase (GDH), 35
Glycine betaine, 239, 242, 243
GOD activity, 33
Goethite, 326, 335, 336
Granular inoculants, 294
Growth-promoting rhizobacteria (PGPR), 105–124

H
Hemoglobin, 232
Heavy metal resistance, 90, 97
Heavy metals, 64, 65, 74, 77, 147, 151
Herb legume, 235
Higher order biofilms, 60
Horticultural
  crop, 207, 214–215
  production, 207, 214–215

I
IAA. *See* Indole-3-acetic acid
Indian mustard, 118, 123
Indole acetamide (IAM) pathway, 113
Indole-3-acetic acid (IAA), 64, 71, 140
Induced systemic resistance (ISR), 120–122
Inocula, 53
Inoculants, 134, 135, 140, 141
Inoculation, mycorrhizal, 210, 213–215, 217, 218
Inoculum
  mycorrhizal, AM, 211, 216, 219
  tuning, 215, 216, 218, 219
Intensification
  diversity loss, 163, 169, 172
  production sustainability, 172
  resilience capacity, 172
  SOM, 172
Intercropping, 212, 213
Ionic ratios, 269
Ionic status, 268–269
Itai-itai, 86

K
Kaolinite, 335, 336
α-Ketobutyrate, 63, 65, 71
Kudzu, 340, 341

L
Lead, 87, 88, 94
Legumes, 63–77
Lettuce, 337
*Leucaena,* 75, 76, 328, 342û344
Ligand, 115–117
Lipochitooligosaccharides, 232, 245
Liquid inoculums, 297
*Lotus,* 230, 236, 242
*Lotus japonicus*, 68
*Lupinus,* 336
Lytic enzymes, 122–123

M
*Macroptilium atropurpureum*, 68
Maize, 334
Mechanisms of action, 135–137, 140
*Medicago truncatula,* 230
Mediterranean forest ecosystems, 256, 257
Mercury, 87, 90, 94
Meristem, 66, 69
*Mesorhizobium,* 65, 72, 229, 234–237, 240, 241
Metabolic cooperation, 52, 58
Metal toxicity, 109
Microarray(s), 231, 239, 240
Microbial community structure, 303–308, 317
Microbial diversity, 302–304, 306, 312, 313
Microbial pesticides, 186–189
Microbial RNA, 312
Micropropagation, 214
Mint, 343

Mobilization, 135, 137–138
Modulation enzyme, 119–120
Mollisol, 330, 332, 336, 340, 341, 343
Monoculture
  aboveground biodiversity, 173
  belowground biodiversity, 173
  nematode infestation, 173
  soil sickness, 173
  vulnerable, 173
Montmorillonite, 336
*Mortierella,* 332, 336, 341, 343
Most probable number (MPN), 304
MPN. *See* Most probable number
Mycorrhiza, 325–344
Mycorrhizal, 229, 243, 244
  industry, 215, 217, 219
  responsiveness, 206–210, 213, 214
  symbiosis, 206, 210–212, 219
Mycorrhizal dependency (MD), 329
Mycorrhizal fungi, 58
Mycorrhizosphere, 340
Mycotrophic, 207, 210, 212

N
National Botanical Research Institute P (NBRIP) medium, 26
N content, 292, 296
Nematode(s), 186, 187, 192
$N_2$ fixation, 135, 137, 138
$N_2$-fixing bacteria, 51, 53
*nif. See* Nitrogen fixation
Nitrogen
  macronutrient, 269–272
  transport systems, 269
Nitrogenase(s), 151, 233, 241–243
Nitrogenase activity, 53
Nitrogen fixation *(nif),* 231, 269–273, 276
*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine (NTG), 24
Nod factor(s), 66, 68, 69, 230, 232
*Nod* gene, 230, 232, 246
Nodulation, 270, 271, 282
Nodule, 64–70, 73, 75–77
Nodule-like structures, 51, 54, 58
Nurse plant, 255–262

O
*Ochrobacterium*, 123
Organic acids (OA), 27–29, 33, 41, 71
Oxalic acid, 334, 336, 344
Oxidative stress, 87, 89, 97
Oxisol, 326, 328, 336, 337, 340, 341, 343

P
Papaya, 338
*Parasponia,* 232, 234
Pathogen
  root, 206, 212
  soil-borne, 208
Pathogenic, 135, 147
PCNB. *See* Pentachloronitrobenzene
PCR. *See* Polymerase chain reaction
Peanut, 242
Peat, 244, 245
*Penicillium,* 330, 332, 334, 336, 343
*Penicillium bilaji,* 332
Pentachloronitrobenzene (PCNB), 316
Pesticide(s), 207, 211
PGPB, 3–4
  ACC deaminase, 4, 8, 9, 14
  auxin, 4
  direct plant-growth promotion, 3
  indirect plant-growth promotion, 3
PGPF, 5
  arbuscular mycorrhizae, 5, 10, 14
  ectomycorrhizae, 7, 12, 13
  endophytic fungi, 7
PGPR. *See* Plant growth-promoting rhizobacteria
PGRs. *See* Plant growth regulators
*Phaseolus,* 237, 241
Phosphatase(s), 30, 135, 137, 140, 151
Phosphate
  depletion zone, 327, 329
  desorption, 328, 333, 337, 338, 343
  diffusion, 327, 329
  fertilization, 327, 344
  fixation, 326–328, 330, 332, 338, 340, 341, 343
  fixation categories, 327
Phosphatic fertilizers, 87
Phosphobacterin, 330
Phospholipid fatty acid (PLFA), 108
Phosphorus, 23, 24

availability, 206, 208, 209
P-deficient, 213
Photosynthesis, 86
Photosynthetic carbon, 308
Phytohormone(s), 69, 76, 107–110, 112, 135, 138, 139, 151
Phytoremediation, 90
Pigeon pea, 334
*Pisum,* 235
Planktonic cells, 52
Plant growth hormones, 58
Plant growth-promoting rhizobacteria (PGPR), 64, 65, 70–72, 74, 77, 133–153
Plant growth regulators (PGRs), 111–113, 134, 135, 138
Plant health-promoting rhizobacteria (PHPR), 106
Plant–plant interactions, 262
Polymerase chain reaction (PCR), 305, 306
Precursor, 138, 147
Proline, 242, 243, 248
Prosopis, 229, 236, 237
Protein, 66, 72, 73, 75
Proteomics, 96–98
Provide ™, 341
*Pseudomonas,* 65, 72, 74, 90, 94, 95, 330, 334, 336, 338–340
Pseudonodules, 51, 54, 58
PSM mechanisms
acid production, 334
ammonium assimilation, 333, 336
organic acid, 330, 333, 334, 336, 338, 341, 344
Pi-desorption, 337–338, 343
P-solubilizing fungi, 51, 53
Pyrroloquinoline quinone (PQQ), 34, 35

Q
Quorum sensing, 52

R
Reactive oxyzen species (ROS), 87–89
Reforestation, 255–262
Responsiveness
to AM fungi, 206–209, 213, 214, 216
to mycorrhiza, 206–210, 214, 216
Rhizobacteria, 64, 133–153
Rhizobiotoxine, 69
*Rhizobium*, 63–77
*Rhizobium leguminosarum*, 65
Rhizoplane, 135, 307, 309
Rhizosphere, 58, 134, 135, 138, 141, 151
Ribosomal DNA, 235, 245
Rice, 334, 339
Rock phosphate (RP), 325, 328, 337, 342
RP. *See* Rock phosphate
R:S ratio, 309
Ryegrass, 337

S
Salinity, 229, 230, 238–244, 248
effects, 2
ethylene rise, 2, 8
osmotic stress, 2
plant tolerance, 5, 14
Salt stress, 238, 239, 241–244, 246, 248
Salt tolerance, 230, 239–241, 243, 246
Seeds
productivity, 268, 274–277
protein content, 268, 276–277
Semiarid, 237, 240
*Sesbania,* 229, 236, 237
*Sesbania rostrata*, 68
Sex pheromones, 195
Siderophore(s), 64, 76, 95, 106, 108–111, 113–119, 135, 138, 140, 151
Silver, 90, 94
*Sinorhizobium,* 75, 229, 230, 234–237, 240
Soil
desertified, 206, 218
erosion, 206, 208
Soil aggregates, 302
Soil fertility, 106, 124, 301, 302, 312, 313, 317, 318
Soil health
concept, 163–164
diagnosis, 162, 163
indicators, 162–169, 174, 179, 180
indices (SHI), 168, 176
Soil health assessment methodology
data screening, 167
indicator integration, 168
MDS validation, 168
redundancy, 167

Soil health importance
  animal health, 171
  crop production, 168–169
  crop quality, 169–170
  environment health, 171
  human health, 170
  production sustainability, 170
Soil health indicator
  criteria, 164–165
  selection, 164–165
  significance, 165–167
Soil health management
  cover crops, 176–177, 179
  crop and farm diversification, 178
  crop rotation, 175–176
  farmer's friendly SHI, 179
  government policy, 175
  legislation, 174–175, 179
  organic husbandry, 178–179
  soil test-based fertilization, 163, 165, 176, 179
  tillage conservation, 176–177
Soil pH, 307–312
Soil solution Pi, 325, 330
Soybean, 68, 76, 339
Stabilization, 90, 91
Strengite, 335
Stress, 134, 139, 140, 147–151
Substrate, 134, 135, 138
Sugars, 71
Sunflower, 339
Sustainability, 133, 134, 139, 141, 152
Symbiotic, 227–242, 244, 245, 247, 248
Symbiotic nitrogen fixation, 63, 76
Systemic resistance, 139

T
Tobacco, 338
Tomato, 339
Transgenic, 147
Transmission electron microscopy (TEM), 94
Tree legume, 229, 235–237
Tricalcium phosphate (TCP), 24, 29
*Trichoderma*, 28, 33, 38, 40
*Trifolium,* 329

V
Variscite, 326, 335
Vegetative organs
  ion accumulation, 277–281
  nitrogen nutrition, 277–281
  plant growth, 277
Vertisol, 337
*Vicia,* 234–236, 244
Viruses, 186, 187, 189–190, 195, 199, 201, 202

W
Wheat, 287–298, 332, 339–341, 343
Wild, 227–229, 234–237, 241–243, 248
Woody legume, 237

X
Xenobiotic, 134