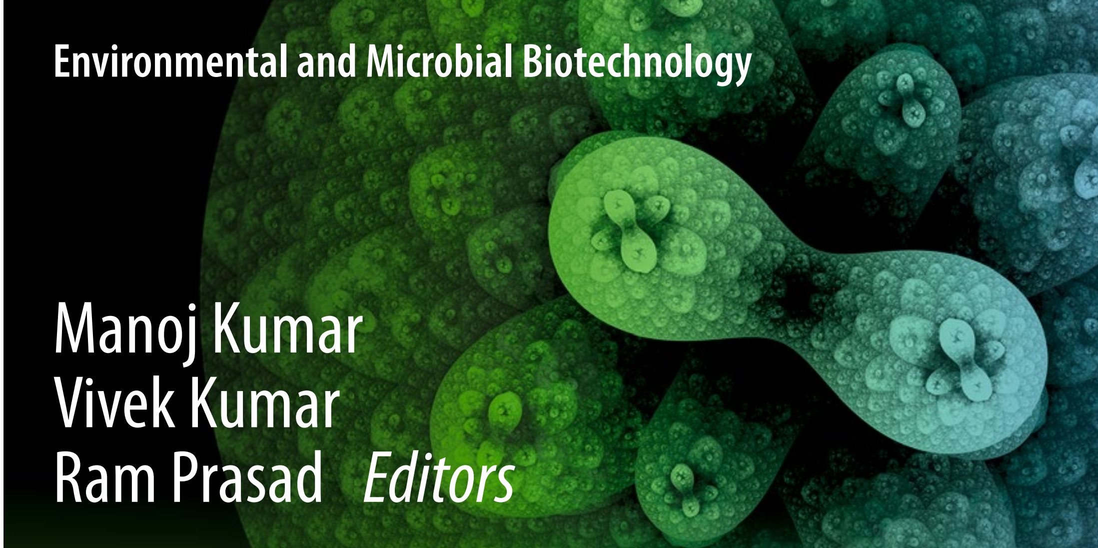
Environmental and Microbial Biotechnology



Manoj Kumar
Vivek Kumar
Ram Prasad *Editors*

Phyto-Microbiome in Stress Regulation

Environmental and Microbial Biotechnology

Series Editor

Ram Prasad, Department of Botany, Mahatma Gandhi Central University,
Motihari, Bihar, India

Innovative and novel advances in microbial biotechnology are providing great understandings in to the machineries of nature, presenting fascinating prospects to apply principles of biology to different arenas of science. Sustainable elucidations are emerging to address the concerns on improving crop productivity through microbes, depleting natural resources, environmental pollution, microbial degradation of pollutants, nanomaterials, nanotoxicity & safety issues, safety of food & agricultural products etc. Simultaneously, there is an increasing demand for natural bio-products of therapeutic and industrial significance (in the areas of healthcare, environmental remediation, microbial biotechnology). Growing awareness and an increased attention on environmental issues such as climate change, energy use, and loss of non-renewable resources have carried out a superior quality for research that provides potential solutions to these problems. Emerging microbiome approaches potentially can significantly increase agriculture productivity & human healthcare and henceforth can contribute to meet several sustainable development goals.

The main objectives have provided an impetus for research on plants and microorganisms that produce novel bio-products with variable properties and understanding their mechanisms of action at cellular and molecular level. Hence, research activities of the environmental and microbial Biotechnology are comprehensively focused up on major sectors viz., bioresources, biorefining, bioremediation of organic and inorganic pollutants, environmental risk analysis of microorganisms, environmental assessment using microbiological indicators, enzymes for environment, food & industrial applications, nanomaterials & nanotoxicity, sustainable ecobiotechnology, biofertilizer, biocontrol agents for agriculture improvement and natural products for healthcare applications.

This book series is a state-of-the-art for a wide range of scientists, researchers, students, policy makers and academician involve in understanding and implementing the knowledge on environmental and microbial biotechnology to develop biologics for proper health care to continue life in smooth and sustainable strategy without any adverse effect.

More information about this series at http://www.springer.com/series/16324

Manoj Kumar • Vivek Kumar • Ram Prasad
Editors

Phyto-Microbiome in Stress Regulation

Editors
Manoj Kumar
Department of Life Sciences
Central University of Jharkhand
Ranchi, Jharkhand, India

Vivek Kumar
Swami Rama Himalayan University
Himalayan School of Biosciences
Dehradun, Uttarakhand, India

Ram Prasad
Department of Botany
Mahatma Gandhi Central University
Motihari, Bihar, India

ISSN 2662-1681 ISSN 2662-169X (electronic)
Environmental and Microbial Biotechnology
ISBN 978-981-15-2575-9 ISBN 978-981-15-2576-6 (eBook)
https://doi.org/10.1007/978-981-15-2576-6

© Springer Nature Singapore Pte Ltd. 2020
This work is subject to copyright. All rights are reserved by the Publisher, whether the whole or part of the material is concerned, specifically the rights of translation, reprinting, reuse of illustrations, recitation, broadcasting, reproduction on microfilms or in any other physical way, and transmission or information storage and retrieval, electronic adaptation, computer software, or by similar or dissimilar methodology now known or hereafter developed.
The use of general descriptive names, registered names, trademarks, service marks, etc. in this publication does not imply, even in the absence of a specific statement, that such names are exempt from the relevant protective laws and regulations and therefore free for general use.
The publisher, the authors, and the editors are safe to assume that the advice and information in this book are believed to be true and accurate at the date of publication. Neither the publisher nor the authors or the editors give a warranty, expressed or implied, with respect to the material contained herein or for any errors or omissions that may have been made. The publisher remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This Springer imprint is published by the registered company Springer Nature Singapore Pte Ltd.
The registered company address is: 152 Beach Road, #21-01/04 Gateway East, Singapore 189721, Singapore

Preface

This book presents state-of-the-art research on the many facets of the plant microbiome, including survivability aspects of plants based on ecology, physiology and genomics, as well as molecular mechanisms of plant-microbe interactions. Topics are well thought out which include the significance of microbial assisted plant growth, induced systemic resistance, tolerance to abiotic stress and biological control of plant pathogens.

The respective contributions show how microbes help plants to cope with abiotic stresses and represent significant progress towards understanding the complex regulatory networks critical to host-microbe interaction and plant adaptation in extreme environments. New insights into the mechanisms of microbial actions in inducing plant stress tolerance open new doors for improving the efficacy of microbial strategies and could produce new ways of economically increasing crop yields without harming the environment. As such, this book offers an essential resource for students and researchers with an interest in plant-microbe interaction, as well as several possibilities for employing the plant microbiome for the enhancement of crop productivity under future climate change scenarios.

Phyto-stimulation and bio-control by plant-associated microbes set a revolutionary paradigm in modern findings towards farming culture in developing countries. At the same time, genetically modified (GM) crops harbouring few significant strains, i.e. *Bacillus thuringiensis* (BT) gene(s) to combat biotic stress caused by insect pests, endorse the contemporary theme of bridging research for the agriculture sector. The book captures the cumulative efforts of contributors in a review format, which can be a meaningful addition for modern researchers.

It not only provides details on existing challenges but also offers deeper insights into the possibility of solving problems, compiled as follows, but not limited to:

- Applications of plant-microbe interactions in contaminated agro-ecosystem management.
- Development of future bio-formulations for sustainable environment.
- Heavy metals, hydrocarbons, radioactive materials and bioremediation of dyes.
- Microbes and soil and water toxicants management.
- Role of plants, genetically modified organisms (GMOs) and earthworms in bioremediation or any topic on bioremediation.

Emphasis is given on the most neglected part of scientific exploration that is rare micro-flora of earth where scientists are understanding the mechanism behind survivals; (b) targeted that the efficient plant microbiome and their benefits in agro-ecosystem, water bodies, human health and targeting global community in plant, soil, water and rare microbe synergism field; (c) highly specialized book on biotic and abiotic stresses and its management through microbial technological inputs, will help the academicians, researchers, environmentalists, industrialists, agriculturists and graduate students and (d) experts across the globe expressed the practiced knowledge in the shape of chapters which deal with zest of specific research on microbiome mediated stress tolerance.

Ranchi, Jharkhand, India — Manoj Kumar
Dehradun, Uttarakhand, India — Vivek Kumar
Motihari, Bihar, India — Ram Prasad

Contents

1 Phytostimulation and Biocontrol by the Plant-Associated *Bacillus amyloliquefaciens* FZB42: An Update 1
Rainer Borriss

2 Genetically Modified (GM) Crops Harbouring *Bacillus thuringiensis* (BT) Gene(S) to Combat Biotic Stress Caused by Insect Pests 21
Bhupendra Koul

3 Characterization and Efficiency of Rhizobial Isolates Nodulating *Cytisus monspessulanus* in the Northwest of Morocco: In Relation to Environmental Stresses 63
Taoufik Belechheb, Mohammed Bakkali, Amin Laglaoui, and Abdelhay Arakrak

4 Isolation and Characterization of the Roots and Soil Endomycorrhizae of *Hedysarum pallidum* Desf. in the Northeast of Morocco 73
Rachid M'saouar, Mohammed Bakkali, Amin Laglaoui, and Abdelhay Arakrak

5 Friends and Foes: Phyto-Microbial Interactions in Molecular Perspective 81
Shyam Solanki, Gazala Ameen, Debankur Sanyal, Shalu Jain, Ammar Elakhdar, Shwetank Lall, Kishore Chittem, Leah Brueggeman, Ajay Kumar, and Robert Brueggeman

6 Isolation and Screening of Inorganic Phosphate Solubilizing *Pseudomonas* Strains from the *Lotus creticus* Rhizosphere Soil from the Northwest of Morocco 99
Imane Achkouk, Saida Aarab, Amin Laglaoui, Mohammed Bakkali, and Abdelhay Arakrak

7 Screening and Characterization of Phosphate-Solubilizing Rhizobia Isolated from *Hedysarum pallidum* in the Northeast of Morocco 113
Samia Hamane, Mounir Hassani Zerrouk, Anass E. Lyemlahi, Saida Aarab, Amin Laglaoui, Mohammed Bakkali, and Abdelhay Arakrak

8 Development of Abiotic Stress Tolerance in Crops by Plant Growth-Promoting Rhizobacteria (PGPR) 125
Sivakumar Subiramani, Sathishkumar Ramalingam, Thiruvengadam Muthu, Shivraj Hariram Nile, and Baskar Venkidasamy

9 Plant Growth-Promoting Rhizobacteria (PGPR) and Their Action Mechanisms in Availability of Nutrients to Plants 147
Hassan Etesami and Sina M. Adl

10 Plant Growth and Development Under Suboptimal Light Conditions 205
Ravinderjit Kaur, Gaganpreet Kaur, Kashmir Singh, and Baljinder Singh

11 Microbial Biotechnology: A Key to Sustainable Agriculture 219
S. K. Gosal, Jaspreet Kaur, and Jupinder Kaur

12 Stress Signalling in the Phytomicrobiome: Breadth and Potential 245
Sahana Basu and Gautam Kumar

13 A Simple Procedure for Isolation, Culture of Protoplast, and Plant Regeneration 269
Indu Kumari

14 Plant Antimicrobial Peptides: Next-Generation Bioactive Molecules for Plant Protection 281
Paomipem Phazang, Neelam Prabha Negi, Meenakshi Raina, and Deepak Kumar

15 Nitrogen Stress in Plants and the Role of Phytomicrobiome 295
Garima Malik, Navneet Singh, and Sunila Hooda

16 Halotolerant Microbes for Amelioration of Salt-Affected Soils for Sustainable Agriculture 323
Sanjay Arora

About the Editors

Manoj Kumar is working as Associate Professor and Head of in the Department of Life Sciences, Central University of Jharkhand, Ranchi, India. He is a scientist with sanguine behavior who is adoring about research and development, with a commitment to lifelong learning. Dr. Kumar has pursued his PhD in Plant Biotechnology and continuing his career in multidisciplinary research in Plant Developmental Biology, Plant-Microbe Interaction and Forest Molecular Genetics. He is a referee for many journals, including Phytoremediation, Journal of Soil Sediments etc. He has guided several research scholars at doctoral and masters level, also he is leading a multidisciplinary research group comprised of diverse research background in biological sciences.

Vivek Kumar is an agricultural microbiologist having 20 Years of experiences in teaching, research and guidance, with a pledge to enduring knowledge. Dr. Kumar is working as Associate Professor Department of Microbiology, Himalayan School of Biosciences, Swami Rama Himalayan University, India.

He is serving in Editorial board of reputed international journals and also a reviewer of peer journals. He has published 61 research papers, 19 book chapters, six review articles and two books. Dr. Kumar has also served as Microbiologist for eight years in Department of Soil and Water Research, Public Authority of Agricultural Affairs & Fish Resources, Kuwait.

Ram Prasad is an Assistant Professor at the Amity Institute of Microbial Technology, Amity University, Uttar Pradesh, India. His research interests include plant-microbe interactions, sustainable agriculture, and microbial nanobiotechnology. Dr. Prasad has published more than a hundred research papers, reviews, and book chapters and has five patents issued or pending and edited or authored several books. Dr. Prasad has 12 years of teaching experience and was awarded the Young Scientist Award (2007) and Prof. J.S. Datta Munshi Gold Medal (2009) by the International Society for Ecological Communications, FSAB Fellowship (2010) by the Society for Applied Biotechnology, Outstanding Scientist Award (2015) in the field of Microbiology by Venus International Foundation, BRICPL Science Investigator Award (2017), Award for Excellence in Research from 6th Academic Brilliance Awards-2018, and the American Cancer Society UICC International Fellowship for Beginning Investigators (USA, 2014). In 2014–2015, Dr. Prasad served as a Visiting Assistant Professor at the Department of Mechanical Engineering at Johns Hopkins University, USA, and since 2017 has served as a Research Associate Professor at the School of Environmental Science and Engineering, Sun Yat-Sen University, China.

Check for updates

1 Phytostimulation and Biocontrol by the Plant-Associated *Bacillus amyloliquefaciens* FZB42: An Update

Rainer Borriss

Abstract

Bacillus amyloliquefaciens FZB42, the type strain for representatives of the plant-associated subspecies *plantarum*, stimulates plant growth and suppresses soil-borne plant pathogens. The strain has been sequenced in 2007. The *B. amyloliquefaciens* FZB42 genome reveals an unexpected potential to produce secondary metabolites. In total, 11 gene clusters representing nearly 10% of the genome are devoted to synthesizing antimicrobial metabolites and/or to confer immunity against them. Ability to synthesize nonribosomally, the antibacterial polyketides macrolactin and difficidin and the antifungal lipopeptide bacillomycin D is an unique feature of the subspecies *plantarum*. However, according to latest research, most of the secondary metabolites are not expressed in plant rhizosphere suggesting that the antibiome expressed during the plant-associated state of PGPR Bacilli does not reflect the vast genetic arsenal devoted to the formation of secondary metabolites. There is now strong evidence that plant-associated Bacilli trigger pathways of induced systemic resistance, which protect plants against attacks of pathogenic microbes, viruses, and nematodes.

Keywords

Bacillus amyloliquefaciens · Macrolactin · Antimicrobial–metabolites · *plantarum*

R. Borriss (✉)
Humboldt-Universität zu Berlin, Institut für Biologie, Berlin, Germany

Nord Reet UG, Greifswald, Germany
e-mail: rainer.borriss@rz.hu-berlin.de

© Springer Nature Singapore Pte Ltd. 2020
M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_1

1.1 Introduction

Environmental-friendly biotechnological approaches, such as the use of microbial biopesticides, offer alternatives to chemical control of plant diseases and pests. Among these alternatives, the use of bioformulations, which are manufactured from plant growth-promoting rhizobacteria (PGPR) with biocontrol activity (BC) (Lugtenberg et al. 2013), is steadily increasing. At present, due to the long-term shelf life of their endospores, bacilli are the most widely used bacteria on the biopesticide market. Their use in agriculture has been previously reviewed (Borriss 2011). An update of *Bacillus*-based bioformulations, currently available for the farmer interested on sustainable agriculture, is presented in Table 1.1.

Plant rhizosphere is a highly competitive environment in which bacteria are abundantly present due to availability of nutrients actively secreted by the plant root and mucilage. Some of these bacteria which are living within or in the vicinity of plant roots and supporting plant growth are generally referred to as PGPR (Kloepper et al. 1980). In many cases, the plant growth-promoting activity is linked with their ability to suppress soil-borne plant pathogens (bacteria and microfungi), occurring in the competing microflora. Different mechanisms are discussed in this context. Besides production of antimicrobial ("antibiotics") and nematicidal compounds, also stimulation of plant-induced systemic resistance (ISR, Doornbos et al. 2012), and a beneficial effect on the composition of the host-plant microbiome might contribute to their suppressive effect (Erlacher et al. 2014). In other PGPR, termed "biofertilizer", plant growth promotion by hormone-like compounds and increased accessibility of nutrients dominates. The mechanisms that are involved in this process can include nitrogen fixation, phosphate and mineral solubilization, and the production of macromolecule degrading enzymes (amylases, proteases, hemicellulases), phytohormones (auxin, cytokinin, and gibberellins), and volatile growth stimulants (such as acetoin and 2,3 butanediol) (Borriss 2011).

Bacillus amyloliquefaciens FZB42 is the type strain for a group of plant-associated *Bacillus* spp. classified as *B. amyloliquefaciens* subsp. *plantarum* (Borriss et al. 2011). Its 3918-kb genome, containing an estimated 3693 protein-coding sequences, lacks extended phage insertions, which occur ubiquitously in the closely related but nonplant-associated *Bacillus subtilis* 168 genome. Further analysis revealed that FZB42 is a bacterium with impressive capacity to produce metabolites with antimicrobial activity (Chen et al. 2007). Its antifungal activity is due to nonribosomal synthesis of the cyclic lipopeptides bacillomycin D and fengycin (Koumoutsi et al. 2004), whilst its antibacterial activity is mainly due to nonribosomally synthesized polyketides (Chen et al. 2006), bacilysin (Chen et al. 2009a), and ribosomally synthesized bacteriocins (Scholz et al. 2011, 2014). Recent proteome and transcriptome studies revealed that plant root exudates stimulate expression of genes involved in root colonization and plant–bacteria interactions (Borriss 2015a, b; Fan et al. 2012a, b, 2015; Kierul et al. 2015). Its plant colonizing ability was demonstrated with a GFP-labeled FZB42 strain on maize and *Arabidopsis* using confocal laser scanning microscopy (Fan et al. 2011). Beneficial effects on plant growth and disease suppression were documented for *B. amyloliquefaciens*

Table 1.1 Examples for commercial use of *Bacillus*-based bioformulations in agriculture

Trade name	*Bacillus* strain	Known properties	Company
Kodiak™	*Bacillus subtilis* GB03	EPA-registered (71065–2) biological and seed treatment fungicide	Bayer Crop Science, former Gustafsson LLC
Companion	*Bacillus subtilis* GB03	EPA-registered (71065–2) biofungicide, prevent and control plant diseases. It produces a broad-spectrum Iturin antibiotic that disrupts the cell-wall formation of pathogens, and it triggers an advantageous induced systemic resistance (ISR) in plants, whereby a plant's natural immune system is activated to fight plant diseases	Growth Products Ltd., White Plains, NY 10603
Yield Shield	*Bacillus pumilus* GB34 (=INR7)	EPA-registered biofungicide (264–985), suppression of root diseases caused by Rhizoctonia and Fusarium	Bayer Crop Science, previously Gustafsson
BioYield™	*B. amyloliquefaciens* GB99 + *Bacillus subtilis* GB122	Combination of strong ISR activity (GB99) with phytostimulaton (GB122)	Bayer Crop Science, previously Gustafsson
Subtilex®, INTEGRAL®	*Bacillus subtilis* MBI600	EPA-registered (71840–8.) biofungicide, provides protection against soil-borne pathogens such as *Rhizoctonia solani*, *Pythium* spp. and *Fusarium* spp. to help prevent damping-off and other root diseases	Becker Underwood, Saskatoon, Canada acquired by BASF
VAULT®	*Bacillus subtilis* MBI600	Produced by "BioStacked®" technology, enhancing growth of soy beans and pea nuts	Becker Underwood, Saskatoon, Canada
	Bacillus pumilus BU F-33	EPA-registered (71840-RG, –RE, 2013) plant growth stimulator, induced systemic resistance	Becker Underwood, Saskatoon, Canada
SERENADE Max	*Bacillus subtilis* QST713	EPA-registered (69592–11) biofungicide, Annex 1 listing of the EU agrochemical registration directive (91/414)	Bayer Crop Science, previously AgraQuest
SERENADE SOIL(R)	*Bacillus subtilis* QST713	EPA-registered (69592-EI, 2012) biofungicide for food crops	Bayer Crop Science, previously AgraQuest

(continued)

Table 1.1 (continued)

Trade name	*Bacillus* strain	Known properties	Company
SERENADE Optimum®	*Bacillus subtilis* QST713	EPA-registered (2013) biofungicide/ bactericide for prevention. It works by stopping spore germination, disrupting cell membrane, and inhibiting attachment of the pathogen to leaves. For use in leafy and fruiting vegetables, strawberries and potatoes. Active against fungal (Botrytis, Sclerotinia), and bacterial pathogens (Xanthomonas and Erwinia)	Bayer Crop Science, previously AgraQuest
CEASE®	*Bacillus subtilis* QST713	Aqueous suspension biofungicide, recommended for leafy and fruiting vegetables, herbs and spices, and ornamentals	BioWorks, Inc., Victor, New York, USA
SONATA®	*Bacillus pumilus* QST2808	EPA-registered (69592–13) biofungicide, powdery mildew control	Bayer Crop Science, previously AgraQuest Inc.
RhizoVital®	*Bacillus amyloliquefaciens* FZB42	Biofertilizer, plant growth promoting activity, provides protection against various soil-borne diseases, stimulation of ISR	ABiTEP GmbH, Berlin
RhizoPlus®	*Bacillus subtilis*	Plant growth-promoting rhizobacterium and biocontrol agent. It can be used for potatoes, corn, vegetables, fruits, and also turf	ABiTEP GmbH, Berlin
Taegro®	*Bacillus subtilis* FZB24	EPA-registered biofungicide. FZB24 has been originally isolated by FZB Berlin, the forerunner of ABiTEP GmbH. Registration as a biofungicide for the United States was performed by Taegro Inc. and then sold to Novozymes without agreement with ABiTEP GmbH where the product is still offered	Syngenta, Basel, previously Novozyme, Davis, California and Earth Biosciences
POMEX	*Bacillus subtilis* CMB26	Microbial fungicide, control and inhibition germination effect on powdery mildew, *Cladosporium fulvum* and *Botrytis cinerea*	NIN Co. Ltd.,
	Bacillus subtilis CX9060	EPA-registered 71840-RG-RE (2012) fungicide, bactericide for food crops, turf and ornamentals	Certis Columbia, MD USA

(continued)

Table 1.1 (continued)

Trade name	*Bacillus* strain	Known properties	Company
Easy Start® TE-Max	*Bacillus subtilis E4-CDX*	Rhizosphere bacterium that competes with harmful pathogens for space around the roots of the grass plant. Once established, this unique strain physically protects the roots and inhibits the advance of soil-borne fungi	COMPO Expert GmbH, Münster, Germany
Double Nickel 55™	*B. amyloliquefaciens* D747	EPA-registered (70051-RNI, 2011) a broad spectrum preventive biofungicide for control or suppression of fungal and bacterial plant diseases (Powdery mildew, *Sclerotinia*, *Botrytis*, *Alternaria*, bacterial leaf spot, bacterial spot and speck, Fire blight, *Xanthomonas*, *Monilinia*	Certis Columbia, MD USA
Amylo-X®	*B. amyloliquefaciens* D747	Annex 1 listing of the EU agrochemical registration directive. Launched in Italy by Intrachem Bio Italia SpA for control of *Botrytis* and other fungal diseases of grapes, strawberries, and vegetables, and bacterial diseases such as fire blight in pome fruit and PSA in kiwi fruit	Certis Columbia, MD USA/Intrachem Bio Italia SpA
BmJ WG	*Bacillus mycoides* BmJ	It works entirely as a microbial SAR activator with no direct effect on the plant pathogen itself. Under development	Certis Columbia, MD USA
	Bacillus pumilus GHA 181	EPA-registered fungicide (2012), food crops, seeds, ground cover, and ornamentals	Premier Horticulture
BioNem	*Bacillus firmus* GB-126	EPA-registered (2008), suppressing plant pathogenic nematodes, *Bacillus firmus* creates a living barrier that prevents nematodes from reaching the roots	AgroGreen, Israel acquired by Bayer Crop Science

Note: The U.S. governmental EPA registration does not depend on successful field trials; it is only necessary to demonstrate that no negative effects are connected with the use of the biofungicide. The table is taken from Borriss (2015b)

FZB42 on tomato, cucumber, cotton, tobacco, and lettuce, for example (Grosch et al. 1999; Idriss et al. 2004; Yao et al. 2006; Guel et al. 2008; Wang et al. 2009; Chowdhury et al. 2013). Two review articles published in open access journals in 2015 (Chowdhury et al. 2015b; Wu et al. 2015b) cover the aspects stressed in this contribution in more detail and are recommended for further reading.

1.2 Root Colonization by FZB42 and Its Impact on the Host Plant Microbiome

The ability of FZB42 to colonize the rhizoplane is a precondition for plant growth promotion. Using a GFP-tagged derivative (Fan et al. 2011, 2012a, b), the fate of bacterial root colonization was recently studied. It ruled out that the bacterium behaves distinctly in colonizing root surfaces of different plants. In contrast to maize, FZB42 colonized preferentially root tips when colonizing *Arabidopsis thaliana* (Dietel et al. 2013). On duckweed, *Lemna minor*, FZB42 accumulated preferably along the grooves between epidermal cells of roots and in the concave spaces on ventral sides of fronds. In vitro studies performed with maize seedlings revealed that the segment within 2–8 cm distant from the basal site of the primary root was a most colonized region by FZB42. On the contrary, few bacterial cells could be observed within the range of 2 cm of root tip. In general, the green fluorescent FZB42 cells were decreasingly observed from the upper part of a root down to the root tip. Scanning electron microscopy confirmed the presence of FZB42 on root hairs, where the bacterial cells were usually associated with a wealth of presumed root exudates (Fan et al. 2012a, b). In lettuce, *Lactuca sativa,* seedlings, bacterial colonization occurred mainly on primary roots and root hairs, as well as on root tips and adjacent border cells. Occurrence of labeled bacteria decreased towards the root tips of the lateral roots, and no colonization of the finer roots could be observed (Chowdhury et al. 2015a).

The rhizosphere competence of FZB42 was recently studied using a combination of field and greenhouse trials. FZB42 is able to effectively colonize the rhizosphere (7.45 to 6.61 Log_{10} CFU g^{-1} root dry mass) within the growth period of lettuce in the field. Our results demonstrated that FZB42 is able to effectively reduce the disease severity of bottom rot caused by soil-borne pathogen *Rhizoctonia solani* on lettuce (Chowdhury et al. 2013).

From a practical point of view, it is interesting to note that the application mode of the biocontrol agent is a key factor for efficacy of FZB42. An effective suppression of *R. solani* was found only after two times application of FZB42, before and after transplanting. For the settlement of the inoculated strain in the rhizosphere in a sufficient high number, it might be important that the microflora in the rhizosphere of young plants is not yet stabilized (Berendsen et al. 2012).

As revealed by T-RFLP, application of FZB42, independent of its mode of application, did not shift the composition of rhizosphere bacterial community in a measurable extent—as also shown for *B. amyloliquefaciens* BNM122 on soybean (Correa et al. 2009). By contrast, inoculation with the pathogen did change the rhizosphere microbial community structure. In complementing that study, the effect of FZB42 and the pathogen *R. solani* on the microbial community of lettuce was more deeply analyzed by 454-amplicon sequencing focusing on the presence of gammaproteobacteria (Erlacher et al. 2014). Clear differences between plants infected by *R. solani* compared to noninoculated healthy plants were found, corroborating the results obtained by T-RFLP. A significant increase in gamma-proteobacterial diversity was detected in samples inoculated with the pathogen. However, together with

FZB42, this increase was less distinct, suggesting a selective compensation of the impact of a pathogen on the indigenous plant-associated microbiome by FZB42. The number of DNA fragments corresponding to FZB42 in samples taken in vicinity of plant roots was steadily decreasing. After five weeks, only 55% of the initial number of FZB42 DNA was traceable (Kröber et al. 2014).

1.3 Plant Growth Promotion

Although the ability of FZB42 to support growth of potatoes, maize, cotton, tobacco, leafy and fruiting vegetables, and ornamentals is well documented (Bochow et al. 2001; Yao et al. 2006; Guel et al. 2008; Burkett-Cadena et al. 2008; Chowdhury et al. 2013), the molecular reasons for the "biofertilizer" effect of beneficial plant-associated Bacilli are still not completely understood. However, we know that several factors are involved in the complex interplay between root-colonizing bacteria and plant:

1. Ability to colonize and to persist at plant roots (see previous section). Their ability to suppress soil-borne pathogens might positively affect the indigenous microbiome of the rhizosphere.
2. Stimulation of plant growth by tryptophan-dependent synthesis of indole-3-acetic acid. Inactivation of genes involved in tryptophan biosynthesis and in a putative tryptophan-dependent IAA biosynthesis pathway led to reduction of both IAA concentration and plant growth-promoting activity in the respective mutant strains (Idris et al. 2007).
3. Volatiles, as 2,3-butanediol and 3-hydroxy-2-butanone (acetoin), released by *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a were reported as enhancing plant growth (Ryu et al. 2003). To synthesize 2,3-butanediol, pyruvate is converted to acetolactate by the acetolactate synthase (AlsS), which is subsequently converted to acetoin by the acetolactate decarboxylase (AlsD) (Fig. 1.1). FZB42 mutant strains, deficient in synthesis of volatiles due to mutations interrupting the *alsD* and *alsS* genes, were found impaired in plant growth promotion (Borriss 2011).

Fig. 1.1 Anaerobic and aerobic formation of 2,3-butanediol via acetoin involves acetolactate synthase and decarboxylase encoded by the *alsSD* operon. The *alsS* insertion mutation abolishes synthesis of 2,3-butandiol (Renna et al. 1993; Cruz Ramos et al. 2000). The figure is taken from Chowdhury et al. (2015b)

4. Enhancement of nutrient availability by phytase-producing bacteria. Soil phosphorous is an important macronutrient for plants. Improved phosphorous nutrition is achievable by "mobilization" of phosphorous fixed as insoluble organic phosphate in phytate (myo-inositol-hexakisphosphate) by soil bacteria (Singh and Satyanarayana 2011). The extracellular 3(1)-phytase of the plant growth-promoting *B. amyloliquefaciens* FZB45 hydrolyzed phytate to D/L-Ins(1,2,4,5,6) P5 in vitro. A phytase-negative mutant strain, whose *phyA* gene was disrupted, did not stimulate plant growth under phosphate limitation (Idriss et al. 2002). Further experiments under field conditions revealed that FZB45 can only stimulate plant growth when phytate is present in soils, which are poor in soluble phosphate, suggesting that phytase acts only under certain conditions as a plant growth stimulator (Ramírez and Kloepper 2010).

1.4 Biocontrol

Genome analysis revealed that nearly 10% of the genome is devoted to synthesizing antimicrobial metabolites and their corresponding immunity genes (Chen et al. 2009b). FZB42 harbors 11 gene clusters involved in synthesis of antimicrobial compounds. Nine of them are involved in nonribosomal synthesis of lipopeptides and polyketides and two in conventional synthesis and modification of bacteriocin peptides. In addition, three further gene clusters contain genes mediating immunity against antimicrobial compounds produced by other related *Bacillus* strains (Table 1.2). This antibiotic arsenal makes *B. amyloliquefaciens* FZB42 and related *B. amyloliquefaciens plantarum* strains to an efficient microbial biopesticides, developed to control plant diseases (Borriss 2011).

For a long time, the plant protective activity of PGPR has been correlated with the potential to secrete a wide array of antibiotic compounds upon growth as planktonic cells in isolated cultures under laboratory conditions. We determined expression of the corresponding secondary metabolites by MALDI TOF mass spectrometry from FZB42 cultures grown in liquid Landy medium under laboratory conditions. Except the orphan *nrs* gene cluster, all expected bioactive compounds were synthesized in reasonable amounts, but the iron siderophore bacillibactin was detected only under iron-deprived conditions. In recent years, it became doubtful that synthesis of metabolites by the planktonic cells grown under laboratory conditions does correspond to their capability to produce those compounds also when grown in biofilm-related structures on the surface of plant tissues.

1.4.1 Lipopeptides, Bacillibactin, and Antifungal Activity

Five gene cluster involved in nonribosomal synthesis of cyclic lipopeptides and the iron-siderophore bacillibactin were identified in the genome of FZB42 (Table 1.2). Three of the respective gene clusters were assigned for synthesis of surfactin,

Table 1.2 Genes and gene cluster encoding for secondary metabolites and immunity against bacteriocin in FZB42

Gene cluster	From	To	Size	Metabolite	Effect against	Reference
Sfp-dependent nonribosomal synthesis of lipopeptides						
srfABCD, aat,334,ycx,CycxD,sfp,yczE	342,618	368,776	32.0 kb	Surfactin	Virus	Koumoutsi et al. (2004)
bmyCBAD	1,871,172	1,908,422	39.7 kb	Bacillomycin D	Fungi	Koumoutsi et al. (2004)
fenABCDE	1,931,328	1,968,997	38.2 kb	Fengycin	Fungi	Koumoutsi et al. (2004)
nrsABCDEF	2,868,410	2,885,927	15.0 kb	Orphan NRP1	Unknown, siderophore?	Chen et al. (2007)
dhbABCDEF	3,021,250	3,032,970	12.8 kb	Bacillibactin	Iron deficiency, siderophore	Chen et al. (2007)
Sfp-dependent nonribosomal synthesis of polyketides						
mlnABCDEFGHI	1,391,841	1,445,094	53.9 kb	Macrolactin	Bacteria	Schneider et al. (2007)
baeBCDE,acpK,baeGHIJLMNRS	1,700,345	1,701,022	74.3 kb	Bacillaene	Bacteria	Chen et al. (2006)
dfnAYXBCDEFGHIJKLM	2,276,743	2,346,266	71.1 kb	Difficidin	Bacteria	Chen et al. (2006)
Sfp-independent nonribosomal synthesis						
bacABCDE,ywfG	3,593,877	3,599,784	6.9 kb	Bacilysin	Bacteria	Chen et al. (2009a, b, c)
Ribosomal synthesis of modified peptides (bacteriocins)						
pznFKGHIAJCDBEL	726,469	736,360	9.96 kb	Plantazolicin	Gram-positive bacteria	Scholz et al. (2011)
acnBACDEF	3,044,506	3,048,678	4.49 kb	Amylocyclicin	Closely related bacteria	Scholz et al. (2014)
Immunity, but no synthesis genes						
mrsK2R2FGE	3,769,734	3,774,552	4.82 kb	Mersacidin	Resistance against Y2	He et al. (2012)
bceBASR	2,856,835	2,861,322	4.49 kb	Bacitracin	Resistance against *B. cereus*	Unpubl. Results
spaKREF	3,210,423	3,214,712	4.29 kb	Subtilin	Resistance against *B. subtilis*	Unpubl. Results

The table is taken from Chowdhury et al. (2015b) with modifications

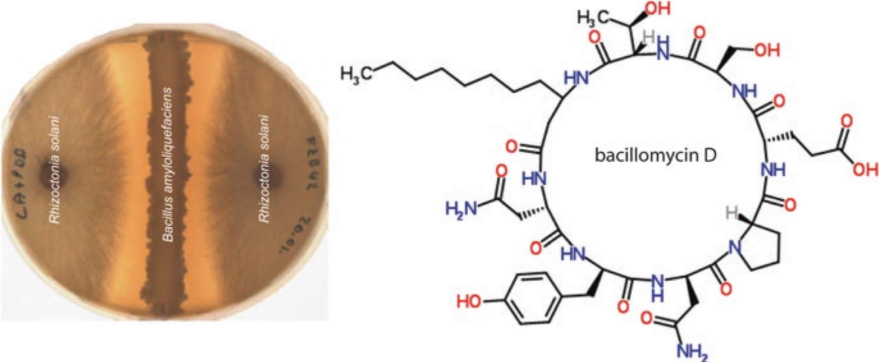


Fig. 1.2 Effect of FZB42 on *Rhizoctonia solani*. A clear inhibition zone indicating growth suppression of the fungal pathogen is visible on agar plates simultaneously inoculated with both microbes. Bacillomycin D was detected as the only prominent compound by MALDI TOF mass spectrometry of samples taken from the surface of the agar plate within the inhibition zone. The figure is taken from Chowdhury et al. (2015b) with slight modifications

fengycin, and bacillomycin D. Bacillomycin D was identified as being the most powerful antifungal metabolite produced by FZB42 (Fig. 1.2).

The heptapeptide moiety of bacillomycinD, belonging to the iturin family of cyclic lipopeptides (LP), is attached to a β-amino fatty acid chain of variable length (C_{14}–C_{17}). The peptide moiety of the heptapeptide surfactin is linked to a β-hydroxyl fatty acid (C_{12}–C_{16}), whilst the fengycin decapeptides are linked to a β–hydroxyl fatty acid chain (C_{14}–C_{18}). Their synthesis is performed by multimodular peptide synthetases and depends on a functional phospho-pantheinyl transferase (Sfp), which transfers 4′- phosphopanthetheine from coenzyme A to the carrier proteins during nonribosomal synthesis.

Within last few years, Ongena and coworkers performed pioneering work for elucidating antibiotic production *in planta* using Matrix-Assisted Laser Desorption/ Ionization Mass Spectrometry Imaging (MALDI MSI). They investigated antibiotic production in a gnotobiotic system in which the plantlet and the associated *B. amyloliquefaciens* S499, a close relative of FZB42, were growing on a gelified medium covering the MALDI target plate. Under these conditions, S499 grows as biofilm on the surface of the plant roots, allowing exact assays of secondary metabolites in the vicinity of root surface. Surfactins were detected in the root environment in much higher relative amounts, which are representing more than 90% of the whole LP production, and their synthesis is rapidly progressing during early biofilm formation. By contrast, synthesis of iturin and fengycin was delayed until the end of the aggressive phase of colonization (Nihorimbere et al. 2012; Debois et al. 2014). Earlier experiments performed with FZB42 colonizing duckweed (*Lemna minor*) plantlets corroborated that surfactin is the most prominent compound which could be detected by MALDI TOF MS in the plant-bacteria system (Idris et al. 2007). Using a gnotobiotic quartz sand system consisting of lettuce plants, the beneficial bacterium FZB42, and the pathogen *R. solani,* it was demonstrated by using alternative techniques (e.g., Fourier Transform Ionen-Cyclotron Massenspectrometry) that

lipopeptides were detectable in the order surfactin > bacillomycinD > fengycin at the plant–bacteria interface (Chowdhury et al. 2015a).

An early surfactin secretion could be of biological relevance since this lipopeptide, although less fungitoxic then iturins and fengycins, is essential for moving on tissues (Kinsinger et al. 2003) and for matrix formation in biofilms (Hofemeister et al. 2004; Lopez et al. 2009a, b). Considering the relative low amounts of the fungitoxic iturins and fengycins in vicinity of plant roots, it might be concluded that their biocontrol effect is possibly less important than expected. The same is true for the iron siderophore bacillibactin, which could not be detected under the conditions of the artificial plant–bacteria associations applied in these studies.

1.4.1.1 Polyketides, Bacilysin, and Bacteriocins Direct Antibacterial Activity

The polyketides, nonribosomally synthesized by FZB42 (Chen et al. 2006; Schneider et al. 2007), have been extensively reviewed previously (Chen et al. 2009b, 2009c; Borriss 2013). The three gene clusters encoding the modularly organized polyketide synthases (PKS) for synthesis bacillaene, macrolactin, and difficidin cover nearly 200 kb and are the largest ones, which are occurring in the FZB42 genome (Table 1.2). Difficidin is the most effective antibacterial compound produced by FZB42^{T}, but also macrolactin and bacillaene possess antibacterial activity. Difficidin is efficient in suppressing plant pathogenic bacterium *Erwinia amylovora,* which causes fire blight disease in orchard trees (Chen et al. 2009a).

Another product of nonribosomal synthesis, the dipeptide bacilysin consisting of anticapsin and alanine moieties, was found to be also involved in suppression of *Erwinia amylovora*. By contrast to the lipopeptides and polyketides mentioned above, bacilysin synthesis is not dependent on the Sfp PP-transferase. A mutant strain CH3, with a disruption of the *sfp* gene and unable to produce any polyketide or lipopeptide, was still able to synthesize bacilysin and to suppress *E. amylovora* (Chen et al. 2009a). Recent experiments, performed by the group of Xuewen Gao, Nanjing Agriculture University, demonstrated that bacilysin, despite difficidin, is efficient in suppressing *Microcystis aeruginosa*: the main causative agent of cyanobacterial bloom in lakes and rivers (Wu et al. 2015a). However, corroborating these results in field trials has to be done. Until now, polyketides and bacilysin have not been detected in plants colonized by *B. amyloliquefaciens* (Debois et al. 2014).

Antimicrobial peptides, ribosomally synthesized as linear precursor peptides, remained unknown in *B. amyloliquefaciens plantarum* for a long time with one remarkable exception: mersacidin, a B-type lantibiotic, was detected in *Bacillus* sp. HIL Y85 (Chatterjee et al. 1992). The strain HIL Y85 was later classified as being *B. amyloliquefaciens plantarum* (Herzner et al. 2011). Nowadays, mersacidin production was also detected in *B. amyloliquefaciens* B9601-Y2 (He et al. 2012). Genes involved in mersacidin self-protection reside also in the genome of FZB42. Transfer of mersacidin biosynthesis genes from HIL Y85 resulted in efficient mersacidin production by the surrogate strain constructed from the FZB42 host (Herzner et al. 2011).

Another representative of the type B lantibiotics, amylolysin from *B. amyloliquefa-ciens* GA1, was recently described. These lantibiotics are active on an array of Gram-positive bacteria, including *Listeria* spp. and methicillin-resistant *S. aureus* by interacting with the membrane lipid II (Arguelles Arias et al. 2013).

The driving force in our search for ribosomally synthesized peptides in FZB42 was the finding that the FZB42 mutant RS06, which is deficient in the Sfp-dependent synthesis of lipopeptides, polyketides, and in the Sfp-independent bacilysin production (Chen et al. 2009a), still produced an antibacterial substance active against *Bacillus subtilis* HB0042. In fact, a metabolite (cpd1335) with a molecular mass of $[M + H]^+ = 1336$ Da was assigned by MALDI TOF MS in FZB42 and in RS06, as well. The compound was named plantazolicin, and the respective gene cluster pzn consisting of 12 genes was identified by cassette mutagenesis. Plantazolicin was characterized as a highly modified peptide undergoing several steps of modification after synthesis. It ruled out that it is a thiazole/oxazole-modified microcin (TOMM) resembling microcin B17 and streptolysin S. Plantazolicin displayed antibacterial activity toward closely related gram-positive bacteria. Due to its extensive degree of modification, Pzn is highly protected from premature degradation by peptidases within the plant rhizosphere (Scholz et al. 2011). Remarkably, human pathogen *Bacillus anthracis* was found sensitive against PZN and underwent massive lysis at 4 μg mL^{-1} (Molohon et al. 2011). The exact structures of plantazolicin A and B were

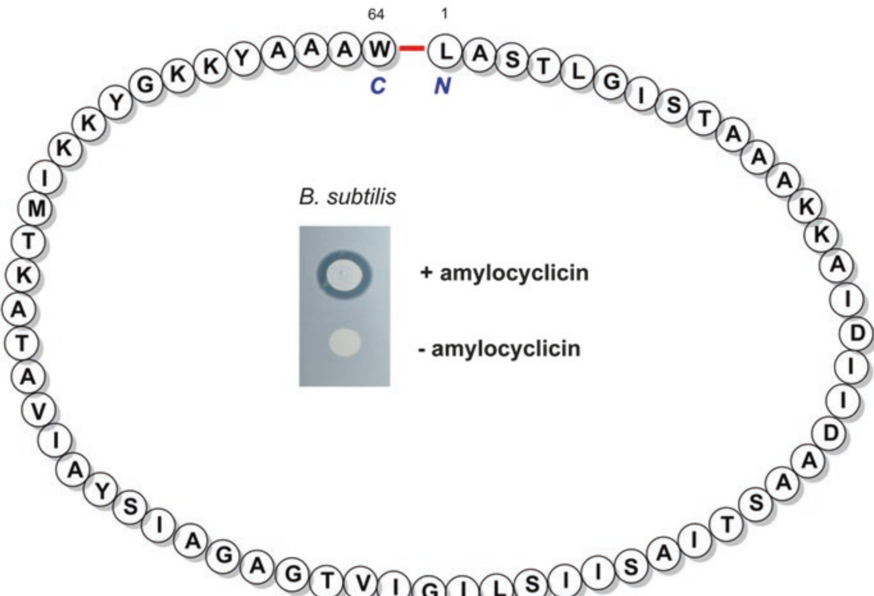


Fig. 1.3 The structure of the mature bacteriocin amylocyclicin bearing a head-to-tail cyclization of L_1 and W_{64}. Amylocyclicin effect on a related *B. subtilis* strain without immunity against the bacteriocin was demonstrated by a spot-on-lawn test performed with an amylocyclicin-producing (top) and -nonproducing strain (bottom). The figure is taken from Chowdhury et al. (2015b)

elucidated, unveiling a hitherto unusual number of thiazoles and oxazoles formed from a linear 14mer precursor peptide (Kalyon et al. 2011).

By transposon mutagenesis of the FZB42 mutant strain RS06, we identified a hitherto unknown gene cluster involved in synthesis and post-translational processing of a novel circular bacteriocin, named amylocyclicin (Fig. 1.3). It ruled out that amylocyclicin inhibits growth of bacterial strains closely related to FZB42, suggesting that this bacteriocin might have a function in competing with other *Bacillus* strains attracted to the plant rhizosphere (Scholz et al. 2014).

1.4.1.2 Nematicidal Activity Is Directed by Plantazolicin

Parasitic nematodes of plants are important plant pathogens that represent a significant financial burden on agriculture. The annual losses in agriculture resulting from this pest amounted to $125 billion worldwide in past years (Sasser and Freckman 1987; Oka et al. 2000). Chemical insecticides of nonselective nature possessing rapid nematicidal effects are widely used as control measures against these pathogens. However, the potential negative impact on the environment and ineffectiveness after prolonged use have led to banning or restricting of the use of most chemical nematicides. Therefore, identification of safe and effective nematicides is urgently needed, and biocontrol measures have recently been given much attention as viable options (Xia et al. 2011). BioNem® prepared from *Bacillus firmus* GB-126 (Table 1.1) was proven for its efficiency in greenhouse and field trials. The numbers of nematode females, eggs, and vermiform life stages at the end of the growing season decreased in the presence of the biocontrol agent, and the cotton yields were similar to those from Aldicarb, the chemical nematicide standard; however, the molecular reason for this effect remained unknown (Castillo et al. 2013).

FZB42 has been shown to reduce nematode eggs in roots, juvenile worms in soil, and plant galls on tomato (Burkett-Cadena et al. 2008). In order to identify specific nematicide-related genes, a random transposon insertion library of FZB42 was screened for relevant genes involved in nematicidal activity and, surprisingly, a gene within the *pzn* gene cluster was identified as a pathogenic factor against nematodes. Further experiments revealed that PZN displayed a moderate nematicidal activity (Liu et al. 2013).

1.5 Induced Systemic Resistance Is Triggered by Plant Growth-Promoting Bacilli

Except surfactin, concentration of antifungal lipopeptides determined *in planta* was found relatively low. Moreover, antibacterial polyketides were not detected so far in vicinity of plant roots colonized by PGPR Bacilli (Debois et al. 2014). Therefore, it is tempting to speculate that induced systemic resistance (ISR) is a main factor for suppressing plant pathogens by PGPR Bacilli. ISR occurs when the plant's defense mechanisms are stimulated and primed to resist infection by pathogens (Van Loon 1997). This activation is distinct from systemic acquired resistance (SAR) in which the response is triggered by pathogenic microorganisms associated with the aerial

portions of the plant. Selected *Bacillus* PGPR strains emit volatiles (VOCs) that can elicit plant defenses. Exposure to VOCs consisting of 2,3-butanediol and acetoin (3-hydroxy-2-butanone) from PGPR *Bacillus amyloliquefaciens* activates ISR in *Arabidopsis* seedlings (Ryu et al. 2004). *Arabidopsis thaliana* plants exposed to *Bacillus subtilis* strain FB17 results in reduced disease severity against *Pseudomonas syringae* compared to plants without FB17 treatment. Exogenous application of acetoin triggers ISR and protects plants against the pathogen in the aerial parts whilst 2,3-butanediol did not (Rudrappa et al. 2010). In this context, it is worth to mention that expression of AlsS of FZB42 involved in synthesis of acetoin (Fig. 1.1) was triggered in the presence of maize root exudate (Kierul et al. unpublished), suggesting that root exudates play a role in eliciting of acetoin biosynthesis in FZB42. It is known that some of the plant metabolites present in root exudates, such as organic acids, trigger the *alsSD* operon (Rudrappa et al. 2010). *B. amyloliquefaciens* FZB24 and FZB42 applied to tobacco roots led to a reduction of tobacco mosaic virus symptoms visible on tobacco leaves and led to decreasing amounts of virus proteins present in leaf tissues. Due to spatial distance between beneficial bacterium and the pathogen, plant ISR, stimulated by the rhizobacterium, might be responsible for this effect (Wang et al. 2009).

The induction of ISR when treated with PGPRs is mediated primarily through plant-signaling molecules such as jasmonic acid (JA), a lipoxygenase pathway product, and ethylene (ET). Salicylic acid (SA) appears to be a critical plant messenger of pathogen exposure and disease resistance in systemic acquired resistance (SAR) (Durner et al. 1997). ISR restricts pathogen multiplication and disease progression through a SA/ET and NPR1-dependent mechanism. In order to determine the signaling pathways triggered by FZB42, the expression of several marker genes in lettuce plants, exposed to FZB42 and the pathogenic fungus *Rhizoctonia solani*, was analyzed by quantitative real-time (RT)-PCR (S. Paul Chowdhury et al.: ´Systemic resistance of *Lactuca sativa* against *R. solani* and secondary metabolite production by FZB42 in an axenic model system´, unpublished). In absence of the pathogen, FZB42 increased expression of PR1 (pathogenesis protein 1, SA marker gene) and PDF1.2 (defensin, JA/ET marker gene), suggesting that SA and ET pathways are involved in upregulating defense response by ISR in lettuce. A similar result was obtained previously, when *Arabidopsis* plantlets were inoculated with *Bacillus subtilis* FB17 and acetoin (Rudrappa et al. 2010). In simultaneous presence of FZB42 and the pathogen *R. solani*, PDF1.2 expression was dramatically enhanced, suggesting a synergistic activation of the JA/ET pathway, whilst the SA pathway—as indicated by a decreased expression of PR-1—was suppressed in presence of both antagonists.

It was found that the circular lipopeptides surfactin and fengycin can act as elicitors of host plant immunity and contribute to increased resistance toward further pathogenesis ingress in bean and tomato plants (Raaijmakers et al. 2010). In bean, purified fengycins and surfactins provided a significant ISR-mediated protective effect on bean plants against the fungal pathogen *Botrytis cinerea*, similar to the one induced by living cells of the producing strain *B. amyloliquefaciens* S499 (Ongena et al. 2007).

We found (Chowdhury et al. 2015a) that the dramatic increase of the defensin 1.2 gene (PDF1.2) expression in simultaneous presence of both antagonists occurred only when wild-type cells of FZB42 were applied. Mutant strains deficient in synthesis of surfactin, fengycin, or acetoin did not stimulate expression of the JA/ET pathway, suggesting that cyclic lipopeptides and acetoin contribute together to the ISR plant response triggered by FZB42.

1.6 Conclusion

An increasing amount of data have been accumulated in course of the last years, suggesting that the antibiome expressed during the plant-associated state of PGPR Bacilli does not necessarily reflect the vast genetic arsenal devoted to the formation of lipopeptides, polyketides, and bacteriocins, which has been elucidated, for example, in the *B. amyloliquefaciens plantarum* FZB42 genome. Obviously, there is a large discrepancy in gene expression of the planktonic cells growing in liquid laboratory cultures and cells growing as biofilms on plant tissue surfaces. Except cyclic lipopeptides no other bioactive compounds such as polyketides were detected in samples taken from the vicinity of plant roots colonized by PGPR *B. amyloliquefaciens* (Debois et al. 2014). Interestingly, surfactin has multiple biological functions in motility, biofilm formation, and cell-to-cell signaling, but is less efficient in direct suppressing of other competing microbes than other lipopeptides or polyketides; it was by far the most prominent compound occurring in the plant rhizosphere, previously being inoculated by PGPR *B. amyloliquefaciens*. For this reason, I conclude that the direct effects exerted by the array of secondary metabolites encoded by the *Bacillus* genome might not be as important and that the biocontrol effects exerted by the Gram-positive bacteria are mainly due to other more indirect effects. I assume that under field conditions, the stimulating effects on plant ISR are more important than direct biocontrol by secreted secondary metabolites. In case of Bacilli, it is very likely that ISR stimulation is a multifactorial process dependent on several compounds produced by the rhizobacteria. Candidate compounds are surfactin, and volatiles, especially acetoin and 2,3 butanediol (Fig. 1.4), since mutants of FZB42, deficient in synthesis of these compounds, were found unable to protect plants from pathogens. Moreover, high expression of defensin, indicating the JA/ET pathway in ISR, was not found when the mutant strains were applied to the plant.

These findings are important for future strategies for screening of powerful PGPR and BC strains. It is known for long time that high efficiency in suppressing fungal or bacterial pathogens do not necessarily reflect the potential of these selected strains for their performance under field conditions. Novel screening procedures have to be developed for functional tests under more appropriate conditions, either directly on plants or at least under conditions allowing biofilm formation on artificial surfaces. However, performance under field conditions remains the most important criterion.

Taken together, the beneficial effect of *Bacillus* PGPR depends, besides their rhizosphere competence, on at least three main factors:

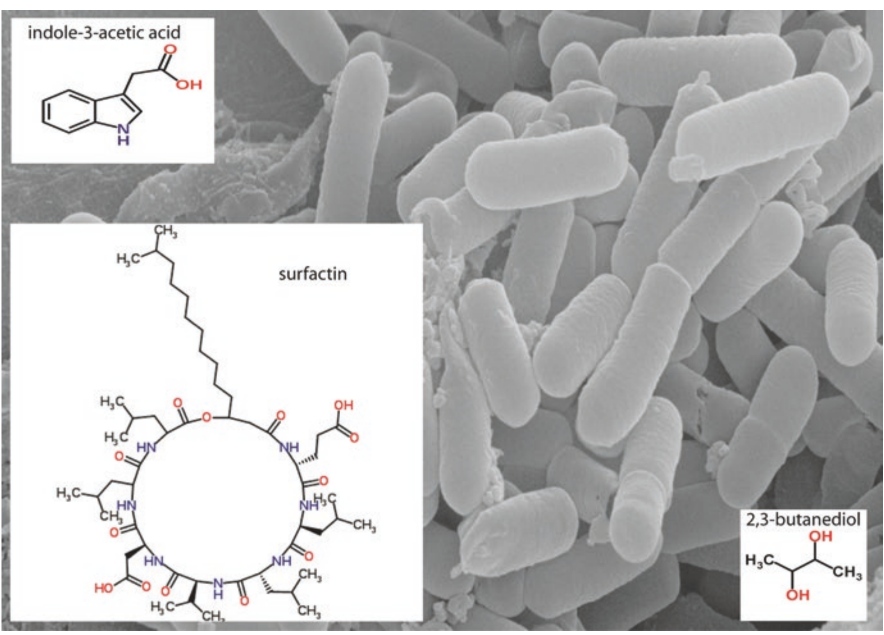


Fig. 1.4 Scanning electron microscopy of FZB42 cells colonizing *Arabidopsis thaliana* roots. Important compounds as surfactin, indole-3-acetic acid, and 2,3-butanediol, which are formed when growing on root surfaces (*in planta*), are indicated

1. Stimulation of plant ISR by bacterial metabolites produced in vicinity of plant roots. Volatiles, such as acetoin and 2,3 butanediol, contribute not only to ISR, but have a direct plant growth-promoting effect, whilst surfactin is important in the early stage of colonization and biofilm formation. In addition, surfactin strengthens the plant ISR response, which suppresses growth of fungal, bacterial, viral, and other plant pathogens.
2. Direct antifungal action by secondary metabolites, such as iturins (e.g., bacillomycin D) and fengycins, secreted into the rhizosphere. However, the suppressing effect exerted by such compounds might be relatively weak, since the amount of such compounds in vicinity of plant root was found relatively low. Until now, antibacterial compounds, such as polyketides, were not detected in this environment.
3. Application of PGPR Bacilli, as FZB42, might compensate, at least in part, undesired changes in the composition of the plant microbiome, caused by the presence of pathogens, as *R. solani.*

Without doubt, other features of PGPR, as production of plant hormones and making available fixed macro- and micro nutrients for plant nutrition, contribute also to the beneficial effect exerted by these microbes, but could not be appropriately treated in this review due to space limitation.

Acknowledgements Many of the recent data, reported in this review, have been obtained in close collaboration with the Helmholtz Center in Munich in frame of the PATHCONTROL project, and the laboratory of Yuewen Gao, Nanjing Agricultural University, China, in frame of a Chinese Collaborative project, financially supported by the BMBF, the German Ministry of Education and Research. I thank especially Soumitra Paul Chowdhury, Anton Hartmann, Joachim Vater, Liming Wu, Xuewen Gao, and Ben Fan (Nanjing Forestry University) for their fruitful collaboration during the last years.

References

Arguelles Arias A, Ongena M, Devreese B, Terrak M, Joris B, Fickers P (2013) Characterization of amylolysin, a novel lantibiotic from *Bacillus amyloliquefaciens* GA1. PLoS One 8(12):e83037. https://doi.org/10.1371/journal.pone.0083037

Berendsen RL, Pieterse CMJ, Bakker PAHM (2012) The rhizosphere microbiome and plant health. Trends Plant Sci 17:478–486

Bochow H, El Sayed SF, Junge H, Stavropoulos A, Schmiedeknecht G. 2001. Use of *Bacillus subtilis* as biocontrol agent. IV. Salt-stress tolerance induction by *Bacillus subtilis* FZB24 seed treatment in tropical field crops, and its mode of action. J Plant Dis Prot 108:21–30. (in German)

Borriss R (2011) Use of plant-associated *Bacillus* strains as biofertilizers and biocontrol agents. In: Maheshwari DK (ed) Bacteria in agrobiology: plant growth responses. Springer, Heidelberg/Dordrecht/London/New York, pp 41–76

Borriss R (2013) Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium Bacillus amyloliquefaciens FZB42. In: de Bruijn FJ (ed) Molecular microbial ecology of the rhizosphere. Wiley Blackwell, Hoboken, NJ, pp 883–898

Borriss R (2015a) Transcriptome and proteome profiling for analyzing fates of global gene expression in plant-beneficial bacilli. Transcriptomics 3:e110. https://doi.org/10.4172/2329-8936.1000e110

Borriss R (2015b) Bacillus, a plant beneficial bacterium. In: Lugtenberg B (ed) Principles of plant-microbe interactions. Springer, Cham, Heidelberg

Borriss R, Chen XH, Rueckert C, Blom J, Becker A, Baumgarth B, Fan B, Pukall R, Schumann P, Sproer C, Junge H, Vater J, Pühler A, Klenk HP (2011) Relationship of *Bacillus amyloliquefaciens* clades associated with strains DSM 7^T and *Bacillus amyloliquefaciens* subsp. *plantarum* subsp. nov. based on their discriminating complete genome sequences. Int J Syst Evol Microbiol 61:1786–1801

Burkett-Cadena M, Kokalis-Burelle N, Lawrence KS, van Santen E, Kloepper JW (2008) Suppressiveness of root-knot nematodes mediated by rhizobacteria. Biol Control 47:55–59

Castillo JD, Lawrence KS, Kloepper JW (2013) Biocontrol of the reniform nematode by *Bacillus firmus* GB-126 and *Paecilomyces lilacinus* 251 on cotton. Plant Dis 97:967–976

Chatterjee S, Chatterjee DK, Lad SJ, Phansalkar MS, Rupp RH, Ganguli BN, Fehlhaber HW, Kogler H (1992) Mersacidin, a new antibiotic from *Bacillus*: fermentation, isolation, purification and chemical characterization. J Antibiot 45:832–838

Chen XH, Vater J, Piel J, Franke P, Scholz R, Schneider K, Koumoutsi A, Hitzeroth G, Grammel N, Strittmatter AW, Gottschalk G, Süssmuth RD, Borriss R (2006) Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB 42. J Bacteriol 188:4024–4036

Chen XH, Koumoutsi A, Scholz R, Eisenreich A, Schneider K, Heinemeyer I, Morgenstern B, Voss B, Hess WR, Reva O, Junge H, Voigt B, Jungblut PR, Vater J, Süssmuth R, Liesegang H, Strittmatter A, Gottschalk G, Borriss R (2007) Comparative analysis of the complete genome sequence of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat Biotechnol 25:1007–1014

Chen XH, Scholz R, Borriss M, Junge H, Mögel G, Kunz S, Borriss R (2009a) Difficidin and bacilysin produced by plant-associated *Bacillus amyloliquefaciens* are efficient in controlling fire blight disease. J Biotechnol 140:38–44

Chen XH, Koumoutsi A, Scholz R, Borriss R (2009b) More than anticipated - production of antibiotics and other secondary metabolites by *Bacillus amyloliquefaciens* FZB42. J Mol Microbiol Biotechnol 16:14–24

Chen XH, Koumoutsi A, Scholz R, Schneider K, Vater J, Suessmuth R, Piel J, Borriss R (2009c) Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. J Biotechnol 140:27–37

Chowdhury SP, Dietel K, Rändler M, Schmid M, Junge H, Borriss R, Hartmann A, Grosch R (2013) Effects of *Bacillus amyloliquefaciens* FZB42 on lettuce growth and health under pathogen pressure and its impact on the rhizosphere bacterial community. PLoS One 8(7):e68818. https://doi.org/10.1371/journal.pone.0068818

Chowdhury SP, Uhl J, Grosch R, Alquéres S, Pittroff S, Dietel K, Schmitt-Kopplin P, Borriss R, Hartmann A (2015a) Cyclic lipopeptides of *Bacillus amyloliquefaciens* subsp. *plantarum* colonizing the lettuce rhizosphere enhance plant defense responses toward the bottom rot pathogen *Rhizoctonia solani*. Mol Plant-Microbe Interact 28(9):984–995. https://doi.org/10.1094/MPMI-03-15-0066-R

Chowdhury SP, Gao X, Hartmann A, Borriss R (2015b) Biocontrol by root-associated Bacillus amyloliquefaciens FZB42 - review. Front Microbiol 6:780. https://doi.org/10.3389/fmicb.2015.00780

Correa OS, Montecchia MS, Berti MF, Ferrari MCF, Pucheu NL et al (2009) *Bacillus amyloliquefaciens* BNM122, a potential microbial biocontrol agent applied on soybean seeds, causes a minor impact on rhizosphere and soil microbial communities. Appl Soil Ecol 41:185–194

Cruz Ramos H, Hoffmann T, Marino M, Nedjari H, Presecan-Siedel E, Dreesen O, Glaser P, Jahn D (2000) Fermentative metabolis of Bacillus subtilis: physiology and regulation of gene expression. J Bacteriol 182:3072–3080

Debois D, Jourdan E, Smargiasso N, Thonart P, de Pauw E, Ongena M (2014) Spatiotemporal monitoring of the antibiome secreted by *Bacillus* biofilms on plant roots using MALDI mass spectrometry imaging. Anal Chem doi: https://doi.org/10.1021/ac500290s, 86, 4431

Dietel K, Beator B, Budiharjo A, Fan B, Borriss R (2013) Bacterial traits involved in colonization of *Arabidopsis thaliana* roots by *Bacillus amyloliquefaciens* FZB42. Plant Pathol J 29:59–66

Doornbos RF, van Loon LC, Bakker PA (2012) Impact of root exudates and plant defense signaling on bacterial communities in the rhizosphere. A review. Agron Sustain Dev 32:227–243

Durner J, Shah J, Klessig DF (1997) Salicylic acid and disease resistance in plants. Trends Plant Sci 2:266–274

Erlacher A, Cardinale M, Grosch R, Grube M, Berg G (2014) The impact of the pathogen *Rhizoctonia solani* and its beneficial counterpart *Bacillus amyloliquefaciens* on the indigenous lettuce microbiome. Front Microbiol 5:175. https://doi.org/10.3389/fmicb.2014.00175

Fan B, Chen XH, Budiharjo A, Bleiss W, Vater J, Borriss R (2011) Efficient colonization of plant roots by the plant growth promoting bacterium *Bacillus amyloliquefaciens* FZB42, engineered to express green fluorescent protein. J Biotechnol 151:303–311

Fan B, Carvalhais LC, Becker A, Fedoseyenko D, von Wirén N, Borriss R (2012a) Transcriptomic profiling of *Bacillus amyloliquefaciens* FZB42 in response to maize root exudates. BMC Microbiol 12:116. https://doi.org/10.1186/1471-2180-12-116

Fan B, Borriss R, Bleiss W, Wu XQ (2012b) Gram-positive rhizobacterium *Bacillus amyloliquefaciens* FZB42 colonizes three types of plants in different patterns. J Microbiol 50:38–44

Fan B, Li L, Chao Y, Förstner K, Vogel J, Borriss R, Wu XQ (2015) dRNA-Seq reveals genomewide TSSs and noncoding RNAs of plant beneficial rhizobacterium *Bacillus amyloliquefaciens* FZB42. PLoS One 10(11):e0142002. https://doi.org/10.1371/journal.pone.0142002

Grosch R, Junge H, Krebs B, Bochow H (1999) Use of *Bacillus subtilis* as a biocontrol agent. III Influence of *Bacillus subtilis* on fungal root diseases and yield in soilless culture. J Plant Dis Protec 106:568–580

Guel A, Kidoglu F, Tuzel Y, Tuzel IH (2008) Effects of nutrition and Bacillus amyloliquefaciens on tomato (Solarium lycopersicum L.) growing in perlite. Span Agric J Res 6:422–429

He P, Hao K, Blom J, Rückert C, Vater J, Mao Z, Wu Y, Hou M, He P, He Y, Borriss R (2012) Genome sequence of the plant growth promoting strain *Bacillus amyloliquefaciens* subsp. *plan-*

tarum B9601-Y2 and expression of mersacidin and other secondary metabolites. J Biotechnol 15:281–291

Herzner AM, Dischinger J, Szekat C, Josten M, Schmitz S, Yakéléba A, Reinartz R, Jansen A, Sahl HG, Piel J, Bierbaum G (2011) Expression of the lantibiotic mersacidin in *Bacillus amyloliquefaciens* FZB42. PLoS One 6(7):e22389. https://doi.org/10.1371/journal.pone.0022389

Hofemeister J, Conrad B, Adler B, Hofemeister B, Feesche J, Kucheryava N, Steinborn G, Franke P, Grammel N, Zwintscher A, Leenders F, Hitzeroth G, Vater J (2004) Genetic analysis of the biosynthesis of non-ribosomal peptide- and polyketide like antibiotics, iron uptake and biofilm formation by Bacillus subtilis A1/3. Mol Gen Genomics 272:363–378

Idris EES, Iglesias DJ, Talon M, Borriss R (2007) Tryptophan dependent production of indole-3-acetic acid (IAA) affects level of plant growth promotion by *Bacillus amyloliquefaciens* FZB42. Mol Plant-Microbe Interact 20:619–626

Idriss EES, Makarewicz O, Farouk A, Rosner K, Greiner R, Bochow H, Richter T, Borriss R (2002) Extracellular phytase activity of *Bacillus amyloliquefaciens* FZB 45 contributes to its plant growth-promoting effect. Microbiology 148:2097–2109

Idriss EE, Bochow H, Ross H, Borriss R (2004) Use of *Bacillus subtilis* and *Bacillus subtilis* FZB37 as biocontrol agent. VI. Phytohormone-like action of culture filtrates prepared from plant growth-promoting *Bacillus amyloliquefaciens* FZB24, FZB42, FZB45. J Plant Dis Protec 111:583–597

Kalyon B, Helaly SE, Scholz R, Nachtigall J, Vater J, Borriss R, Süssmuth RD (2011) Plantazolicin A and B: structure of ribosomally synthesized thiazole/oxazole peptides from *Bacillus amyloliquefaciens* FZB42. Org Lett 13:2996–2999

Kierul K, Voigt B, Albrecht D, Chen XH, Carvalhais LC, Borriss R. (2015). Influence of root exudates on the extracellular proteome of the plant growth-promoting bacterium *Bacillus amyloliquefaciens* FZB42. Microbiology 161(Pt 1):131–147. doi: https://doi.org/10.1099/mic.0.083576-0

Kinsinger RF, Shirk MC, Fall R (2003) Rapid surface motility in *Bacillus subtilis* is dependent on extracellular surfactin and potassium ion. J Bacteriol 185(18):5627–5631

Kloepper JW, Leong J, Teintze M, Schroth M (1980) Enhancing plant growth by siderophores produces by plant-growth-promoting rhizobacteria. Nature 286:885–886

Koumoutsi A, Chen XH, Henne A, Liesegang H, Hitzeroth G, Franke P, Vater J, Borriss R (2004) Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J Bacteriol 186:1084–1096

Kröber M, Wibberg D, Grosch R, Eikmeyer F, Verwaaijen B, Chowdhury SP et al (2014) Effect of the strain Bacillus amyloliquefaciens FZB42 on the microbial community in the rhizosphere of lettuce under field conditions analyzed by whole metagenome sequencing. Front Microbiol 5:636. https://doi.org/10.3389/fmicb.2014.00636

Liu Z, Budiharjo A, Wang P, Shi H, Fang J, Borriss R, Zhang K, Huang X (2013) The highly modified microcin peptide plantazolicin is associated with nematicidal activity of *Bacillus amyloliquefaciens* FZB42. Appl Microbiol Biotechnol 97:10081–10090

Lopez D, Fischbach MA, Chu F, Losick R, Kolter R (2009a) Structurally diverse natural products that cause leakage trigger multicellularity in Bacillus subtilis. Proc Natl Sci USA 106:280–285

Lopez D, Vlamakis H, Losick R, Kolter R (2009b) Cannibalism enhances biofilm development in Bacillus subtilis. Mol Microbiol 74:609–618

Lugtenberg BJJ, Malfanova N, Kamilova F, Berg G (2013) Plant growth promotion by microbes. In: de Brujn FJ (ed) Molecular microbial ecology of the rhizosphere, vol 2. Wiley Blackwell, Hoboken, NJ, pp 561–573

Molohon KJ, Melby JO, Lee J, Evans BS, Dunbar KL, Bumpus SB, Kelleher NL, Mitchell DA (2011) Structure determination and interception of biosynthetic intermediates for the plantazolicin class of highly discriminating antibiotics. ACS Chem Biol 6(12):1307–1313

Nihorimbere V, Cawoy H, Seyer A, Brunelle A, Thonart P, Ongena M (2012) Impact of rhizosphere factors on cyclic lipopeptide signature from the plant beneficial strain amyloliquefaciens S499. FEMS Microbiol Ecol 79:176–191

Oka Y, Koltai H, Bar-Eyal M, Mor M, Sharon E, Chet I, Spiegel Y (2000) New strategies for the control of plant-parasitic nematodes. Pest Manag Sci 56:983–988

Ongena M, Jourdan E, Adam A, Paquot M, Brans A, Joris B, Arpigny JL, Thonart P (2007) Surfactin and fengycin lipopeptdes of *Bacilllus subtilis* as elicitors of induced systemic resistance in plants. Environ Microbiol 9:1084–1090

Raaijmakers J, De Bruin I, Nybroe O, Ongena M (2010) Natural functions of cyclic lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiol Rev 34:1037–1062

Ramírez CA, Kloepper JW (2010) Plant growth promotion by *Bacillus amyloliquefaciens* FZB45 depends on inoculum rate and P-related soil properties. Biol Fert Soils 46:835–844

Renna MC, Najimudin N, Winik LR, Zahler SA (1993) Regulation of the *Bacillus subtilis alsS*, *alsD*, and *alsR* genes involved in post-exponential phase production of acetoin. J Bacteriol 175:3863–3875

Rudrappa T, Biedrzycki ML, Kunjeti SG, Donofrio NM, Czymmek KJ, Paré PW, Bais HP (2010) The rhizobacterial elicitor acetoin induces systemic resistance in *Arabidopsis thaliana*. Commun Integr Biol 3:130–138

Ryu CM, Farag MA, Hu CH, Reddy M, Wei HX, Pare PW, Kloepper J (2003) Bacterial volatiles promote growth in *Arabidopsis*. Proc Natl Acad Sci U S A 100:4927–4932

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Paré PW (2004) Bacterial volatiles induce systemic resistance in Arabidopsis. Plant physiology, March 2004. Vol 134:1017–1026

Sasser JN, Freckman DW (1987) A world perspective on nematology: the role of the society. Society of Nematologists, Hyattsville, MD

Schneider K, Chen XH, Vater J, Franke P, Nicholson G, Borriss R, Suessmuth RD (2007) Macrolactin is the polyketide biosynthesis product of the *pks2* cluster of *Bacillus amyloliquefaciens* FZB42. J Nat Prod 70:1417–1423

Scholz R, Molohon KJ, Nachtigall J, Vater J, Markley AL, Süssmuth RD, Mitchell DA, Borriss R (2011) Plantazolicin, a novel microcin B17/streptolysin S-like natural product from *Bacillus amyloliquefaciens* FZB42. J Bacteriol 193:215–224

Scholz R, Vater J, Budiharjo A, Wang Z, He Y, Dietel K, Schwecke T, Herfort S, Lasch P, Borriss R (2014) Amylocyclicin, a novel circular bacteriocin produced by *Bacillus amyloliquefaciens* FZB42. J Bacteriol 196:1842–1852

Singh B, Satyanarayana T (2011) Microbial phytases in phosphorous acquisition and plant growth promotion. Physiol Mol Biol Plants 17:93–103

Van Loon LC (1997) Induced resistance in plants and the role of pathogenesis-related proteins. Eur J Plant Pathol 103:753–765

Wang S, Wu H, Qiao J, Ma L, Liu J, Xia Y, Gao X (2009) Molecular mechanism of plant growth promotion and induced systemic resistance to tobacco mosaic virus by Bacillus spp. J Microbiol Biotechnol 19(10):1250–1258

Wu L, Wu H, Chen L, Yu XF, Borriss R, Gao X (2015a) Difficidin and bacilysin *Bacillus amyloliquefaciens* FZB42 have antibacterial activity against *Xanthomonas oryzae* from rice pathogens. Sci Rep 5:12975. https://doi.org/10.1038/srep12975

Wu L, Wu HJ, Qiao J, Gao X, Borriss R (2015b) Novel routes for improving biocontrol activity of Bacillus based bioinoculants. Front Microbiol 6:1395. https://doi.org/10.3389/fmicb.2015.01395

Xia Y, Xie S, Ma X, Wu H, Wang X, Gao X (2011) The *purL* gene of *Bacillus subtilis* is associated with nematicidal activity. FEMS Microbiol Lett 322:99–107

Yao AV, Bochow H, Karimov S, Boturov U, Sanginboy S et al (2006) Effect of FZB24 *Bacillus subtilis* as a biofertilizer on cotton yields in field tests. Arch Phytopathol Plant Prot 39:1–6

Check for updates

2 Genetically Modified (GM) Crops Harbouring *Bacillus thuringiensis* (BT) Gene(S) to Combat Biotic Stress Caused by Insect Pests

Bhupendra Koul

Abstract

Insect pests are a menace to the crop plants as they cause 15–22% annual crop loss. *Bacillus thuringiensis* (*Bt*) crystal protein toxin(s) have been observed to be effective against lepidopteran, coleopteran, dipteran and hemipteran insect pests. With the emergence of recombinant DNA technology, computational biology and plant transformation procedures, it is now possible to design, modify and transfer any gene (natural or synthetic) into crop plants especially, to cope with insect pests, herbicide tolerance, various abiotic stresses and to enhance the expression level and nutritional quality. Bt-based biopesticides are an alternative to synthetic pesticides and are insect- specific, effective, eco-friendly and cost-effective. *Agrobacterium*-mediated plant transformation technique utilizes the natural genetic engineering property of *Agrobacterium tumefaciens* which has played a pivotal role in plant genetic engineering and development of stable transgenics, over conventional breeding procedures. Several stable Bt-transgenics (potato, maize, cotton, soybean, canola, squash, rice, etc.) developed by various companies (Monsanto, Dow AgroSciences, Syngenta, Bayer cropScience, etc.) have been approved by Genetic Engineering Appraisal Committee (GEAC), Environment Protection Agency (EPA), and commercialized. The most successful story of Bt-transgenics is that of Bt-cotton (Bollgard: trade name) harbouring *Bt-cry1Ac* like gene. In order to avoid the development of insect resistance, various strategies such as use of hybrid gene, *Bt*-gene pyramiding, refugia strategies, enhanced expression of *Bt*-gene(s) and use of sterile insects are followed as and when required for maintaining the sustainability of *Bt*-technology. In the last few years, after analysing the effectiveness and promising future of this 'green

B. Koul (✉)
Lovely Faculty of Technology and Sciences, Department of Biotechnology, School of Bioengineering and Biosciences, Lovely Professional University, Phagwara, Punjab, India
e-mail: bhupendra.18673@lpu.co.in

© Springer Nature Singapore Pte Ltd. 2020
M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_2

technology,' there has been a remarkable progress in the list of countries accepting the Bt-GM crops.

Keywords
Bacillus thuringiensis · *cry* gene · *Agrobacterium tumefaciens* · Plant transformation · *Bt*-GM crops

2.1 Introduction

The sustainable plant productivity and crop yield(s) in coming years is the major constrain for food and nutritional security for the human population in developing countries, where arable land per capita is shrinking, while human and livestock population is steadily increasing. Plant and crop productivity and yield are the result of interaction of several physiological, biochemical and metabolic processes over a defined period of time, reflected in gain of total biomass or converted harvestable commodity like seeds, fruits or edible plant parts under a set of environmental conditions that consist of several physical, geo-chemical and biological components. Therefore, besides the genetic potential of plant species, the phenotypic performance of crop plants in field profoundly depends on and is influenced by several physical, abiotic and biotic parameters and is highly variable. Hence, plant yield or harvest index is dependent on several factors and several of them are beyond human control and are part of climate change and environment. Among biotic components that influence plant/crop yield perhaps infestation of plant pathogens and insect pest are major issues after the agronomic inputs and practices. The infestation of insect pests alone during field and storage condition may affect up to from 24 to 65 ± 5% loss in grain yield of major crops (Ronald 2011). Control of agricultural insect pests under field and storage conditions largely depend on the wide spread use of synthetic insecticides and pesticides which are harmful to the ecosystem and human population (Hilder and Boulter 1999; Wahab 2009). Alternative to conventional chemical insecticides, application of microbial insecticides containing different microbial preparations and delta endotoxins (Cry proteins) from *Bacillus thuringiensis* (*Bt*) have emerged as ecofriendly and sustainable method for control of agricultural insect pests in the last 50–60 years (Sanahuja et al. 2011). Attempts are being made to use alternative bioinsecticides in field as well as in storage conditions to minimize the losses in grain yield. In recent past, with the development of diverse biotechnological tools and techniques of recombinant DNA and genetic engineering, it is now possible to transfer and express a desired gene in its native or modified form into the identified organism including plants, animals and microbes. Among the battery of genetically modified organisms (GMOs), the transgenic plants, expressing genes from either trans- or cis-origin, are the latest introduction for sustainable crop and plant yield (Park et al. 2011).

The most widely used and well-documented example of transgenic plant in agriculture practice is the Bt-cotton, where Bt-toxin crystalline proteins of Cry1A family are expressed starting from the native wild *cry1Ab* and *cry1Ac* genes of *Bacillus thuringiensis* to highly modified synthetic version that are expressed in cotton followed by maize and soybean which are released for commercial cultivation (Perlak et al. 2001; James 2012). Since then, transgenics of major crop plants like cotton, maize, soybean, canola, tomato, rice, squash, potato, papaya, sugarcane and mustard have been developed for insect-pest resistance, herbicide tolerance and resistance to viruses and have been grown in more than 30 countries over 181.5 million hectares in 2016. About 17.3 million farmers over the world have been benefited by transgenic technology and are growing biotech crops. Interestingly, recently, five conservative European countries, namely Spain, Portugal, Czechia, Romania and Slovakia, have agreed to cultivate Bt-maize. Therefore, the transgenic technology has been adopted by both developed and developing countries like the United States, China, Brazil, Argentina, Canada and India and African countries, for different traits.

Bacillus thuringiensis (*Bt*) is a gram-positive soil bacterium which can produce crystalline inclusions during the second phase of sporulation. These inclusions eventually develop into hydrophobic crystalline structures consisting of several toxin proteins that are of insecticidal nature against a wide spectrum of agricultural insect pests (Whiteley and Schnepf 1986). Most of the crystal proteins are protoxins of proteinaceous nature and are proteolytically converted into smaller toxic polypeptides in the midgut region of corresponding agricultural insect. This activated toxin interacts with the midgut epithelial cells of susceptible insects (Hofmann et al. 1988; Bravo et al. 2007, 2011; Vachon et al. 2012; Pardo Lopez et al. 2013) and biochemically generate the pores in the cells of brush border membrane, thus disturbing the osmotic balance and eventually the septicemia in the target insect leading into death of the insect (Knowles and Ellar 1988; Bravo et al. 2007, 2011). Several specific high-affinity binding sites on insect membranes to *B. thuringiensis* toxins have been documented for specificity of different toxin peptides generated by different strains/species/isolates of *B. thuringiensis* owing to different genes coding for the corresponding crystal protein (Schnepf et al. 1998; Hofte and Whiteley 1989).

Since the first cloning of an insecticidal crystal protein (ICP) gene (*cry*) from *B. thuringiensis* by Schnepf and Whiteley (1981), a large number of *cry* genes from different strains/species of *Bt* have been cloned, identified and characterized (Crickmore et al. 1998; deMaagd et al. 2001). Till date, more than 500 different *cry* genes from *B. thuringiensis* have been characterized and systematically documented in the literature and enlisted in website maintained by Crickmore and his group (www. glfc.cfs.nrcan.gc.ca/bacillus). These insecticidal genes code specific toxins effective against insect orders belonging to Lepidoptera, Diptera and Coleoptera. Some are effective against other insect orders like Hymenoptera, Homoptera, Orthoptera, and Mallophaga, nematodes, mites and protozoa as well (Feitelson et al. 1992; Bravo et al. 2007). *B. thuringiensis* strains have a genome size of 2.4–5.7 million bp, and most of these bacterial strains possess both circular and

sometimes linear extra chromosal elements; however, the *cry* genes are mostly located on the large plasmid (Gonzalez et al. 1981; Gonzalez et al. 1982). A large number of *cry* genes producing insecticidal toxins effective against common agricultural insect pests have been identified, cloned and expressed in different plant species to develop insect pest resistance genetically modified transgenic plants (James 2012).

Since the first introduction of *cry* gene into model plant tobacco for expressing insect-resistant trait (Barton et al. 1987; Vaeck et al. 1987), several major crop species have been genetically modified for expression of different insecticidal *cry* genes affective against different order of insects (Fischhoff et al. 1987; Perlak et al. 1990; Perlak and Fischhoff 1993; Fujimoto et al. 1993; Koziel et al. 1993; Adang et al. 1993; Nayak et al. 1997; Sanyal et al. 2005). The initial studies with introduction and expression of native full-length *cry* genes from *B. thuringiensis* into plants have shown very poor expression of toxin production, and the produced toxin was unstable in the plant system (Perlak et al. 1990; Schnepf et al. 1998). Several biochemical and genetical reasons have been attributed for poor stability and low expression of Bt-toxins in transgenic plants.

The earlier studies with transfer of Bt-*cry* genes showing poor expression were attributed to silencing of foreign gene, instability of RNA transcripts of insecticidal crystal protein genes (Murray et al. 1989), early termination of the transcript due to existence of polyadenylation at multiple sites in coding region of native Bt-*cry* genes (Diehn et al. 1996, 1998) and rapid degradation of mRNA (Perlak et al. 1991; Adang et al. 1993; DeRocher et al. 1998). The evidence to these factors was associated to earlier reports for lack of a correlation between promoter activity and mRNA accumulation (Fischhoff et al. 1987; Vaeck et al. 1987). The analytical results of tobacco transgenics expressing full-length native *cry1Ac* showed majority of transcript shorter than anticipated full length of the gene (Barton et al. 1987). These studies based on expression of full-length native *cry1Ac* and *cry1Ab* insecticidal genes lead to characterization of several polyadenylation sequences along with cryptic termination sequences in native *Bt*-*cry* genes.

These early reports suggested reinvesting the *Bt*-*cry* gene for its structure and functioning in the plant system. Subsequently, by analysing the nucleotide sequences of several *cry* genes, it was evident that crystal protein genes of *B. thuringiensis* were destined for expression in prokaryotic cell and of typical prokaryotic architecture in having codon sequences preferable to prokaryotes and gene length for optimum expression and stability of toxin in hydrophobic state and nucleotide sequences and GC content suitable to prokaryotes. These observation lead to several modifications in Bt-*cry* genes which included truncation of 3′ end of gene to eliminate hydrophobicity of the endotoxin, removable of polyadenylation, mRNA instability and criptic termination sequences, for higher expression of Bt-*cry* genes in plants (Fischhoff et al. 1987; Vaeck et al. 1987; Perlak et al. 1991). A major modification in the *cry* gene was incorporated to modify and introduce plant-preferred codons in the truncated version of Bt-*cry* genes (Delannay et al. 1989; Perlak et al. 1990, 1991).

These studies eventually led to major modification in designing of synthetic version of truncated *cry1Ac* and *cry1Ab* genes comprising of about ~1845 bp, where maximum care was taken to possibly use plant-preferred codons, elimination of all the termination sequences and mRNA instability components (Perlak et al. 1991; Sardana et al. 1996; Cheng et al. 1998). The designed genes were successfully shown to express the Cry1Ab and Cry1Ac toxins in different plant species, and promising transgenic plants of various species were developed (Perlak et al. 1991; Stewart Jr et al. 1996; Singsit et al. 1997; Perlak et al. 2001; Sanyal et al. 2005). Based on these developments and further molecular investigation of *cry* toxin and its interaction with different receptor on susceptible insect resulted in development of hybrid and fusion *cry* genes for wider host range and enhanced toxicity against agriculturally important target insects (Datta et al. 1998; Wu et al. 2000; Naimov et al. 2003; Singh et al. 2004; Ho et al. 2006; Rajamohan et al. 2006). To enhance host range of Cry toxin and to address the growing resistance development in target insects against these toxins, several mutations have been incorporated in the toxin for effective binding to receptor (Bravo and Soberon 2008; Soberon et al. 2013). Similarly, translation fusions of two cry genes or additional sequences for wider host range have been designed (Bohorova et al. 2001; Mehlo et al. 2005).

Lepidoptera is the most devastating group of field insects causing significant damages to large number of crop plants. Among them, *Helicoverpa armigera*, *Heliothis virescens*, *Ostrinia nubilalis*, *Spodoptera* spp., *Plutella xylostella* and *Pectinovophora gossypiella* are the important insects infesting several important crops like cotton, cabbage, okra, tomato, cauliflower, chickpea, maize and soybean. Two Bt-genes, *cry1Ab* and *cry1Ac*, have been documented for coding most effective toxin showing maximum mortality in range of 20–80 ng toxin/mg of fresh weight. Both these genes are most widely used for developing insect-resistant phenotype. To make these two toxins highly effective and efficient against target insect pests, several modifications have been incorporated including truncation, codon optimization, point mutations and application of 5′ regulatory sequences for over expression of the toxins at desired level in different plant species. The mechanism for pore formation and recognition of different receptors and their affinity to these toxins have been well documented. The native and modified versions of full length *cry1Ab* (3.5 kb) and *cry1Ac* (3.5 kb) and their synthetic modified truncated versions of 1.8 kb size have been widely used for developing the transgenic plants of different species exhibiting resistance against a number of insect pests (Cheng et al. 1992; Koziel et al. 1993; Stewart Jr et al. 1996; Alam et al. 1998; Cheng et al. 1998; Perlak et al. 2001; Sanyal et al. 2005; Mehrotra et al. 2011; Sanahuja et al. 2011). Except for selection of a unique event of transgenic cotton expressing a full-length native *cry1Ac* gene with few modifications and transgenic maize expressing *cry1Ab,* which have gone for commercial cultivation (Koziel et al. 1993; Perlak et al. 2001; Ferry et al. 2004; James 2012), most of the transgenics of different plant species are limited to demonstration under laboratory conditions. Despite several modifications incorporated in native wild type *cry1Ab* and *cry1Ac* genes which share more than 94 ± 0.5% sequence homology, their over-expression, however, in different plant species to recover promising transgenic plants with sufficient level of Bt-toxin(s)

have been a matter of concern (Diehn et al. 1996; DeRocher et al. 1998). The most widely used successful transgenic event of Bt-cotton (Monsanto to 531) resistant to bollworm complex of *Heliothis virescens/Helicoverpa armigera*, *Pectinophora gossypiella* and *Helicoverpa zea* was developed with native full-length *cry1Ac* gene having some specific minor modification (Perlak et al. 2001). The event has been designated as Bollgard I and been grown commercially in large areas in several countries (James 2012). Subsequently, to check the possibility of insect developing resistance against Bt-cotton technology, a second version of transgenic cotton plant designated as Bollgard II, expressing two different *cry* genes such as *cry1Ac* and *cry2Ab*, has been developed and released for commercial cultivation (Purcell et al. 2004; Ferry et al. 2004). Interestingly, native *cry1Ac* coding gene was documented for very poor expression in higher plants owing to high AT content and presence of several pre-termination sequences. This situation necessitated the truncation and enrichment of GC content, since plants in general have a higher GC content than that found in bacterial genes (Murray et al. 1989), and particularly delta-endotoxin *cry* genes have higher AT content. Modifying the coding sequences to increase GC content, 3′ truncation and possible elimination of polyadenylation or termination sequences of the native *cry* genes resulted into dramatic increase in the expression of the insecticidal toxin proteins (Delannay et al. 1989; Perlak et al. 1991; Carozzi et al. 1992). A highly modified *cry1Ab* gene-coding toxin protein of 648 amino acid of the native proto-toxin of 1155 amino acids was expressed in maize to develop resistance against European corn borer (ECB), *Ostrinia nubilalis* (Hubner), a major pest of maize (Carozzi et al. 1992; Koziel et al. 1993). Comparative nucleotide and amino acid sequences of prominent *cry1A* group of genes (*cry1Aa*, *Ab* and *Ac*) coding insecticidal crystal proteins affective against large number of Lepidopteran insects showed distinct homology and similarities in 5′ coding sequences for toxin molecules comprising of pore forming and receptor-binding domains except for the specific changes in the sequences coding for the receptor-recognizing domains of the toxin molecules (Haider and Ellar 1987; Schnepf et al. 1998; Bravo and Soberon 2008). This comparative and exhaustive sequence analysis was further executed to other group of insecticidal crystal protein genes to reflect the diversity and evolution of different *cry* genes coding different insecticidal toxin proteins effective against specific insects (Feitelson et al. 1992; DeMaagd et al. 2001; Sanahuja et al. 2011).

Among *cry1A* group of genes, the response of the toxins against lepidopteran insects has been found in the order *cry1Ac* > *cry1Ab* and least in *cry1Aa* gene. This is further attributed to the molecular structure of insecticidal Cry toxin and its affinity to bind with a different receptor on the midgut of susceptible insects and attachment of toxin molecules with different epitopes of same or different receptors on BBMV cells (Estela et al. 2004; Bravo et al. 2007). Considering the close resemblance and high homology of nucleotide sequences of *cry1Ab* and *cry1Ac* gene and based on the architecture of toxin-coding sequences, completely synthetic version of both the 1.8 kb genes has been developed and extensively used for optimal expression in higher plants (Sardana et al. 1996; Cheng et al. 1998). The comparative sequence analyses of both *cry1Ab* and *cry1Ac* genes have shown three blocks of 668, 403 and 279 bp which are identical in both the case while the fourth block of

495 bp comprises sequence variations that seem to code for the receptor-binding domain of the toxin protein and may be the possible reason for differential toxicity. Considering the high homology and similarities between modified synthetic *cry1Ab* and *cry1Ac* genes for enhanced expression of toxin in higher plant, achieving promising number of transgenic with high level of toxin expression is not a routine process. Despite the successful commercial release of Bt-cotton expressing *cry1Ac* gene but recovery of stable transgenic plant with high level of Cry1Ac toxin is still confined to laboratory level around the globe. Only restricted plant species have been documented for high level expression of *Cry1Ac* toxin compared to number of transgenic plants developed with Cry1Ab toxin. The modified-*cry1Ab* gene has been successfully introduced and expressed to sufficient level in several plant species like maize (Koziel et al. 1993; Singh et al. 2005), rice (Fujimoto et al. 1993; Wunn et al. 1996; Wu et al. 1997; Cheng et al. 1998; Alam et al. 1998, 1999; Tu et al. 2000; Marfà et al. 2002), cotton (Perlak et al. 1990), brinjal (Kumar et al. 1998), soybean (Parrott and Clemente 2004), tomato (Kumar and Kumar 2004), sugar beet (Jafari et al. 2009) and chickpea (Mehrotra et al. 2011). However, restricted plant species have been transformed with *cry1Ac* gene to develop stable transgenic plants of cotton (Perlak et al. 1990, 2001; Rawat et al. 2011), tobacco (Barton et al. 1987; Vaeck et al. 1987), tomato (Fischhoff et al. 1987; Mandaokar et al. 2000), chickpea (Kar et al. 1997; Sanyal et al. 2005), peanut (Singsit et al. 1997) and canola (Stewart Jr et al. 1996).

2.2 Genetic Transformation

Genetic transformation is the deliberate alternation and modification of the genome of an organism (bacteria, plant, animal) by introduction of one or few specific foreign genes using other than conventional procedures, and the modified organism is termed as transformed or transgenic organism. Genetic transformation of plants is becoming an indispensable aid to plant physiologists, biochemists and biotechnologists in understanding the role of individual and application of these procedures for crop improvement with newer traits. Scientists of Calgene Inc. of Davis, California, used the antisense RNA technology to inactivate the gene (polgalacturonase [PG]) responsible for softening the tomato to produce first genetically modified tomato 'Flavr-Savr' in 1991 and was approved by the US FDA in 1994.

2.3 Gene Transfer Method

2.3.1 Direct DNA Transfer Methods

The direct DNA transfer method has been proved to be simple and effective for introducing foreign DNA into plant genomes (Fig. 2.1). Among these methods, the most frequently used one is the microprojectile bombardment procedure where

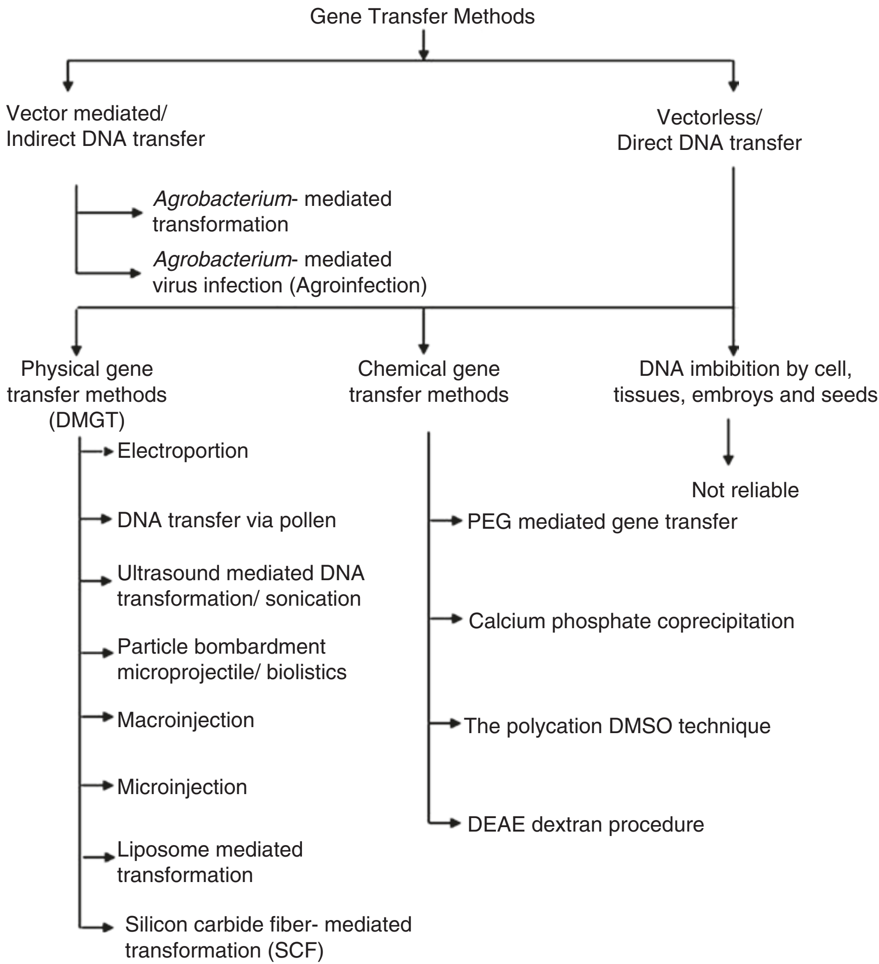


Fig 2.1 Schematic representation of the various gene transfer strategies

transforming DNA is coated onto metal microcarriers like tungsten or gold that are accelerated with high velocity either by gun powder device or through compressed inert gases. The microcarriers acquire sufficient kinetic energy to allow them to penetrate to the intact plant, animal or bacterial cell wall and plasma membrane without killing the cells.

2.3.2 Indirect DNA Transfer Method

As with other dicotyledonous crops, *Agrobacterium*-mediated transformation is the most widely used method for gene transfer. Among the various vectors used in plant transformation, the Ti plasmid of *Agrobacterium tumefaciens* has been widely used.

This bacteria is known as 'natural genetic engineer' of plants, because these bacteria have natural ability to transfer T-DNA of their plasmids into plant genome upon infection of cells at the wound site and cause an unorganized growth of a cell mass known as crown gall. Ti plasmids are used as gene vectors for delivering useful foreign genes into target plant cells and tissues. The foreign gene is cloned in the T-DNA region of Ti plasmid in place of unwanted sequences.

2.4 *Agrobacterium*-Mediated Genetic Transformation

Agrobacterium tumefaciens is a gram-negative, soil phytopathogen of family Rhizobiaceae that causes the disease 'crown gall' in a wide variety of dicotyledonous plants (Fig. 2.2). Crown gall is a plant tumour, a lump of undifferentiated tissue, which often forms at the area of crown, the junction between the root and the stem of the infected plants. The pathogenic property of this bacterium was recognized much earlier (Smith and Townsend 1907).

A. *tumefaciens*: induces crown gall disease.

A. *rhizogenes*: induces hairy root disease.

A. *radiobacter*: an avirulent strain.

During the infection at wound site, the bacterium transfers a small part of its own plasmid DNA called T-DNA (transfer DNA) into the plant cell that results in two key events.

1. The plant cell begins to proliferate and form tumours and receive the ability to grow in cultures, which even do not have any growth regulator.
2. They begin to synthesize an unusual arginine derivative called opines (octopine, nopaline, etc.) which are not found in normal tissues.

Bacteria can be classified as octopine, nopaline, agropine, succinamopine or chrysopine strains (octopine is condensation product of arginine and pyruvic acid). The metabolism of opines is a central feature of crown gall disease. The type of opine produced is not determined by the host plant but by the bacterial strain. In general, the bacterium induces the synthesis of an opine, which it can catabolize and

Fig. 2.2 (**a**) Electron micrograph of *A. tumefaciens* (**b**) A plant root with crown galls, (**c**) A plant showing symptoms of hairy roots

use as its sole energy source for carbon and nitrogen. Clearly, an interesting interrelationship is evolved, where *A. tumefaciens* subvert the plant's metabolism to make amino acids, which can be utilized only by the bacteria as a food and energy source.

2.4.1 Ti Plasmid of *Agrobacterium*

The ability of *Agrobacterium tumefaciens* to induce crown gall disease in plants is controlled by genetic information carried on a large conjugative plasmid (of about 200 kb size) called Ti plasmid for its tumour-inducing capacity. Virulence is lost when the bacterium is cured for the plasmid, and cured strains have lost the capacity to utilize octopine or nopaline. Ti plasmids have temperature-sensitive replication, i.e. high temperature (more than 30 °C) leads to curing of plasmids. Ti plasmids have regions for replication (origin of replication), conjugal transfer, virulence and T-DNA.

Three bacterial genetic elements are required for T-DNA transfer to plants.

1. 25 bp direct repeated flanking and defining the T-DNA
2. Virulence (*vir*) genes encoded by the Ti plasmid in a region outside of the T-DNA.
3. Number of chromosomal genes, of which some are important for attachment to the bacterium to the plant cell

2.4.2 Organization of T-DNA

T-DNA (transfer DNA) is about 23 kb segment of Ti plasmid, which is transferred into the plant genome during *Agrobacterium* infection. T-DNA contains the gene for constitutive synthesis of auxins, cytokinins and opines and is defined on both the sides by 24 bp direct inverted repeat called border sequences, which are required for T-DNA excision and transfer. The deletion of either border sequence completely blocks the transfer of T-DNA into the plant cell. However, mutational analysis shows that only the right repeat is absolutely required for T-DNA transfer and they function in *cis* and polar fashion. The T-DNA is organized into two distinct regions called TL (left T-DNA) and TR (right T-DNA). Both TL and TR are always transferred together in nopaline plasmids and integrated into the plant genome as a single segment. But in octopine plasmids, the TL and TR are transferred independently so that a single cell may contain one or both of these segments. T-DNA has three genes, which are involved in crown gall formation. Two of these genes, *iaa*M and *iaa*H encodes tryptophan 2-monooxygenase and indoleacetamide hydrolase, respectively, which together convert tryptophan into indole 3-acetic acid (IAA); the locus was earlier called 'shooty' locus, and the genes were designated as *tms1* (tumour with shoots) and *tms2*. The third gene, *ipt*, encodes a zeatin-type cytokinin, isopentenyl transferase; the locus was earlier designated as 'rooty' locus and

designated as *tmr* (tumour having roots). T-DNA also contains genes involved in opine biosynthesis near the right border. All the genes present in T-DNA contain eukaryotic regulatory sequences. As a result, these genes are expressed only in plant cells, and they are not expressed either in *Agrobacterium* or in *E. coli*.

2.4.3 Organization of *vir* Region

The *vir* region of the Ti plasmid contains 8 operons, which together span to about 40 kb of DNA and possesses 25 genes. This region mediates the transfer of T-DNA in both *cis* and *trans* fashion into plant genome, and hence is essential for virulence and transfer of T-DNA (Hooykaas and Mozo 1994). Among the eight *vir* operons, four operons, viz., *vir*A, *vir*B, *vir*D and *vir*G are essential for virulence, while the remaining four operons play an accessory role in transfer of T-DNA. *Vir*A and *vir*G, which are constitutively expressed, regulate the expression of other *vir* loci. Signal transduction proceeds via activation of *vir*G by *vir*A, in response to the activation of *vir*A by plant phenolics like acetosyringone and α-hydroxy acetosyringone. After activation, *vir*G dimerizes and activates the transcription of other *vir* genes (Zambryski et al. 1989). The functions of different *vir* genes are given in Table 2.1.

2.4.4 T-DNA Transfer Process

T-DNA transfer begins with the introduction of bacteria into a plant wound (Fig. 2.3). Wounding is a necessary event in the process and may, at least is part, be required for the synthesis by the plant, certain compounds that induce the expression of the vir genes. Two of the most active substances identified are acetosysingone and β-hydroxy acetosysingone. T-DNA transfer process starts by binding of *vir*D1 gene product to the right border (RB) sequence, *vir*D1 has the topoisomerase activity that facilitates the action of protein *vir*D2, as endonuclease; in nicking, at the right border and covalently binds to the 5′ end. The 3′ end produced at the site

Table 2.1 Functions of different *vir* genes

Vir region	No. of genes	Function
*vir*A	1	Encodes a sensor protein; receptor for acetosyringone and functions as an autokinase; also phosphorylates *vir*G protein; constitutive expression
*vir*B	11	Membrane proteins; role in conjugal tube formation
*vir*C	2	Helicase activity
*vir*D	4	*Vir*D1, has topoisomerase activity and *vir*D2 is an endonuclease
*vir*E	2	Single strand binding protein (SSBP)
*vir*F	1	Not well understood
*vir*G	2	DNA binding protein, induces the expression of all *vir* operon; constitutive expression
*vir*H	2	Not well known

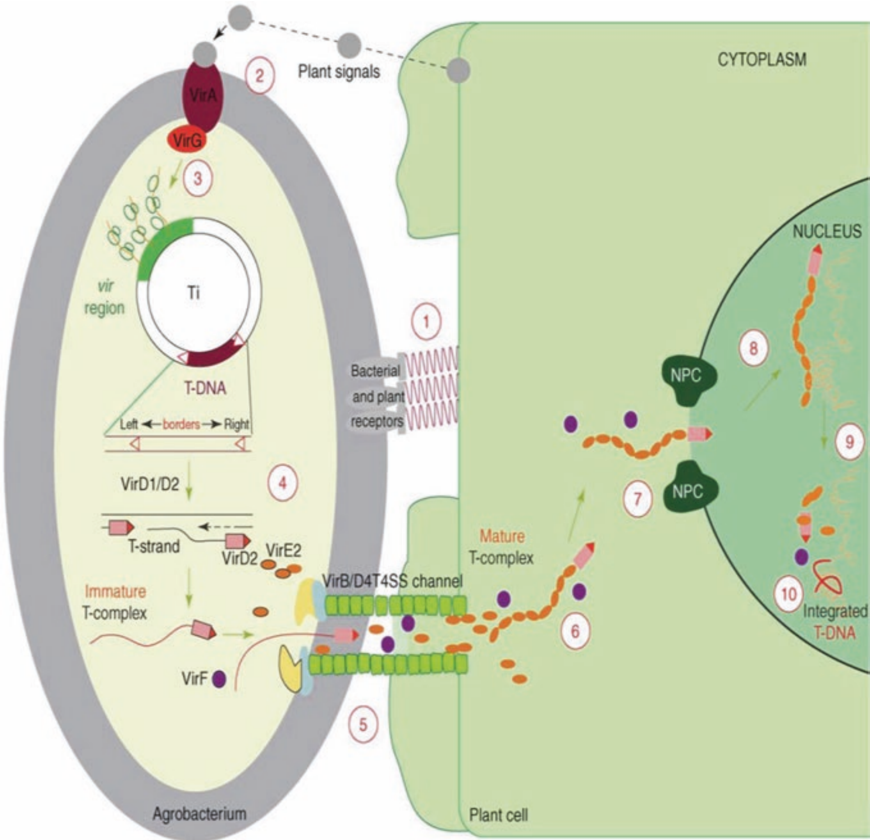


Fig. 2.3 Model for *Agrobacterium*-mediated genetic transformation of plants (Tzfira and Citovsky 2006). The transformation process comprises of 10 major steps and begins with recognition and attachment of the *Agrobacterium* to the host cells (*1*). Sensing of specific plant signals by the *Agrobacterium* VirA/VirG two-component signal-transduction system (*2*). Following activation of the vir gene region (*3*), a mobile copy of the T-DNA is generated by the VirD1/D2 protein complex (*4*) and delivered as a VirD2–DNA complex (immature T-complex), together with several other Vir proteins, into the host-cell cytoplasm (*5*). Following the association of VirE2 with the T-strand, the mature T-complex forms, travels through the host-cell cytoplasm (*6*) and is actively imported into the host-cell nucleus (*7*). Once inside the nucleus, the T-DNA is recruited to the point of integration (*8*), stripped of its escorting proteins (*9*) and integrated into the host genome (*10*)

of nick serves as a primer for replacement synthesis of DNA in the $5' \rightarrow 3'$ direction as a result of which the T-strand is displaced from the DNA duplex.

The *vir*E2 protein is a single-strand DNA-binding protein and about 600 copies of it binds to the single-stranded T-DNA, thus protecting it from nuclease action. *Vir*B operon encodes membrane-bound proteins, which participate in conjugal tube formation between the bacterial and plant cells to provide a channel for T-DNA transfer, whereas *vir*B11 has ATPase activity, which generates energy needed for the delivery of T-DNA into the plant cells (Zambryski et al. 1989). The nuclear

localization signals present on the *vir*D2 and *vir*E2 proteins drive the T-DNA towards the nucleus of the plant cell. This mechanism accounts for the polarity; *cis*-acting nature of the border repeat sequences also explains the importance of right border repeat in T-DNA transfer. Apart from Ti plasmid, chromosomal virulence genes (*chv*) are also involved in T-DNA transfer from *Agrobacterium* to plants. The *chv* genes are required for the synthesis of cyclic glucans, which are involved in plant cell-binding *Chv* A, *chv*B and *psc* A that are involved in the synthesis and export of cyclic β-1,2-glucan. A more direct role in attachment has been demonstrated for rhicadhesin, a calcium-binding protein located on bacterial cell surface. The induction of *Agrobacterium vir* genes in response to plant wound-specific compounds implies that a bacterial recognition system must detect the plant signal and transmit the information inside the bacterial cells. This process is mediated by products of *vir* A and *vir* G.

2.4.5 Vectors Derived from Ti Plasmids

Large size, absence of unique restriction sites and tumourigenic properties of Ti plasmids precluded the use of wild-type Ti plasmids as vectors. Presently, plant transformation vectors have been produced by replacing tumour-including genes with dominant selectable markers and desired traits. These types of vectors are known as disarmed vectors; with functional *vir* genes and T-DNA border sequences. Such non-oncogenic plant transformation vectors are either co-integrated or binary types.

2.4.6 Co-integrate Vector System

Vectors that recombine via DNA homology into a resident Ti plasmid are often referred to as integrative or cointegrative vectors. In this type of vector systems, both T-DNA and *vir* regions are present in the same Ti plasmids. Gene of interest can be inserted in between T-DNA borders by a co-integration event between the homologous sequences present in the cloning vector and T-DNA region of Ti plasmid. Efficiency of co-integrate system relies on the frequency of conjugal transfer and homologous recombination.

2.4.7 Binary Vector System

The binary vector system consists of two autonomously replicating plasmids within *A. tumefaciens* a shuttle (more commonly referred to as binary) vector that contains gene of interest between the T-DNA border and a helper Ti plasmid that provides the *vir* gene products. The *vir* gene can act in trans and encode proteins, which are required for the transfer of T-DNA. The standard components of binary vector are:

1. Multiple cloning site
2. A broad host range origin of replication functional in both *E. coli* and *A. tumefaciens*
3. Selectable markers for both bacteria and plants
4. T-DNA border sequences (although only right border is absolutely essential)

2.4.8 Selectable Markers

Selection of transformed cells is a key factor in developing successful methods for genetic transformation. This is done by certain selectable marker genes that are present in the vector along with the gene of interest. Selectable markers are an integral part of plant transformation strategies (Table 2.2).

Each selectable marker presents some favourable and some unfavourable features. Therefore, the choice of a marker should be based on the plant species and other considerations in the study. The NPT II gene from transposon *Tn5* confers resistance to the amino glycoside antibiotics kanamycin, neomycin and G 418. The NPT II gene product, *neomycin phosphotransferase*, inactivates these antibiotics through its phosphorylation (Bevan et al. 1983). This marker is a most widely used system for plant selection and screening as no endogenous level is reported so far in green plants.

2.4.9 Advantages of *Agrobacterium*-Mediated Plant Transformation

It is a natural means of DNA transfer and is perceived as a more acceptable technique over long conventional breeding procedures. It is capable of infecting intact plant cells, tissues and organs. Transformed plants can be regenerated more rapidly. It is capable of transferring large fragments of DNA very efficiently without substantial rearrangements of the transgene. Integration of DNA is relatively a precise

Table 2.2 Selectable markers genes used for gene transfer

Selectable marker genes	Substrates used for selection
Neomycin phospotransferase (*npt*II)	G 418, kanamycin, neomycin, paromycin
Hygromycin phospotransferase (*npt*)	Hygromycin B.
Gentamycin acetyl transferase	Gentamycin
Streptomycin phospotransferase	Streptomycin
Dihydrofolate reductase (*dhfr*)	Methotrexate
Phosphinothricin acetyl transferase	L-Phosphinothricin (PPT)
5-Enolpyruvyl shikimate 3 phosphate (EPSP) syhthase (*aro*A)	Glyphosafe
Acetolactate synthase mutant form (*als*)	Sulphonyl urea, imidazolinones
Bromoxynil nitrilase (*bxn*)	Bromoxynil

process; it serves as an ideal insertional mutagenesis vehicle as it introduces one to several copies of the transferred DNA into the intact genome at one or few loci. The integrated DNA gives consistent maps and appropriate segregation ratios. The stability of the gene(s) and the respective trait(s) have been found to be stable over many generations. All of these features make this technique reliable for commercialization of transgenic plants. A wide range of explants have been successfully transformed using Agrobacterium, although cotyledons have been most commonly used (McCormick et al. 1986). Other explants like vegetative leaves and hypocotyl (McCormick et al. 1986) stem have also been used with high transformation frequency both with binary as well as co-integrate Ti plasmid vectors used in these experiments.

2.4.10 Disadvantages of *Agrobacterium*-Mediated Plant Transformation

There is limitation of host range as it cannot transform many important food crops. Cells and tissues that are able to regenerate are difficult to transform. The embryogenic cells are placed in deeper layers and are thus not amenable to T-DNA transfer.

2.5 Factors Affecting Plant Transformation

A successful gene transfer procedure is mainly dependant on the following factors: (1) simple, reproducible, genotype-independent and cost-effective regeneration protocol for (2) target tissues, which are both competent for transformation and regeneration, (3) an efficient DNA delivery method, (4) procedure to select for transgenic tissues and (5) the ability to recover fertile plants avoiding somaclonal variation in transgenic plants (Velcheva et al. 2005; Thi Van et al. 2010).

Availability of high-frequency genotype-independent in vitro regeneration system amenable to *Agrobacterium*-mediated transformation is the major pre-requisite for developing transgenic lines (Birch 1997). A number of factors influencing genetic transformation such as genotype, type of explant, explant orientation, wounding procedure, co-cultivation duration, the role of phenolic compounds, *Agrobacterium* strain, bacterial cell density, etc. play an important role in determining overall transformation efficiency. The optimization of selection and screening procedures are crucial for improving transformation efficiency and most importantly developing non-chimeric transgenic plants.

2.6 *Bacillus thuringiensis* (*Bt*) Endotoxin Crystal Protein Genes for Insect Resistance

Agricultural pests are mostly controlled by the use of synthetic pesticides and rarely by cultural practices. Therefore, the excessive and reckless use of agrochemicals has been a subject of public concern as it has led to harmful consequences on the environment and carcinogenicity to non-targets organisms.

The reliance on gene transfer technology to transfer insect-resistance genes of diverse origin into crop plants provides an economical, feasible and eco-friendly alternative to the extensive use of chemicals pesticides. Insect-resistant transgenic plants may be raised by introducing foreign genes encoding either δ-endotoxin, protease inhibitors (PI), lectins, amylase inhibitors, etc. (Boulter 1993; Gatehouse et al. 1997). The most widely used, well-documented and reliable approach in this context is the insecticidal crystal protein (ICP) genes of *Bacillus thuringiensis* (*Bt*) which code for δ-endotoxin (Whiteley and Schnepf 1986). Gram-positive spore-forming entomopathogenic bacteria of Bacillaceae family particularly *Bacillus thuringiensis* produce a large variety of protein toxins to aid them to invade, infect and kill their hosts. This bacterium produces an insecticidal crystal protein which forms inclusion bodies of bipyramidal, cuboidal, flat rhomboid or a composite with two or more crystal types during sporulation (Bajwa and Kogan 2001). ICPs are one of the several classes of endotoxins produced during sporulation, and δ-endotoxins (delta endotoxins) are the most effective than other classes of α-, β- and γ-endotoxins (alpha, beta and gamma) to agricultural insect pests. The genes coding these toxins are called *cry* genes.

Although the Cry proteins exhibit diversity, they are specific to the target insect orders: lepidoptera (moths and butterflies), diptera (mosquitoes and flies) and coleopteran (weevils and beetles), and few new toxins have been identified to kill hymenopterans (bees and wasps) and nematodes (Schnepf et al. 1998; Pigott and Ellar 2007; Bravo et al. 2007). Considering a large number of *cry* genes and diversity of encoded toxins against different groups of insects and microbes, several nomenclatures and classification of ICP genes have been proposed (Hofte and Whiteley 1989; Sanchis et al. 1988; Crickmore et al. 1998; Crickmore et al. 2011).

Table 2.3 Classification of *cry* genes on the basis of their activity spectrum[a]

Protein	Subspecies (strain)	Activity spectrum	Protoxin/active molecular mass in kDa
Cry I	*Cry*I *Kurstak*i (HD-1), *aizawai*, *sotto*	Lepidopteran	130–160/ca.60
*Cry*II	*Cry*II *Kurstak*i (HD-1), *Kurstak*i (HD-263)	Lepidopteran and dipteran (mosquito)	70–71/ca.65
*Cry*IIIA	*Tenebrionsis*	Coleopteran (chrusomelids)	73/ca.65
*Cry*IIIB	*Japonicus*	Coleopteran (scrarabaeids)	73/ca.55
*Cry*IV	*Israelensis*	Diptera (mosquito, black flies and nematodes)	72–134/ca.46–48

[a]Hofte and Whiteley (1989); Rukmini et al. 2000)

However, new toxin-encoding genes are being identified and the number is increasing therefore, nomenclature and name of the new *cry* genes is assigned according to the extent of evolutionary divergence, as projected by phylogenetic tree algorithms. The large and variable family of insecticidal proteins of BT was earlier classified on the basis of their activity, into five major classes, as shown in Table 2.3. Later, Crickmore et al. (1998) suggested a common platform for nomenclature of Bt-*cry* genes and broadly classified them into 22 groups of *cry* genes and two groups of cytolytic (*cyt*) parasporal inclusion protein genes that exhibited hemolytic activity.

According to Crickmore et al. (2011), Cry toxins have been classified on the basis of their primary amino acid sequence and more than 500 different *cry* gene sequences have been classified into 70 subgroups. These *cry* gene sequences have been divided into four phylogentically unrelated protein families with different modes of action: three domain Cry toxins (3D), mosquitocidal Cry toxins (Mtx), binary-like (Bin) and the Cyt toxins. Among these toxins, the family of three-domain Cry toxins represents the largest group with more than 53 different subgroups.

As mentioned before, Bt-toxins are extremely specific to the target insect pests, non-toxic to animals including non-target insects and human beings, non-hazardous and eco-friendly (DeMaagd et al. 2001). These characteristics led to the advancement of bioinsecticides, and formulations based on Bt-spores to control agricultural insects have been developed and used extensively. Besides production of insecticidal δ-endotoxins by *B. thuringiensis*, some of the bacterial species are documented to express toxins during the non-sporulating state called 'Vip,' or vegetative insecticidal protein, which are toxic to insects and microbes (Gatehouse 2008). Both Cry and Cyt toxins interact with very specific receptors on susceptible insect pests. The primary mode of Cry protein is to recognize the receptor on insect midgut epithelial cells and lyse the cells by inserting the domain I and resulting into pore formation.

The three-domain Cry toxins are globular molecules harbouring three distinct domains connected by single linkers. The domain I at the N-terminal end comprises a series of α-helices arranged in a cylindrical formation while domain II comprises a triple β-sandwich for receptor binding. Most of the *Bt*-toxins are expressed as protoxin of higher molecular weight and are non-toxic; however, their proteolytic products are of smaller size and are highly toxic to the susceptible insects. The main difference between the 65 and 130-kDa three-domain Cry toxin is a C-terminal extension that is found in the 130-kDa protoxins, which is cleaved by proteases present in the larval midgut and is therefore dispensable for toxicity (DeMaagd et al. 2001). The N-terminal region of all three-domain *cry* genes codes for the N-terminal fragment of protoxin which comprises 20–60 residues, while the active toxin is composed of approximately 600–620 amino acid residues. The X-ray crystallographic studies of different trypsin-activated Cry toxins, such as Cry1Aa (Lepidopteran specific), Cry3Aa, Cry3Bb and Cry8Ea (Coleopteran specific), Cry4Aa and Cry4Ba (Dipteran specific) and Cry2Aa protoxin (Dipteran-lepidopteran specific), have been determined (Li et al. 1991; Grochulski et al. 1995; Galitsky et al. 2001; Morse et al. 2001; Boonserm et al. 2005, 2006; Guo et al. 2009).

Cry proteins are modular in structure, consisting of three different functional domains as I, II and III (Schnepf et al. 1998). N-terminal part of the toxin fragment comprising six amphipathic helices (α-1, 2, 3, 4, 6, 7) with a central hydrophobic helix (α-5) makes the domain I of δ-endotoxins (Li et al. 1991; Grochulski et al. 1995). Two alternative models, viz. 'Penknife Model' (Hodgman and Ellar 1990) and 'umbrella model' (Li et al. 1991), were proposed to explain the pore-forming mechanism of domain I of δ-endotoxins. Following insertion of the toxin, helix α-1 is removed due to protease digestion, and it is the only helix that does not bind to BBMV vesicles as synthetic peptide mimicking studies show that α-5 helix and α-4-α-5 helix loop is important for toxin aggregation and ion channel formation (Gerber and Shai 2000). It has been proposed that after the toxin binds to the receptor, there occurs a change in the conformation of this domain allowing the hydrophobic surfaces of the helices to face the exterior of the bundle, leading to insertion into the membrane and the formation of ion channels (Knowles 1994). Domain II is made of three antiparallel β-sheets, oriented parallel to the α-helices of domain I. Domain III is made of two antiparallel β-sheets into β-sandwich structure which is involved in several functions such as stability, as receptor binding, specificity determination and ion channel gating (Schnepf et al. 1998). Arginine-rich block in domain III of δ-endotoxin is called 'arg face,' through which domain III makes contact with domain I and regulates ion channel conductance (Saraswathy and Kumar 2004).

The results of phylogenetic analysis suggest that domain I sequences seem common only for a subgroup of toxin proteins. Shuffling of the functional domains was observed only for domain II and III in some toxins. Toxins with dual specificity for lepidopteran and coleopteran insects are examples of domain III shuffling among coleopteran and lepidopteran-specific toxins. The phylogenetic analysis of the Cry toxin family shows that the great variability in the biocidal activity has resulted from two fundamental evolutionary processes: (i) independent evolution of the three functional domains and (ii) domain swapping among different toxins. These two processes have generated toxin proteins with similar modes of action but with diverse specificities. It is suggested that sequence divergence in combination with domains swapping by homologous recombination might have caused extensive range of specificities and evolution of different Bt-toxins (DeMaagd et al. 2001; Bravo et al. 2007).

After ingestion of Bt-ICP by a target insect, Bt-protoxin first passes through the peritrophic matrix (PM) diffusing into the midgut brush border, where it is digested to yield toxin of smaller molecular mass that mediates insect death (Gill et al. 1992; Knowles 1994). The PM is a single semiporous tube consisting of several layers of mucin like glycoproteins and chitin microfibrils (Nation 2002; Ma 2005). It serves as a barrier against the entry of virus, bacteria and bacterial products, such as Bt-protoxin (Nation 2002). Receptor binding is a key factor for specificity, specific binding involves two steps: one that is reversible and other is irreversible. Recent data suggested that toxicity correlates with irreversible binding (Aronson and Shai 2001). Irreversible binding might be related to insertion of the toxin into the membrane but could also reflect a tighter interaction of the toxin with the receptor. The delta endotoxin-binding receptors in the larval midgut are identified as glycoprotein. Domain II

loops showed immunoglobulin-like structural folds, and carbohydrates are used as recognition epitopes by these folds (Li et al. 1991). CryIAc toxin specifically binds to a 120 kDa aminopeptidase-N (APN) receptor and binding interaction is mediated by Gal NAc, presumably covalently attached to the APN. Knight et al. (1994) have shown that O-glycans associated with a C-terminal O-glycosylated 'Stalk' structure in the APN molecule are the most likely site for CryIAc toxin binding determined by lectin binding and carbohydrate compositional analysis.

Cadherin-like proteins also serve as receptors for CryIAc toxins in lepidopteran insects. Cadherin is critical for initial binding with toxin followed by further proteolytic changes, oligomerization, binding to APNs in lipid rafts and insertion into the cell membrane for forming pores (Hua et al. 2004). Regions of domain II of CryIA toxins bind to specific sites on Bt-R_1 Cadherin-like protein. Three CryIAb toxin-binding regions in *Manduca sexta* Bt-R_1 have been mapped to aa^{865}–aa^{875} (Site 1), aa^{1331}–aa^{1342} (Site 2) and aa^{1363}–aa^{1464} (Site 3). The first site ^{865}NITIHITDTNN^{875} is involved in binding loop 2 and second site ^{1331} IPLPASILTVTV^{1342} binds to loop α-8 located on CR11. Ectodomain CR12 (Site 3) is a critical Cry1Ab receptor epitope and is the minimum region found to be crucial to confer cell susceptibility to Cry1Ab to the same level as full-length Bt-R_1 (Hua et al. 2004; Xie et al. 2005).

2.7 Mechanism of Action of Three-Domain Cry Toxins in Lepidoptera

The activated toxin of 60 kDa goes through a complex sequence of binding events with different insect gut Cry-binding proteins (receptors), leading to membrane insertion and pore formation (Bravo et al. 2004; Pigott and Ellar 2007; Pacheco et al. 2009). Two models have been proposed which demonstrate the series of events that occur during receptor–Bt-protein interaction: [A] pore formation model and [B] signal transduction model.

2.7.1 Pore Formation Model

According to the pore formation model, binding to Bt-R_1 (receptor) is possibly the first event in the interaction with the microvilli membrane. This initial binding promotes a conformational change in the toxin-facilitating proteolytic cleavage of helix α-1, by a membrane-bound protease followed by formation of pre-pore oligomeric structure. The oligomeric toxin then binds to the APN which induces a conformation change and a molten globule state of the toxin which is inserted into lipid rafts inducing pore formation and cell swelling (Bravo et al. 2007). After insertion into the membrane bilayers, the toxin inhibits k^+ transport and amino acid assimilation in the gut lumen, causing imbalance in pH, ion and other macro molecules and culminate into insect death (Ma 2005). According to a recent report of Pardo Lopez et al. (2013), which is an extension of pore-formation model, the first binding/interaction of activated Cry1A toxins is a low-affinity interaction with

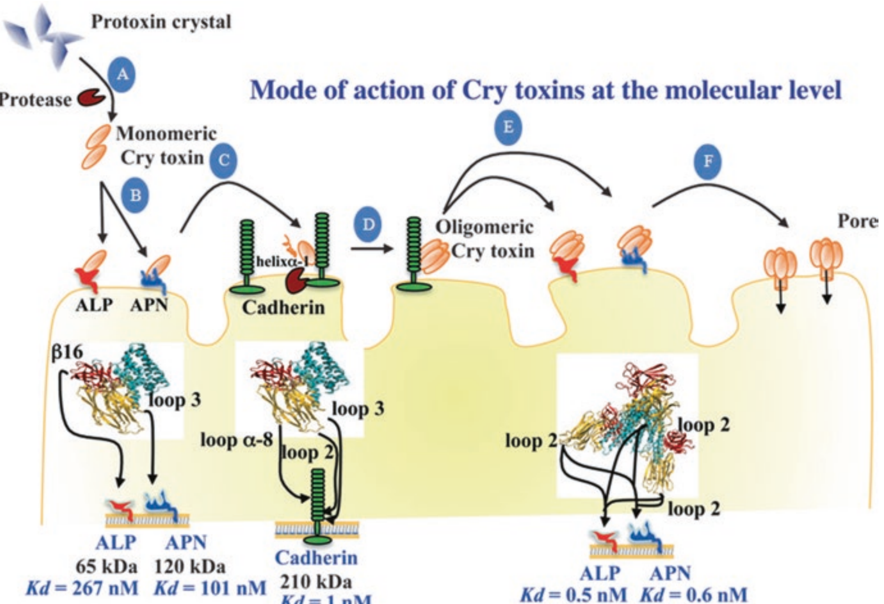


Fig. 2.4 Schematic representation of the mechanism of action of three-domain Cry toxins in Lepidoptera at the molecular level. (*A*) the larvae ingest the three domain-Cry protoxin, which is solubilized in the midgut lumen due to high pH and reducing conditions and get activated by gut proteases, thus generating the toxin fragment. (*B*) the monomeric three domain-Cry toxin binds ALP and APN receptors, in a low-affinity interaction, the toxin is then located in close proximity to the membrane (*C*) the monomeric three domain-Cry toxin binds the cadherin receptor in a high-affinity interaction and this interaction induces proteolytic cleavage of the N-terminal end of the toxin, including helix α-1 of domain I (*D*) the cleaved three domain-Cry toxin is then able to oligomerize in a toxin pre-pore oligomer (*E*) the oligomeric three domain-Cry structure binds to ALP and APN receptors with high affinity (*F*) the pre-pore inserts into the membrane causing pore formation

ALP (alkaline phosphatase) and APN receptors (aminopeptidase-N) (K_d = 101 nM for APN and 287 nM for ALP). The interaction with APN occurs through exposed loop 3 of domain II and interaction with ALP through strand β-16 of domain III (Masson et al. 1995; Pacheco et al. 2009; Arenas et al. 2010). ALP and APN are highly abundant proteins anchored to the membrane by a glycosyl phosphatidylinositol anchor (Upadhyay and Singh 2011). The interaction with ALP and APN concentrates the activated toxin in the microvilli of the midgut cells due to which the toxin is able to bind in a high affinity interaction to the cadherin receptor (K_d = 1 nm; Vadlamudi et al. 1995; Gómez et al. 2006, Pacheco et al. 2009; Arenas et al. 2010). A schematic representation of mechanism of action of three-domain Cry toxin in Lepidopterans at the molecular level has been shown in Fig. 2.4.

2.7.2 Signal Transduction Model

Another model which is signal transduction suggests that Bt-toxicity could be related to G-protein-mediated apoptosis following the receptor binding (Zhang et al. 2006). Binding of Cry toxin to Bt-R_1 mediates cell death by activating a signalling pathway involving stimulation of the stimulatory G-protein-α-subunit (G-αs) and adenylyl cyclase (AC), which increases the cyclic adenosine monophosphate (AMP) levels, and activation of protein kinase A (PKA). Activation of AC/PKA signalling pathway initiates a series of cytological events that include membrane blebbing, appearing of nuclear ghosts and cell swelling followed by cell lysis (Zhang et al. 2006). Diagrammatic view of the two models of Cry toxin action has been shown in Fig. 2.5.

Broderick et al. (2006) have put up an interesting observation that *B. thuringiensis* toxicity depends on the interaction with microorganisms of the normal gut community. Elimination of gut microbial community by oral administration of antibodies abolished insecticidal toxicity, and re-establishment of an enterobacter sp., that normally resides in the midgut microbial community has restored *B. thuringiensis*-mediated killing.

Transgenic plants expressing *B. thuringiensis* toxins have been used successfully to provide resistance against selected agricultural insects. Since the development of first transgenic tobacco and tomato plants with native Bt-*cry* gene (Vaeck et al. 1987; Fischhoff et al. 1987; Barton et al. 1987) considerable progress has been made to develop promising transgenic plants with highly modified Bt-*cry* genes for stability of mRNA and high-level expression (Gatehouse 2008). A large number of stable transgenic plants of different families, expressing various Bt-*cry* genes have been developed which exhibit significant protection to insect damages in lab and field (Hilder and Boulter 1999; Sharma et al. 2000; Tabashnik et al. 2003).

2.8 BT-GM Crops

A large number of crop plants expressing Bt-insecticidal endotoxin have been successfully transformed by *Agrobacterium*-mediated approach. Major reports on development of insect-resistant plants are summarized in Table 2.4.

Stable transgenic plants of tobacco (Barton et al. 1987), tomato (Delannay et al. 1989; Gordon-Kamm et al. 1990), rice (Koziel et al. 1993; Datta et al. 1998;), soybean (Parrott et al. 1994; Stewart Jr et al. 1996), groundnut (Singsit et al. 1997), pigeonpea (Surekha et al. 2005) and chickpea (Kar et al. 1997; Sanyal et al. 2005) have been developed. Recently, very high level of expression of Bt-cry2Aa2 protein in chloroplast up to 35.5% of total protein (DeCosa et al. 2001) and expression and inheritance of multiple transgenes (gene pyramiding) in rice (Cheng et al. 1998; Maqbool et al. 2001) and cabbage (Cao et al. 2001; Zhao et al. 2003) have been documented, for efficient management of insects and as insect-resistance management strategy. The global status of approved and commercially available Bt-GM crops is shown in Table 2.5.

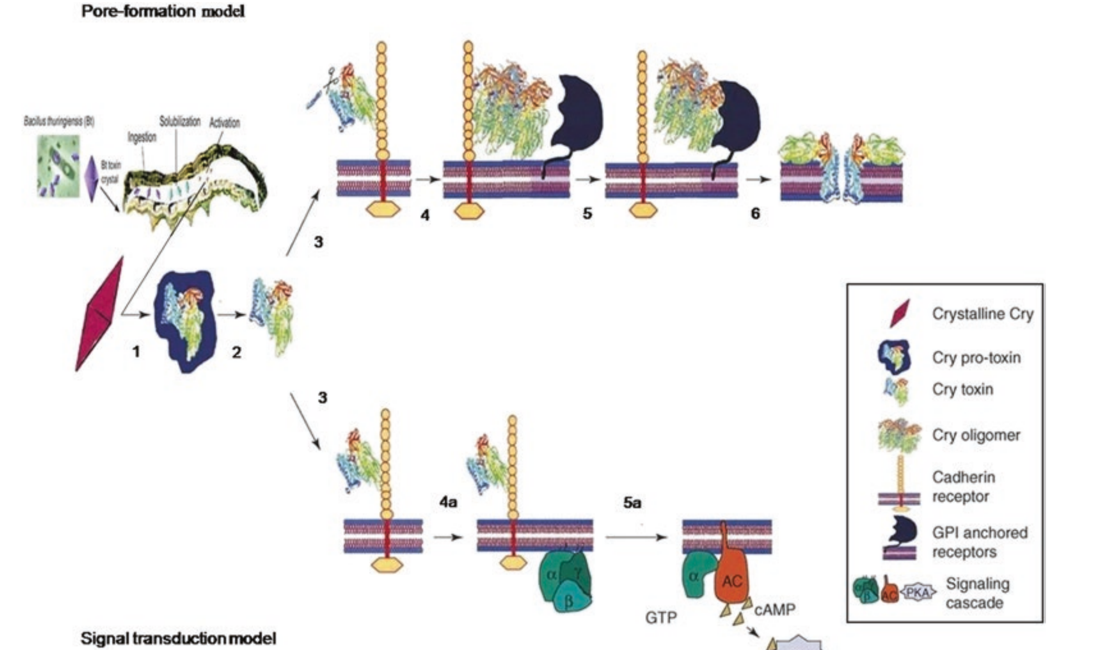


Fig. 2.5 Models of the mode of action of Cry toxins and resulting mechanism for resistance (Bravo and Soberon 2008). Two different mechanisms can be distinguished: the pore formation model (top) and the signal transduction model (bottom), which both include similar initial steps for toxin solubilization in midgut lumen (*1*) activation by midgut proteases (*2*) and binding to primary receptor cadherin. In the pore formation model (top), step (*3*) induces the cleavage of helix α-1 and triggers toxin oligomerization (*4*) the toxin oligomer then binds to a secondary receptor, such as aminopeptidase or alkaline phosphatase, which are anchored by a glycosylphosphatidylinositol anchor in the membrane, and (*5*) finally, the toxin inserts itself into the membrane, thereby forming a pore that kills the insect cells. (*6*) The signal transduction model (bottom) proposes that the interaction of the Cry toxin with a cadherin receptor triggers an intracellular cascade pathway that is mediated by activation of protein G (*4a*) which, in a subsequent step (*5a*), activates adenylyl cyclase. This signal then increases the levels of cyclic adenosine monophosphate, which activates protein kinase A and leads to cell death

Table 2.4 Agriculturally important plants transformed with *Bt*-genes for insect resistance

Crop	Botanical name	Gene	Useful trait	Expression	Reference(s)
	Arachis hypogea	*cry1Ac*	Efficacy against lesser cornstalk borer	0.18%	Singsit et al. (1997)
	Brassica napus	*cry1Ac*	Resistance to *H. zea* Boddie and *S. exigua* Hubner	0.4%	Stewart Jr et al. (1996)
	Brassica oleracea	*cry1Ab*	Resistance to diamond back moth larvae	0.5.ng g^{-1} f.w.	Cao et al. (2001), Bhattacharya et al. (2002)
		cry1C	*Plutella xylostella*	–	Zhao et al. (2001)
	Cajanus cajan	*cry1EC*	Resistance to *Spodoptera litura*	–	Surekha et al. (2005)
		cry1Ab	Protection from *Helicoverpa armigera*	–	Verma and Chand (2005)
		cry1Ab	Protection from *H. armigera*	–	Sharma et al. (2006)
	Cicer arietinum	*cry1Ac*	Resistance against pod borer *Heliothis armigera*	0.003%	Kar et al. (1997)
		cry1Ac	Pod borer insect *H. armigera*	14.5–23.5 ng. Mg^{-1}	Sanyal et al. (2005)
		cry1Ac	Protection from *H. armigera* and *S. litura*	6–20 ng.Mg^{-1}	Indurker et al. (2010)
	Coffea canephera/ Coffea arabica	*cry1Ac*	Resistance to leaf miner	>0.1%	Leroy et al. (2000)
	Glycine max	*cry1Ac*	Resistance to bollworm (*H. zea Boddie* Boddie) and bud worm (*H. virescens* F.)	0.02%	Stewart Jr et al. (1996)
	Gossypium hirsutum	*cry1Ab* *cry1Ac*	Resistance to cotton bollworm (*H. armigera* Hubner)	0.05–0.1%	Perlak et al. (1990)
		cry2Ab	Resistance to pinkboll worm (*Pectinophora gossypiella*)	–	Tabashnik et al. (2002)

(continued)

Table 2.4 (continued)

Crop	Botanical name	Gene	Useful trait	Expression	Reference(s)
	Ipomoea batatas	*cryIIIA δ-endotoxin*	Resistance against sweet potato weevil (*Cylas formicarius*)	–	Morán et al. (1998)
	Lycopersicon esculentum	*cry1Ac*	Resistance to tobacco hornworm (*Manduca sexta* L.)	0.001%	Fischhoff et al. (1987)
		Bt(k)	Resistance to tobacco hornworm (*M. sexta* L.), tomato pinworm (*Keiferia lycopersicella*) and tomato fruit worm (*Heliothis zea*)	1 ng mg^{-1} TSP	Delannay et al. (1989)
		cry1Ac	Resistance to fruitworm (*H. armigera* Hubner)	0.06–0.42%	Mandaokar et al. (2000)
		cry1Ab	Protection against fruitborer (*H. armigera* Hubner)	–	Kumar and Kumar (2004)
	Meidcago sativa	*cryIC*	Resistance to *S. litura* and *S. exigua*	0.01–2%	Strizhov et al. (1996)
	Nicotiana tabacum	*cry1Aa*	Resistance to tobacco hornworm (*M. sexta* L.)	–	Barton et al. (1987)
		δ-Endotoxin var. kurstaki HD1	Resistance to lepidopteran insects	–	Barton et al. (1987)
		cry1Ab	Resistance to tobacco hornworm (*M. sexta* L.) and budworm (*H. virescens* Fabricius)	0.001%	Vaeck et al. (1987)
		cry1Ac	Resistance to tobacco hornworm (*M. sexta* L.)	0.03%	Perlak et al. (1991)
		cry1Ab	Resistance to lepidopteran pests	400 ng^{-1}μg g^{-1}f.w.	Carozzi et al. (1992)
		cry1Ac	Resistance to tobacco budworm (*H. virescens* Fabricius)	3–5%	McBride et al. (1995)
		cry1C	Resistance to *S. litura* and *S. exigua*	0.01–0.2%	Strizhov et al. (1996)

(continued)

Table 2.4 (continued)

Crop	Botanical name	Gene	Useful trait	Expression	Reference(s)
		cry1Ia5	Protection against *Heliothis armigera*	0.06%	Selvapandiyan et al. (1998)
		cry1Aa2	Resistance to *H. virescens, H. zea, S. exigua*	2–3%	Kota et al. (1999)
		cry2Aa2	Resistance to cotton bollworm (*H. zea* Boddie)	35.5%	DeCosa et al. (2001)
		δ-Endotoxin	Control of polyphagous pest *S. litura*	–	Singh et al. (2004)
		cry2Aa2	Effective control of *H. virescens*	0.21%	Zaidi et al. (2005)
		cry1Ac	Control of *H. virescens* and *M. sexta*	0.083%	Gulbitti-Onarici et al. (2009)
	Oryza sativa	*cry1Ab*	Resistance to striped stem borer (*Chilo suppressalis* Walker), and leaf folder (*Cnaphalocrocis medinalis* Guenee)	0.05%	Fujimoto et al. (1993)
		cry1Ac	Resistance to yellow stem borer (*S. incertulas* Walker)	–	Nayak et al. (1997)
		cry1Ab	Resistance to yellow stem borer (*S. incertulas*)	–	Wu et al. (1997)
		cry1Ab/Ac	Resistance to striped stemborer & yellow stem borer	3%	Cheng et al. (1998)
		cry1Ab	Resistance to yellow stem borer (*S. incertulas* Walker)	–	Datta et al. (1998)
		cry2A	Effective control of yellow stemborer and rice leaf folder	5%	Maqbool et al. (1998)
		cry1B	Resistance to striped stem borer	0.01–0.4%	Breitler et al. (2004)

(continued)

Table 2.4 (continued)

Crop	Botanical name	Gene	Useful trait	Expression	Reference(s)
		cry1Ab	Resistance to 8 lepidopteran rice pests	1%	Shu et al. 2000
		cry1Ab *cry1Ac* Hybrid	Resistance to leaf folder (*C. medinalis* Guenee) and yellow Stem borer (*S. incertulas* Walker)	0.01–0.2%	Tu et al. (2000)
		cry1Ab	Resistance to eight lepidopteran rice pests	–	Shu et al. (2000)
		cry1Ac	Resistance to yellow stem borer (*S. incertulas*)	0.1%	Khanna and Raina (2002)
		cry1Ab	Resistance to rice leaffolder *C. medinalis*	–	Ye et al. (2003)
		cry1Ab/Ac	Resistance to stem borer	–	Ramesh et al. (2004)
		cry2A	Resistance to lepidopteran rice pest	9.65–12.11 μg g^{-1}f.w.	Chen et al. (2005)
	Populus tremuloides	*cry1Aa*	Resistance to forest tent caterpillar (*Malacosoma disstria* Lasiocampidae) and gypsy moth	–	McCown et al. (1991)
		cry3A	*Chrysomela tremulae* F. (Col.)	–	Cornu et al. (1996)
	Saccharum officinarum	*cry1Ab*	Resistance to stem borer (*Diatraea saccharalis* F.)	–	Arencibia et al. (1997)
		cry1Ac	Control against stemborer in field trials	50 ng mg^{-1} TSP	Weng et al. (2010)
	Solanum melongena	*cryIIIb*	Resistance to Colorado potato beetle (*Leptinotarsa decemlineata* Say)	–	Arencibia et al. (1997)
		cryIIIA	Resistance to Colorado potato beetle (*L. decemlineata* Say)	–	Jelenkovic et al. (1998)

(continued)

Table 2.4 (continued)

Crop	Botanical name	Gene	Useful trait	Expression	Reference(s)
		cry1Ab	Significant insecticidal activity against *Leucinodes orbonalis*	0.02%	Kumar et al. (1998)
	Solanum tuberosum	*cry1Ab*	Resistance to tuber moth (*Phthorimaea operculella* Zeller)	–	Peferoen et al. (1990); Rico et al. (1998)
		cryIIIA	Tolerance to Colorado beetle (*L. decemlineata* Say)	–	Adang et al. (1993); Perlak and Fischhoff (1993); Coombs et al. (2002)
		*cry*V Bt *cry1Ab*	Resistance to potato tuber moth (*P. operculella* Zeller)	–	Douches et al. (1998)
		cry9Aa2	Resistance to potato tuber moth	–	Gleave et al. (1998)
		cry1Ab	Resistance to *H. armigera*	0.005–0.04%	Chakrabarti et al. (2000)
		cry1Ac	Resistance to *Tecia solanivora*	0.02–17 μg g^{-1} f.w.	Valderrama et al. (2007)
	Vigna aconitifolia	*cry1Ac*	Protection from *H. armigera*	–	Kamble et al. (2003)
	Zea mays	*cry1Ab*	Resistance to European corn borer (*Ostrinia nubilalis* Hubner)	0.4%	Koziel et al. (1993)
		cry1Ab	Resistance to *O. nubialis*	14–213 ng g^{-1} f.w.	Fearing et al. (1997)
		cry1Ab	Protection against *S. littoralis*	46.8–85.3 $ngcm^{-2}$	Dutton et al. (2005)

Table 2.5 Global status of approved and commercially available Bt-crops

Crop	Transgenic event(s)	Trade name	Company	Trait genes	Trait targets
Cotton	LLCotton25, MON 15985	FiberMax Libertylink Bollguard II*	Bayer cropScience	*bar*, *cry 1Ac*, *cry2Ab*	Lepidopteran pests, weeds
Cotton	DAS-21023-5, DAS-24236-5	Wide strike*	Dow AgroSciences	*pat*, *cry1Ac*, *cry1Fa*	Lepidopteran pests, weeds
Cotton	DAS-21023-5, DAS-24236-5, MONO1445-2	Wide strike* Roundup/Ready*	Dow AgroSciences	*pat*, *cry1Ac*, *cry1Fa*, *CP4EPSPS*	Lepidopteran pests, weeds
Cotton	DAS-21023-5, DAS-24236-5, MON88913-8	Wide strike*/ Roundup Ready* Flex	Dow AgroSciences	*pat*, *cry1Ac*, *cry1Fa*, *CP4EPSPS*	Lepidopteran pests, weeds
Cotton	MON531, MON1445-2	Roundup Ready*, Bollguard	Monsanto	*cry1Ac*, *CP4EPSPS*	Lepidopteran pests, weeds
Cotton	MON88913-8, MON15985	Bollguard II*, Roundup/Ready* Flex	Monsanto	*CP4EPSPS*, *cry1Ac*, *cry1Ab*	Lepidopteran pests, weeds
Maize	TC1507	Herculex CB	Dow AgroSciences and Pioneer Hi Bred	*cry1Fa*, *pat*	Lepidopteran pests (European corn borer), weeds
Maize	TC1507	Herculex CB	Dow AgroSciences and Pioneer Hi Bred	*cry1Fa*, *pat*	Lepidopteran pests (European corn borer), weeds
Maize	DAS-59122-7	Herculex RW	Dow AgroSciences and Pioneer Hi Bred	*cry34Ab/cry35Ab1*, *pat*	Coleopteran pests (corn rootworms), weeds

Crop	Transgenic event(s)	Trade name	Company	Trait genes	Trait targets
Maize	TC1507, DAS-59122-7	Herculex XTRA	Dow AgroSciences and Pioneer Hi Bred	*cry1Fa*, *cry34Ab1*, *cry35Ab1*, *pat*	Lepidopteran and coleopteran pests, weeds
Maize	DAS-59122-7, TC1507, NK603	Herculex XTRA/ Roundup/Ready∗ 2	Dow AgroSciences and Pioneer Hi Bred	*pat*, *CP4EPSPS*, *cry34Ab1*, *cry35Ab1*, *cry1Fa 2*	Lepidopteran and coleopteran pests, weeds
Maize	MON89034	Yield guard VT pro	Monsanto	*cry1A105*, *cry2Ab2*	Lepidopteran pests
Maize	MON88017	Yield guard VT	Monsanto	*CP4EPSPS*, *cry3Bb1*	Coleopteran pests (corm rootworms), weeds
Maize	MON810, MON88017	Yield guard VT Triple	Monsanto	*cry1A105*, *cry2Ab2*, *cry3Bb*	Lepidopteran and coleopteran pests, weeds
Maize	MON89034, MON88017	Genuity VT Triple Pro	Monsanto	*cry1A105*, *cry2Ab2*, *cry3Bb*	Lepidopteran and coleopteran pests, weeds
Maize	MON89034, TC1507, MON88017, DAS-59122-7	Genuity Smartstax TM	Monsanto and Dow AgroSciences	*pat*, *CP4EPSPS*, *cry 1Fa2*, *cry1A105*, *cry2Ab*, *cry3Bb1*, *cry34Ab1*, *cry35Ab1*	Lepidopteran and coleopteran pests, weeds
Maize	BtII, GA21	Agrisure GT/CB/ LL	Syngenta	*cry1Ab*, *pat*, mutant maize *EPSPS*	Lepidopteran pests (European corn borer), weeds
Maize	BtII, MIR604	Agrisure CB/LL/ RW	Syngenta	*cry1Ab*, m*cry3Aa*, *pat*	Lepidopteran and coleopteran pests, weeds
Maize	GA21, BtII, MIR604	Agrisure 3000GT (GT/CB/LL/RW)	Syngenta	*pat*, *cry1Ab*, m*cry3Aa*, mutant maize *EPSPS*	Lepidopteran and coleopteran pests, weeds

2.9 Management of Resistance Development

Since most of the insect-resistant transgenic plants released for commercial cultivation harbour single insecticidal Bt-*cry* gene and the target insect populations are consistently being exposed to the single toxin protein, the possibility of insects evolving resistance to single Bt-toxin is high (Zhao et al. 2005; Gunning et al. 2005). There are reports on development of resistance to *cry1Ab* in open field populations of the diamond black moth, *Plutella xylostella* (Tabashnik et al. 1993; Ballester et al. 1994) and resistance to *cry1Aa*, *cry1Ab*, *cry1Ac* and *cry1F* have been reported in laboratory selection experiments (Tabashnik et al. 1997). In recent years, several Bt-cotton hybrid lines expressing *cry1Ac* have been approved for commercial cultivation in India, and due to small farm holdings, diverse cropping system and immigration of insects to alternative hosts, the possibility of developing heterogeneous insect population is very high (James 2012). Moreover, pink bollworm resistant to Bt-cotton harbouring the Bt-*cry1Ac* gene has been reported in the fields in India, where farmers rarely follow the refugia strategy (Tabashnik et al. 2010). Several strategies have been proposed for the management of resistance development in field insects, including the application of diverse mixture of toxins, high expression of Bt-toxin, weedy refugia, hybrid and pyramiding of different Bt-toxin genes and use of sterile insect (Gatehouse 2008; Tabashnik et al. 2010). Few key reports that have demonstrated the beneficial aspect of gene pyramiding in transgenic plants have been summarized in Fig. 2.6.

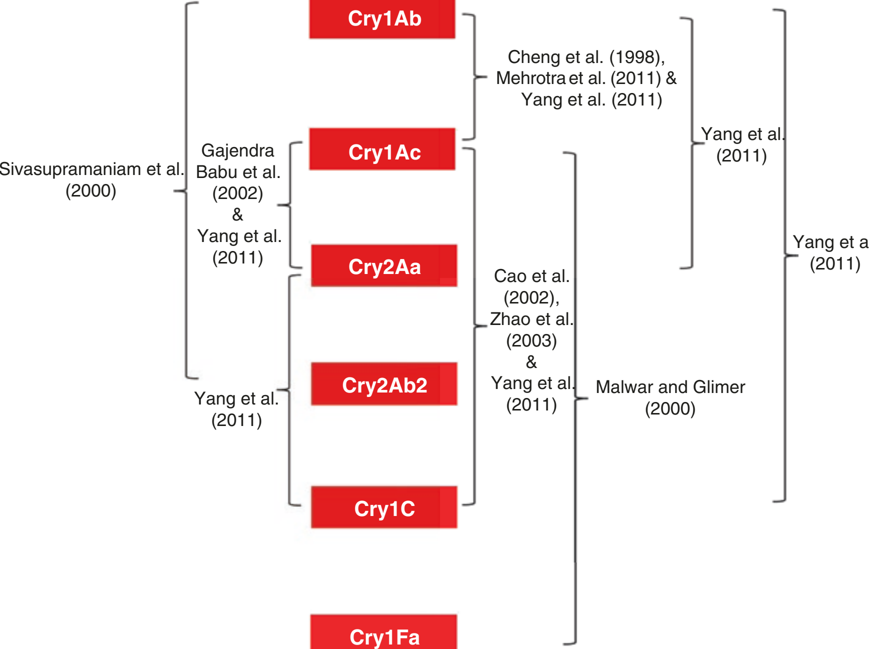


Fig. 2.6 Bt-gene pyramiding as a preventive and resistance management strategy

In recent years, transgenic plants expressing two dissimilar insect toxins have been developed, and the most successful example is Bt-cotton 'Bollgard II' expressing *cry1Ac* and *cry2Ab2* genes (Perlak et al. 2001; Zhao et al. 2005). The efficacy and sustainability of transgenic plants towards prevention of resistance development in insects rely on the pyramiding and co-expression of two or more diverse transgenes, without affecting the yield parameters (Zhao et al. 2003; Gatehouse 2008).

2.10 Conclusions

The transfer of Bt-*cry* gene(s) into plants has provided potentially powerful alternative strategies for the protection of crops against major agricultural field insects. The toxin encoded by *cry1A* gene(s) is highly effective against Lepidopteran group of insects that causes major damages to crop plants. A comparative interaction of Bt-toxins Cry1Aa, Cry1Ab and Cry1Ac encoded by corresponding genes with larval midgut binding sites (receptors) of *Helicoverpa armigera* has shown their competition for common binding sites to different epitopes of the receptors in the order of Cry1Ac > 1Ab > 1Aa and exerting corresponding toxicity (Estela et al. 2004; Bravo et al. 2007).

The *cry1Ac* gene has been extensively modified and codon optimized along with other modifications for over-expression in different plant species like tobacco, cotton, tomato, potato, chickpea and rice (Sharma et al. 2004; Ferry et al. 2004). The most successful story is the commercialization of transgenic cotton expressing the *cry1Ac* gene as Bollgard I in 1996 and Bollgard II with *cry1Ac* and *cry2Ab* in the year 2000 that has offered significant benefits over the application of synthetic insecticides and yield to the farmers (Perlak et al. 2001). The expression of native (wild type) full-length *cry1Ac* gene in plants was very low due to instability and premature termination of transcript (DeRocher et al. 1998; Perlak et al. 1990). Several modifications have been incorporated in the *cry* gene for over-expression, and the major breakthrough has been in the designing of synthetic versions of the gene with codon modifications to remove the putative polyadenylation sequences and use of plant preferred codons for high-level expression in plants (Perlak et al. 1990).

The 5′ and 3′ UTR leader sequences play an important role in transgene expression by regulating transcription and translation initiation of the foreign gene (Tyc et al. 1984; Lu et al. 2008). In particular, the use of viral leaders 5′ UTR has shown to greatly increase the accumulation of recombinant proteins (Dowson Day et al. 1993). The most preferred are tobacco mosaic virus Ω sequence (TMV), tobacco etch virus (TEV) and alfalfa mosaic virus (AMV) leader sequences (Datla et al. 1993; Gallie et al. 1995; Wang et al. 2001) which have been used for optimization of expression of several foreign proteins in plants (Haq et al. 1995; Agarwal et al. 2008; Wang et al. 2008). The 3′ UTR contains message for transcript polyadenylation that directly affects mRNA stability (Chan and Yu 1998). Heterologous 3′ UTR from plant or plant viruses have been used to stabilize the transcript formation (Hood et al. 1997; Staub et al. 2000; Ko et al. 2003).

The use of a synthetic truncated version (1.85 kb) of the *cry* genes coding toxin portion has been demonstrated to be most effective for Bt-transgenics against Lepidopteran insects (Perlak et al. 1990; Sardana et al. 1996). However, the most promising transgenic event of cotton (Monsanto 531) which has been commercialized is developed with full-length modified *cry1Ac* gene (Perlak et al. 2001; Purcell et al. 2004). All insect-resistant transgenic cotton varieties derived from this single event are performing well under field conditions in different agroclimatic regions across the globe (James 2012). Interestingly, development of stable transgenic plants of tomato expressing Cry1Ab toxin has been documented for insect protection (Kumar and Kumar 2004; Fischhoff et al. 1987; Srivastava 2007). Moreover, frequency and recovery of promising transgenic plant expressing Bt-toxin coded by full-length gene is also extremely low. But a question arises as to why the full-length synthetic gene was used, while the initial trials were performed with its truncated version? The answer to this is the modified full-length Cry1Ac toxin, although exhibits lower expression levels, efficiently induces oligomerization, prepore formation and insecticidal activity compared to modified truncated Cry1Ac toxin, at higher expression levels. These results suggest the importance of modified full-length *cry1Ac* gene for stability and integrity of the insect-resistance trait compared to truncated version of *cry1Ab* or *cry1Ac* gene(s) alone (Koul 2013). In reality, the commercially released Bt-cotton was developed with full-length *cry1Ac*-like gene whose nucleotide alignment study revealed that 'Monsanto 531' *cry* gene sequence is a hybrid gene where the sequence 1–1398 bp is that of *cry1Ab* gene. It was done in order to provide a blend of binding characteristics offered by Cry1Ab as well as pore formation characteristics offered by Cry1Ac, in the aforementioned successful *cry1Ac*-like gene, for raising transgenic cotton and its commercialization.

References

Adang MJ, Brody MS, Cardineau G, Eagan N, Roush RT, Shewmaker CK, Jones A, Oakes JV, McBride KE (1993) The reconstruction and expression of a *Bacillus thuringiensis* cryIIIA gene in protoplasts and potato plants. Plant Mol Biol 21:1131–1145

Agarwal S, Singh R, Sanyal I, Amla DV (2008) Expression of modified gene encoding functional human *α*-1-antitrypsin protein in transgenic tomato plants. Transgenic Res 17:881–896

Alam MF, Datta K, Abrigo E, Vasquez A, Senadhira D, Datta SK (1998) Production of transgenic deepwater indica rice plants expressing a synthetic *Bacillus thuringiensis* cry1A(b) gene with enhanced resistance to yellow stem borer. Plant Sci 135:25–30

Alam MF, Datta K, Abrigo E, Oliva N, Tu J, Virmani SS, Datta SK (1999) Transgenic insect-resistant maintainer line (IR 68899B) for improvement of hybrid rice. Plant Cell Rep 18:572–575

Arenas I, Bravo A, Soberon M, Gomez I (2010) Role of alkaline phosphate from *Manduca sexta* in the mechanism of action of *Bacillus thuringiensis* Cry1Ab toxin. J Biol Chem 258:12497–12503

Arencibia A, Vázquez RI, Prieto D, Téllez P, Carmona ER, Coego A, Hernández L, De la Riva GA, Selman-Housein G (1997) Transgenic sugarcane plants resistant to stem borer attack. Mol Breed 3(4):247–255

Aronson AI, Shai Y (2001) Why *Bacillus thuringiensis* insecticidal toxins are so effective: unique features of their mode of action. FEMS Microbiol Lett 195:1–8

Bajwa WI, Kogan M (2001) *Bacillus thuringiensis* based biological control of insect pests. http://www.ppc.orst.edu/dir/microbial/bt/

Ballester V, Escriche B, Mensua JL, Riethmacher GW, Ferre J (1994) Lack of cross-resistance to other *Bacillus thuringiensis* crystal proteins in a population of *Plutella xylostella* highly resistant to *cry*1A(b). Biocontrol Sci Tech 4:437–443

Barton KA, Whiteley HR, Yang NS (1987) *Bacillus thuringiensis* δ-endotoxin expressed in transgenic *Nicotiana tabaccum* provides resistance to lepidopteran insects. Plant Physiol 85:1103–1109

Bevan MW, Flavell RB, Chilton MD (1983) A chimaeric antibiotic resistance gene as a selectable marker for plant cell transformation. Nature 304:184–187

Bhattacharya N, Bhat SR, Kirti PB, Chopra VL (2002) Development of insect-resistant transgenic cabbage plants expressing a synthetic *cry1A(b)* gene from *Bacillus thuringiensis*. Curr Sci 83:146–150

Birch RG (1997) Plant transformation: problems and strategies for practical application. Annu Rev Plant Physiol Plant Mol Biol 48:297–326

Bohorova N, Frutos R, Royer M, Estanol P, Pacheco M, Rascon Q, Mclean S, Hoisington D (2001) Novel synthetic *Bacillus thuringiensis cry1B* gene and cry1B-*cry1Ab* translational fusion confer resistance to south western corn borer, sugarcane borer and fall army worm in transgenic tropical maize. Theor Appl Genet 103:817–826

Boonserm P, Davis D, Ellar J, Li J (2005) Crystal structure of the mosquito-larvicidal toxin Cry4Ba and its biological implications. J Mol Biol 348:363–382

Boonserm P, Mo M, Angsuthanasombat C, Lescar J (2006) Structure of the functional form of the mosquito larvicidal Cry4Aa toxin from *Bacillus thuringiensis* at a 2.8-angstrom resolution. J Bacteriol 188:3391–3401

Boulter D (1993) Insect pest control by copying nature using genetically-engineered crops. Biochemistry 34:1453–1466

Bravo A, Soberon M (2008) How to cope with insect resistance to Bt toxins? Cell 26:573–579

Bravo A, Gomez I, Conde J, Munoz-Garay C, Sanchez J, Miranda R, Zhuang M, Gill SS, Soberon M (2004) Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. Biochem Biophys Acta 1667:38–46

Bravo A, Gill SS, Soberon M (2007) Mode of action of *Bacillus thuringiensis* cry and Cyt toxins and their potential for insect control. Toxicon 49(4):423–435

Bravo A, Likitvivatanavong S, Gill SS, Soberon M (2011) *Bacillus thuringiensis*: a story of a successful bioinsecticides. Insect Biochem Mol Biol 4:423–431

Breitler JC, Vassal JM, del Mar Catala M, Meynard D, Marfà V, Melé E, Royer M, Murillo I, San Segundo B, Guiderdoni E, Messeguer J (2004) Bt rice harbouring *cry* genes controlled by a constitutive or wound-inducible promoter: protection and transgene expression under Mediterranean field conditions. Plant Biotechnol J 2(5):417–430

Broderick NA, Raffa KF, Handelsman J (2006) Midgut bacteria required for *Bacillus thuringiensis* insecticidal activity. Proc Natl Acad Sci U S A 103:15196–15199

Cao J, Shelton AM, Earle ED (2001) Gene expression and insect resistance in transgenic broccoli containing a *Bacillus thuringiensis cry1Ab* gene with the chemically inducible PR-1a promoter. Mol Breed 8:207–216

Carozzi NB, Warren GW, Desai N, Jayne SM, Lotstein R, Rice DA, Evola S, Koziel MG (1992) Expression of a chimeric CaMV35S *Bacillus thuringiensis* insecticidal protein gene in transgenic tobacco. Plant Mol Biol 20:539–548

Chakrabarti SK, Mandaokar AD, Shukla A, Pattanayak D, Naik PS, Sharma RP, Kumar PA (2000) *Bacillus thuringiensis cry1Ab* gene confers resistance to potato against *Helicoverpa armigera* (Hubner). Potato Res 43(2):143–152

Chan MT, Yu SM (1998) The 3′ untranslated region of a rice alpha-amylase gene functions as a sugar-dependent mRNA stability determinant. Proc Natl Acad Sci U S A 95:6543–6547

Chen M, Liu X, Wang Z, Song J, Qi Q, Wang PG (2005) Modification of plant *N*-glycans processing: the future of producing therapeutic protein by transgenic plant. Med Res Rev 25:343–360

Cheng J, Bolgard MG, Saxena RC, Sticklen MB (1992) Production of insect resistant potato by genetic transformation with a delta-endotoxin gene from *Bacillus thuringiensis* var. kurstaki. Plant Sci 81:83–91

Cheng X, Sardana R, Kaplan H, Altosaar I (1998) *Agrobacterium*-transformed rice plants expressing synthetic *cry1A(b)* and *cry1A(c)* genes are highly toxic to striped stem borer and yellow stem borer. Proc Natl Acad Sci U S A 95:2767–2772

Coombs J, Douches D, Li W, Grafius E, Pett W (2002) Combining engineered (Bt-cry3A) and natural resistance mechanisms in potato for control of Colorado potato beetle. J Am Soc Hort Sci 127:62–68

Cornu D, Leple JC, Bonade M, Ross A, Augustin S, Delplanque A, Jouanin L, Pilate G (1996) Expression of proteinase inhibitor and a *Bacillus thuringiensis* δ-endotoxin in transgenic poplars. In: Proceedings IUFRO meeting on somatic cell genetics and molecular genetics of trees. Kluwer, Dordrecht, pp 131–136

Crickmore N, Ziegler DR, Feitelson J, Schnepf E, Van Rie J, Lereclus D, Baum J, Dean DH (1998) Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. Microbiol Mol Biol Rev 62:807–813

Crickmore N, Zeigler DR, Schnepf E, Van Rie J, Lereclus D, Baum J, Bravo A, Dean DH (2011) *Bacillus thuringiensis* toxin nomenclature. http://www.lifesci.sussex.ac.uk/home/Neil_Cricknore/Bt/index.html

Datla RS, Bekkaoui F, Hammerlindl JK, Pilate G, Dunstan DI, Crosby WL (1993) Improved high level constitutive foreign gene expression in plants using an AMV RNA4 untranslated leader sequence. Plant Sci 94:139–149

Datta K, Vasquez A, Tu J, Torrizo L, Alam MF, Oliva N, Abrigo E, Khush GS, Datta SK (1998) Constitutive and tissue-specific differential expression of *cry1A(b)* gene in transgenic rice plants conferring resistance to rice insect pests. Theor Appl Genet 97:20–30

DeCosa B, Moar W, Lee SB, Miller M, Daniell H (2001) Over-expression of the Bt *cry*2Aa2 operon in chloroplasts leads to transformation of insecticidal crystals. Nat Biotechnol 19:71–74

Delannay X, LaVallee BJ, Proksch RK, Fuchs RL, Sims SR, Greenplate JT, Marrone PG, Dodson RB, Augustine JJ, Layton JG, Fischhoff DA (1989) Field performance of transgenic tomato plants expressing the *Bacillus thuringiensis* var kurstaki insect control protein. Nat Biotechnol 7:1265–1269

DeMaagd RA, Bravo A, Crickmore N (2001) How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. Trends Genet 17:193–199

DeRocher EJ, Vargo-Gogola TC, Diehn SH, Green PJ (1998) Direct evidence for rapid degradation of *Bacillus thuringiensis* toxin mRNA as a cause of poor expression in plants. Plant Physiol 117:1445–1461

Diehn SH, De Rocher EJ, Green PJ (1996) Problems that can limit the expression of foreign genes in plants: lessons to be learned from *B.t.*-toxin genes. In: Setlow JK (ed) Genetic engineering: principles and methods, vol 18. Plenum Press, New York, pp 83–99

Diehn SH, Chiu W-L, DeRocher EJ, Green PJ (1998) Premature polyadenylation at multiple sites within a *Bacillus thuringiensis* toxin gene-coding region. Plant Physiol 117:1433–1443

Douches DS, Westedt AL, Zarka K, Schroeter B, Grafius EJ (1998) Potato transformation to combine natural and engineeered resistance for controlling tuber moth. Hort Sci 33:1053–1056

Dowson Day MJ, Ashurst JL, Mathias SF, Watts JW, Wilson TM, Dixon RA (1993) Plant viral leaders influence expression of a reporter gene in tobacco. Plant Mol Biol 23:97–109

Dutton A, Romies J, Bigler F (2005) Effects of Bt maize expressing *cry1Ab* and Bt spray on *Spodoptera littoralis*. Entomol Exp Appl 114:161–169

Estela A, Escriche B, Ferre J (2004) Interaction of *Bacillus thuringiensis* toxins with larval midgut binding sites of *Helicoverpa armigera* (Lepidoptera: Noctuidae). App Environ Microbiol 70:1378–1384

Fearing PL, Brown D, Vlachos D, Meghji M, Privalle L (1997) Quantitative analysis of CryIA(b) expression in B.t. maize plants, tissues, and silage and stability of expression over successive generations. Mol Breed 3:169–176

Feitelson JS, Payne J, Kim L (1992) *Bacillus thuringiensis*: insect and beyond. Biotech 10:271–275

Ferry N, Edwards MG, Gatehouse JA, Gatehouse AMR (2004) Plant-insect interactions: molecular approaches to insect resistance. Curr Opin Biotechnol 15:155–161

Fischhoff DA, Bowdish KS, Perlak FJ, Marrone PG, McCormick SM, Niedermeyer JG, Dean DA, Kusano-Kretzmer K, Mayer EJ, Rochester DE, Rogers SG, Fraley RT (1987) Insect tolerant transgenic tomato plants. Nat Biotechnol 5:807–813

Fujimoto H, Itoh K, Yamomoto M, Kyojuka J, Shimamoto K (1993) Insect resistant rice generated by introduction of a modified δ-endotoxin gene of *Bacillus thuringiensis*. Nat Biotechnol 11:1151–1155

Galitsky N, Cody V, Wojtczak A, Ghosh D, Luft JR, Pangborn W, English L (2001) Structure of the insecticidal bacterial δ-endotoxin Cry3Bb1 of *Bacillus thuringiensis*. Acta Cryst D 57:1101–1109

Gallie DR, Tanguay RL, Leathers V (1995) The tobacco etch viral 5′ leader and poly(A) tail are functionally synergistic regulators of translation. Gene 165:233–238

Gatehouse JA (2008) Biotechnological prospects for engineering insect-resistant plant. Plant Physiol 146:881–887

Gatehouse AMR, Davidson GM, Newell CA, Merryweather A, Hamilton WDO, Burgess EPJ, Gilbert RJC, Gatehouse JA (1997) Transgenic potato plants with enhanced resistance to the tomato moth *Laccanobia oleracea*: growth room trials. Mol Breeding 3:49–63

Gerber D, Shai Y (2000) Insertion and organization within membranes of delta-endotoxin pore-forming domain, helix-4-loop-helix 5 and inhibition of its activity by a mutant helix 4 peptide. J Biol Chem 275:23602–23607

Gill SS, Cowles EA, Pietrantonio FV (1992) The mode of action of *Bacillus thuringiensis* endotoxins. Annu Rev Entomol 37:615–636

Gleave AP, Mitra DS, Markwick NP, Morris BAM, Beuning LL (1998) Enhanced expression of the *Bacillus thuringiensis cry9Aa2* gene in transgenic plants by nucleotide sequence modification confers resistance to potato tuber moth. Mol Breed 4:459–472

Gómez I, Arenas I, Benitez I, Miranda-Rios J, Becerri B, Grande R, Almagro JC, Bravo A, Soberon M (2006) Specific epitopes of domain II and III of *Bacillus thuringiensis* Cry1Ab toxin involved in the sequential interaction with cadherin and aminopeptidase-N receptors in *Manduca sexta*. J Biol Chem 281:34032–34039

Gonzalez JM, Dulmage HT Jr, Carlton BC (1981) Correlation between specific plasmids and delta-endotoxin production in *Bacillus thuringiensis*. Plasmid 5:351–365

Gonzalez JM, Brown BJ Jr, Carlton BC (1982) Transfer of Bacillus thuringiensis plasmids coding for delta-endotoxin among strains of *B. thuringiensis* and *B. cereus*. Proc Natl Acad Sci U S A 79:6951–6955

Gordon-Kamm WJ, Spencer TM, Mangano ML, Adams TR, Daines RJ, Start WG, O'Brien JV, Chambers SA, Adams WR, Willetts NG, Rice TB, Mackey CJ, Krueger RW, Kausch AP, Lernaux PG (1990) Transformation of maize cells and regeneration of fertile transgenic plants. Plant Cell 2:603–618

Grochulski P, Masson L, Borisova S, Pusztai-Carery M, Schwartz JL, Brousseau R, Cygler M (1995) Bacillus thuringiensis Cry1A(a) insecticidal toxin: crystal structural and channel formation. J Mol Biol 254:447–464

Gulbitti-Onarici S, Zaidi MA, Taga I, Ozcan S, Altosaar I (2009) Expression of Cry1Ac in transgenic tobacco plants under the control of a wound-inducible promoter (AoPR1) isolated from *Asparagus officinalis* to control *Heliothis virescens* and *Manduca sexta*. Mol Biotechnol 42(3):341–349

Gunning RV, Dang HT, Kemp FC, Nicholson IC, Moores GD (2005) New resistance mechanism in *Helicoverpa armigera* threatens transgenic crops expressing *Bacillus thuringiensis* Cry1Ac toxin. Appl Environ Microbiol 71:2558–2563

Guo S, Ye S, Liu Y, Wei L, Xue J, Wu H, Song F, Zhang J, Wu X, Huang D, Rao Z (2009) Crystal structure of *Bacillus thuringiensis* Cry8Ea1: an insecticidal toxin toxic to underground pests, the larvae of *Holotrichia parallela*. J Struct Biol 168:259–266

Haider MZ, Ellar DJ (1987) Analyses of the molecular basis of insecticidal specificity of *Bacillus thuringiensis* crystal delta-endotoxin. Biochem J 248:197–201

Haq TA, Mason HS, Clements JD, Artnzen CJ (1995) Oral immunization with recombinant bacterial antigen produced in transgenic plants. Science 268:714–716

Hilder VA, Boulter D (1999) Genetic engineering of crop plants for insect resistance-a critical review. Crop Prot 18:177–191

Ho NH, Baisakh N, Oliva N, Datta K, Frutos R, Datta SK (2006) Translational fusion hybrid Bt genes confer resistance against yellow stem borer in transgenic Elite Vietnamese rice (*Oryza sativa* L.) cultivars. Crop Sci 46:781–789

Hodgman TC, Ellar DJ (1990) Models for the structure and function of the *Bacillus thuringiensis* delta-endotoxins determined by compilational analysis. DNA Seq 1:97–106

Hofmann C, Vanderbruggen H, Hofte H, Van Rie J, Jansens S, Van Mellaert H (1988) Specificity of *Bacillus thuringienis* δ-endotoxin is correlated with the presence of high-affinity binding sites in the brush border membrane of target insect midguts. Proc Natl Acad Sci U S A 85:7844–7848

Hofte H, Whiteley HR (1989) Insecticidal crystal proteins of *Bacillus thuringiensis*. Microbiol Rev 53:242–255

Hood E, Witcher D, Maddock S, Meyer T, Baszezynski C, Bailey M (1997) Commercial production of Avidin from transgenic maize: characterization of transformant, production, processing, extraction and purification. Mol Breed 3:291–306

Hooykaas PJJ, Mozo T (1994) *Agrobacterium* molecular genetics. Plant Molecular Biology Manual B, vol 3. Klwer Academic Publishers, Belgium, pp 1–9

Hua G, Jurat-Fuentes JL, Adang MJ (2004) Bt-R1a extracellular cadherin repeat 12 mediates *Bacillus thuringiensis* Cry1Ab binding and cytotoxicity. J Biol Chem 279:28051–28056

Indurker S, Misra HS, Eapen S (2010) *Agrobacterium*-mediated transformation in chickpea (*Cicer arietinum* L.) with an insecticidal protein gene: optimisation of different factors. Physiol Mol Biol Plants 16(3):273–284

Jafari M, Norouzi P, Malboobi MA, Ghareyazie B, Valizadeh M, Mohammadi SA, Mousavi M (2009) Enhanced resistance to a lepidopteran pest in transgenic sugar beet plants expressing synthetic cry1Ab gene. Euphytica 165:333–344

James C (2012) Global status of commercialized biotech/GM crops. ISAAA Brief No. 44. ISAAA, Ithaca, NY

Jelenkovic G, Billings S, Chen Q, Lashomb J, Hamilton G, Ghidiu G (1998) Transformation of eggplant with synthetic cryIIIA gene produces a high level of resistance to the Colorado potato beetle. J Am Soc Hortic Sci 123:19–25

Kamble S, Misra HS, Mahajan SK, Eapen S (2003) A protocol for efficient biolistic transformation of mothbean *Vigna aconitifolia* L. Jacq. Marechal. Plant Mol Biol Rep 21:457–457

Kar S, Basu D, Das S, RamKrishnan NA, Mukherjee P, Nayak P (1997) Expression of cry1Ac gene of *Bacillus thuringiensis* in transgenic chickpea plants inhibits development of pod borer (*Heliothis armigera*) larvae. Transgenic Res 6:177–185

Khanna HK, Raina SK (2002) Elite Indica transgenic rice plants expressing modified Cry1Ac endotoxin of *Bacillus thuringiensis* show enhanced resistance to yellow stem borer (*Scirpophaga incertulas*). Transgenic Res 11(4):411–423

Knight P, Crickmore N, Ellar DJ (1994) The receptor for *Bacillus thuringiensis* CryIA(c) delta-endotoxin in the brush border membrane of the lepidopteran *Manduca sexta* is aminopeptidase N. Mol Microbiol 11:429–436

Knowles B (1994) Mechanisms of action of *Bacillus thuringiensis* insecticidal endotoxins. Adv Insect Physiol 24:275–308

Knowles BH, Ellar DJ (1988) Differential specificity of two insecticidal toxins from *Bacillus thuringiensis* var aizawai. Mol Microbiol 2:153–157

Ko K, Tekoah Y, Rudd PM, Harvey DJ, Dwek RA, Spitsin S (2003) Function and glycosylation of plant-derived antiviral monoclonal antibody. Proc Natl Acad Sci U S A 100:8013–8018

Kota MDH, Varma S, Gareznski F, Moar WJ (1999) Overexpression of *Bacillus thuringiensis* (Bt) cry2Aa2 protein in chloroplasts confers resistance to plants against susceptible and Bt-resistant insects. Proc Natl Acad Sci U S A 96:1840–1845

Koul B (2013) Expression of insecticidal toxin coded by modified full-length and truncated Bt-*cry1Ac* genes in transgenic tomato for assessment of their stability and efficacy against target insects, PhD thesis. Banasthali Vidyapith, Rajasthan, India

Koziel MG, Beland GL, Bowman C, Carozzi NB, Crenshaw R, Crossland L, Dawson J, Desai N, Hill M, Kadwell S, Launis K, Lewis K, Maddox D, McPherson K, Meghji MR, Merlin E, Rhodes R, Warren GW, Wright M, Evola SV (1993) Field performance of elite transgenic maize plants expressing an insecticidal protein derived from *Bacillus thuringiensis*. Biotech 11:194–200

Kumar H, Kumar V (2004) Tomato expressing Cry1A(b) insecticidal protein from *Bacillus thuringiensis* protected against tomato fruit borer, *Helicoverpa armigera* (Hubner) (Lepidoptera: Noctuidae) damage in the laboratory, greenhouse and field. Crop Prot 23:135–139

Kumar PA, Mandaokar A, Sreenivasu K, Chakrabarti SK, Bisaria S, Sharma SR, Kaur S, Sharma RP (1998) Insect-resistant transgenic brinjal plants. Mol Breed 4:33–37

Leroy T, Henry AM, Royer M, Altosaar I, Frutos R, Duris D, Philippe R (2000) Genetically modified coffee plants expressing the *Bacillus thuringiensis cry1Ac* gene for resistance to leaf miner. Plant Cell Rep 19:382–389

Li J, Carroll J, Ellar DJ (1991) Crystal structure of insecticidal δ-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. Nature 353:815–817

Lu J, Sivamani E, Azhakanandam K, Samadder P, Li X, Qu R (2008) Gene expression enhancement mediated by the 5' UTR intron of the rice rubi3 gene varied remarkably among tissues in transgenic rice plants. Mol Gen Genomics 279:563–572

Ma G (2005) The molecular biology of tolerance to *Bacillus thuringiensis* endotoxin in *Helicoverpa armigera*: a novel mechanism and its genetic transmission. PhD Thesis, The University of Adelaide, Waite Campus, Australia

Mandaokar AD, Goyal RK, Shukla A, Bisaria S, Bhalla R, Reddy VS, Chaurasia A, Sharma I, Altosaar I, Kumar PA (2000) Transgenic tomato plant resistant to fruit borer (*Helicoverpa armigera* Hubner). Crop Prot 19:307–312

Maqbool SB, Husnain T, Riazuddin S, Masson L, Christou P (1998) Effective control of yellow stem borer and rice leaf folder in transgenic rice indica varieties basmati 370 and M7 using the novel δ-endotoxin cry2A *Bacillus thuringiensis* gene. Mol Breed 4(6):501–507

Maqbool SB, Riazuddin S, Loc NT, Gatehouse AMR, Gatehouse JA, Christou P (2001) Expression of multiple insecticidal genes confers board resistance against a range of different rice pests. Mol Breed 7:85–93

Marfà V, Melé E, Gabarra R, Vassal JM, Guiderdoni E, Messeguer J (2002) Influence of the developmental stage of transgenic rice plants (cv. Senia) expressing the cry1B gene on the level of protection against the striped stem borer (*Chilo suppressalis*). Plant Cell Rep 20:1167–1172

Masson L, Lu YJ, Mazza A, Brousseau R, Adang MJ (1995) The Cry1A(c) receptor purified from *Manduca sexta* displays multiple specificities. J Biol Chem 270:20309–20315

Mcbride KE, Svab Z, Schaaf DJ, Hogan PS, Stalker DM, Maliga P (1995) Amplification of a chimeric *Bacillus thuringiensis* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. Bio/Technology 13:362–365

McCormick S, Jeanne N, Fry J, Barnason A, Horsch R, Fraley R (1986) Leaf disc transformation of cultivated tomato (*L. esculentum*) using *Agrobacterium tumefaciens*. Plant Cell Rep 5:81–84

McCown BH, McCabe DE, Russell DR, Robison DJ, Barton KA, Raffa KF (1991) Stable transformation of Populus and incorporation of pest resistance by electric discharge particle acceleration. Plant Cell Rep 9:590–594

Mehlo L, Gahakwa D, Nghia PT, Loc NT, Capell T, Gatehouse JA, Gatehouse AMR, Christou P (2005) An alternative strategy for sustainable pest resistance in genetically enhanced crops. Proc Natl Acad Sci 102:7812–7816

Mehrotra M, Singh AK, Sanyal I, Altosaar I, Amla DV (2011) Pyramiding of modified *cry1Ab* and *cry1Ac* genes of *Bacillus thuringiensis* in transgenic chickpea (*Cicer arietinum* L.) for improved resistance to pod borer insect *Helicoverpa armigera*. Euphytica 182(1):87–102

Morán R, García R, López A, Zaldúa Z, Mena J, García M, Armas R, Somonte D, Rodríguez J, Gómez M, Pimentel E (1998) Transgenic sweetpotato plants carrying the delta-endotoxin gene from *Bacillus thuringiensis* var. tenebrionis. Plant Sci 139:175–184

Morse RJ, Yamamoto T, Stroud RM (2001) Structure of Cry2Aa suggests an unexpected receptor binding epitope. Structure 9:409–417

Murray EE, Lotzer J, Eberle M (1989) Codon usage in plant genes. Nucleic Acids Res 17(2):477–498

Naimov S, Dukiandijiev S, de Maad RA (2003) A hybrid *Bacillus thuringiensis* delta-endotoxin gene gives resistance against a coleopteran and lepidopteran pest in transgenic potato. Plant Biotechnol J 1:51–57

Nation J (2002) Insect physiology and biochemistry, 1st edn. CRC Press, Boca Raton, FL

Nayak P, Basu D, Das S, Basu A, Ghosh D, Ramakrishnan NA, Ghosh M, Sen SK (1997) Transgenic elite indica rice plants expressing Cry1Ac δ-endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scripophaga incertulas*). Proc Natl Acad Sci U S A 94:2111–2116

Pacheco S, Gómez I, Arenas I, Saab-Rincon G, Rodríguez-Almazán C, Gill SS, Bravo A, Soberón M (2009) Domain II loop 3 of *Bacillus thuringiensis* Cry1Ab toxin is involved in a "ping pong" binding mechanism with *Manduca sexta* aminopeptidase-N and cadherin receptors. J Biol Chem 284:32750–32757

Pardo Lopez L, Soberon M, Bravo A (2013) *Bacillus thuringiensis* insecticidal three-domain cry toxins: mode of action, insect resistance and consequences for crop protection. FEMS Microbial Rev 37:3–22

Park JR, McFarlane I, Phipps RH, Ceddia G (2011) The role of transgenic crops in sustainable development. Plant Biotechnol J 9:2–21

Parrott WA, Clemente TE (2004) Transgenic soybean. In: Boerma HR, Specht JE (eds) Soybeans: improvement, production, and uses, Agronomy monograph no. 16, 3rd edn. American Society of Agronomy-Crop Science Society of America-Soil Science Society of America, Madison, WI, pp 265–302

Parrott WA, All JN, Adang MJ, Bailey MA, Boerma HR, Stewart CNJ (1994) Recovery and evaluation of soybean plants transgenic for a *Bacillus thuringiensis* var. Kurstaki insecticidal gene. In Vitro Cell Dev Biol 30:144–149

Peferoen M, Jansens S, Reynaerts A, Leemans J (1990) Potato plants with engineered resistance against insect attack. In: Vayda M, Park W (eds) Molecular and cellular biology of the potato. CAB, Tucson, pp 193–204

Perlak FJ, Deaton RW, Armstrong TA, Fuchs RL, Sims SR, Greenplate JT, Fischhoff DA (1990) Insect-resistant cotton plants. Nat Biotechnol 8:939–943

Perlak FJ, Fischhoff DA (1993) Insect resistant cotton: from the laboratory to the marketplace. In: Kim L (ed) Advanced engineered pesticides. Marcel Dekker, New York, pp 199–211

Perlak FJ, Fuchs RL, Dean DA, McPherson SL, Fischhoff DA (1991) Modification of the coding sequences enhances plant expression of insect control protein genes. Proc Natl Acad Sci U S A 88:3324–3328

Perlak FJ, Oppenhuizen M, Gustafson K, Voth R, Sivasupramaniam S, Heering D, Carey B, Ihrig RA, Roberts JK (2001) Development and commercial use of Bollgard cotton in the USA-early promises versus today's reality. Plant J 27:489–501

Pigott CR, Ellar DJ (2007) Role of receptors in *Bacillus thuringiensis* crystal toxin activity. Microbiol Mol Biol Rev 71(2):255–281

Purcell JP, Oppenhuizen M, Wofford T, Reed AJ, Perlak FJ (2004) The story of Bollgard cotton. In: Christou P, Klee H (eds) Handbook of plant biotechnology. John Wiley & Sons, New York, NY, pp 1147–1163

Rajamohan F, Alzate O, Cotrill JA, Curtiss A, Dean DH (2006) Protein engineering of *Bacillus thuringiensis* delta-endotoxin: mutations at domain II of Cry1Ab enhance receptor affinity and toxicity toward gypsy moth larvae. Proc Natl Acad Sci 93:14338–14343

Ramesh S, Nagadhara D, Reddy VD, Rao KV (2004) Production of transgenic indica rice resistant to yellow stem borer and sap-sucking insects, using super-binary vectors of *Agrobacterium tumefaciens*. Plant Sci 166:1077–1085

Rawat P, Singh AK, Ray K, Chaudhary B, Kumar S, Gautam T, Kanoria S, Kaur G, Kumar P, Pental D, Burma PK (2011) Detrimental effect of expression of Bt endotoxin Cry1Ac on in vitro regeneration, in vivo growth and development of tobacco and cotton transgenics. J Biosci 36:363–376

Rico E, Ballester V, Mensua JL (1998) Survival of two strains of *Phthorimae opercutella* (Lepidoptera: Gelechiidae) reared on transgenic potatoes expressing a *Bacillus thuringiensis* crystal protein. Agronomie 18:151–155

Ronald P (2011) Plant genetics, sustainable agriculture and global food security. Genetics 188:11–20

Rukmini V, Reddy CY, Venkateswerlu G (2000) *Bacillus thuringiensis* crystal δ-endotoxin: role of proteases in the conversion of protoxin to toxin. Biochimie 82:109–116

Sanahuja G, Banakar R, Twyman RM, Capell T, Christou P (2011) *Bacillus thuringiensis*: a century of research, development and commercial applications. Plant Biotechnol J 9:283–300

Sanchis V, Lereclus D, Menou G, Chaufaux J, Lecadet MM (1988) Multiplicity of delta endotoxin genes with different insecticidal specificities in *Bacillus thuringiensis* aizawai. Mol Microbiol 2:393–404

Sanyal I, Singh AK, Kaushik M, Amla DV (2005) *Agrobacterium*-mediated transformation of chickpea (*Cicer arietinum* L.) with *Bacillus thuringiensis cry*1Ac gene for resistance against pod borer insect *Helicoverpa armigera*. Plant Sci 168:1135–1146

Saraswathy N, Kumar PA (2004) Protein engineering of δ-endotoxins of *Bacillus thuringiensis*. Electron J Biotechnol 7(2)

Sardana R, Dukiandjiev S, Giband M, Cheng X, Cowan K, Sauder C, Altosaar I (1996) Construction and rapid testing of synthetic and modified toxin gene sequences Cry1A (b & c) by expression in maize endosperm culture. Plant Cell Rep 15:677–681

Schnepf HE, Whiteley HR (1981) Cloning and expression of the *Bacillus thuringiensis* crystal protein gene in *Escherichia coli*. Proc Natl Acad Sci U S A 78:2893–2897

Schnepf HE, Crickmore N, Van Rie J, Lereculus D, Baum J, Feitelson J, Zeigler DR, Dean DH (1998) *Bacillus thuringiensis* and its pesticidal crystal proteins. Microbiol Mol Biol Rev 62:775–806

Selvapandiyan A, Reddy VS, Kumar PA, Tewari KK, Bhatnagar RK (1998) Transformation of *Nicotiana tabacum* with a native cry1Ia5 gene confers complete protection against *Heliothis armigera*. Mol Breed 4(6):473–478

Sharma KK, Seetharama N, Ortiz R, Sharma HC (2000) Prospects for using transgenic resistance to insects in crop improvement. Electron J Biotechnol 3:76–95

Sharma HC, Sharma KK, Seetharama N, Crouch JH (2004) Genetic engineering of crops for insect control: potential and limitations. CRC Crit Rev Plant Sci 23:47–72

Sharma KK, Lavanya K, Anjalah A (2006) *Agrobacterium tumefaciens*-mediated production of transgenic pigeonpea (*Cajanus cajan* [L.] mill sp.) expressing the synthetic BT *cry1Ab* gene. Invitro Cell Dev Biol 42:165–173

Shu Q, Ye G, Cui H, Cheng X, Xiang Y, Wu D, Gao M, Xia Y, Hu C, Sardana R, Altosaar I (2000) Transgenic rice plants with a synthetic *cry1Ab* gene from *Bacillus thuringiensis* were highly resistant to eight lepidopteran rice pest species. Mol Breed 6(4):433–439

Singh PK, Kumar M, Chaturvedi CP, Yadav D, Tuli R (2004) Development of a hybrid δ-endotoxin and its expression in tobacco and cotton for control of a polyphagous pest *Spodoptera litura*. Trans Res 14:1–14

Singh R, Channappa RK, Deeba F, Nagaraj NJ, Sukavaneaswaran MK, Manjunath TM (2005) Tolerance of Bt corn (MON810) to maize stem borer *Chilo partellus* (Lepidoptera: Crambidae). Plant Cell Rep 24:556–560

Singsit C, Adang MJ, Lynch RE, Anderson WF, Wang A, Cardineau G, Ozias-Akins P (1997) Expression of *Bacillus thuringiensis cry1A*(c) gene in transgenic peanut plants and its efficacy against lesser cornstalk borer. Trans Res 6:169–176

Smith EF, Townsend CO (1907) A plant-tumor of bacterial origin. Science 25:671–673

Soberon M, Lopez-Diaz JA, Bravo A (2013) Cyt toxins produced by *Bacillus thuringiensis*: a protein conserved in several pathogenic microorganisms. Peptides 41:87–89

Srivastava S (2007) Expression and performance of modified *Bacillus thuringiensis* insecticidal *cry1A* genes in transgenic tomato for insect resistance. Ph.D. thesis, University of Lucknow, Lucknow, India

Staub JM, Garcia B, Graves J, Hajdukiewicz PTJ, Hunter P, Nehra N, Paradkar V, Schlittler M, Carroll JA, Spatola L, Ward D, Ye GN, Russell D (2000) High-yield production of a human therapeutic protein in tobacco chloroplasts. Nat Biotechnol 18:333–338

Stewart CN Jr, Adang MJ, All JN, Boerma HR, Cardineau G, Tucker D, Parrott WA (1996) Genetic transformation, recovery and characterization of fertile soybean transgenic for a synthetic *B. thuringiensis cry1Ac* gene. Plant Physiol 112:121–129

Strizhov N, Keller M, Mathur J, Koncz-Kálmán Z, Bosch D, Prudovsky E, Schell J, Sneh B, Koncz C, Zilberstein A (1996) A synthetic cryIC gene, encoding a *Bacillus thuringiensis* delta-endotoxin, confers Spodoptera resistance in alfalfa and tobacco. Proc Natl Acad Sci U S A 93(26):15012–15017

Surekha C, Beena MR, Arundhati A, Singh PK, Tuli R, Dutta-Gupta A, Kirti PB (2005) *Agrobacterium*-mediated genetic transformation of pigeonpea (*Cajanus cajan* L. Millsp.) using embryonal segments and development of transgenic plants for resistance against *Spodoptera*. Plant Sci 169:1074–1080

Tabashnik BE, Finson N, Johnson MW, Moar WJ (1993) Resistance to toxins from *Bacillus thuringiensis* subsp. *Kurstaki* causes minimal cross-resistance to *B. thuringiensis* subsp. *aizawai* in the diamondback moth (Lepidoptera: Plutallida). Appl Environ Microbiol 59:1332–1335

Tabashnik BE, Liu YB, Finson N, Masson L, Heckel DG (1997) One gene in diamondback moth confers resistance to four Bacillus thuringiensis toxins. Proc Natl Acad Sci U S A 94:1640–1644

Tabashnik BE, Dennehy TJ, Sims MA, Larkin K, Head GP, Moar WJ, Carrière Y (2002) Control of resistant pink bollworm by transgenic cotton with *Bacillus thuringiensis* toxin Cry2Ab. Appl Environ Microbiol 68:3790–3794

Tabashnik BE, Carrière Y, Dennehy TJ, Morin S, Sisterson MS, Roush RT, Shelton AM, Zhao JZ (2003) Insect resistance to transgenic Bt crops: lessons from the laboratory and field. J Eco Entomol 96:1031–1038

Tabashnik BE, Sisterson MS, Ellsworth PC, Dennehy TJ, Antilla L, Liesner L, Whitlow M, Staten RT, Fabrick JA, Unnithan GC, Yelich AJ, Ellers-Kirk C, Harpold VS, Li X, Carriere Y (2010) Suppressing resistance to Bt cotton with sterile insect release. Nat Biotechnol 28:1304–1307

Thi Van D, Ferro N, Jacobsen HJ (2010) Development of a simple and effective protocol for *Agrobacterium tumefaciens* mediated leaf disc transformation of commercial tomato cultivars. GM Crops 1–5:312–321

Tu J, Zhang G, Datta K, Xu C, He Y, Zhang Q, Khush GS, Datta SK (2000) Field performance of transgenic elite commercial hybrid rice expressing Bacillus thuringiensis δ-endotoxin. Nat Biotechnol 18:1101–1104

Tyc K, Konarska M, Gross HJ, Filipowicz W (1984) Multiple ribosome binding to the 5′-terminal leader sequence of tobacco mosaic virus RNA. Assembly of an 80S ribosome X mRNA complex at the AUU codon. Eur J Biochem 140:503–511

Tzfira T, Citovsky V (2006) *Agrobacterium*-mediated genetic transformation of plants: biology and biotechnology. Curr Opinion Biotech 17:147–154

Upadhyay SK, Singh PK (2011) Role of alkaline phosphatase in insecticidal action of Cry1Ac against *Helicoverpa armigera* larvae. Biotechnol Lett 33:2027–2036

Vachon V, Laprade R, Schwartz JL (2012) Current models of the mode of acion of *Bacillus thuringiensis* insecticidal crystal proteins: a critical review. J Invertebr Pathol 111:1–12

Vadlamudi RK, Weber E, Ji I, Ji TH, Bulla LA Jr (1995) Cloning and expression of a receptor for an insecticidal toxin of *Bacillus thuringiensis*. J Biol Chem 270:5490–5494

Vaeck M, Reynaerts A, Hofte H, Jansens S, De Beuckeleer M, Dean C, Zabeau M, Van Montagu M, Leemans J (1987) Transgenic plants protected from insect attack. Nature 328:33–37

Valderrama AM, Velá squez N, Rodríguez E, Zapata A, Zaidi M, Altosaar I, Arango R (2007) Resistance to *Tecia solanivora* (Lepidoptera: Gelechiidae) in three transgenic andean varieties of potato expressing *Bacillus thuringiensis* Cry1Ac protein. J Econ Entomol 100(1):172–179

Velcheva M, Faltin Z, Flaishman M, Eshdat Y, Perl A (2005) A liquid culture system for *Agrobacterium*-mediated transformation of tomato (*Lycopersicon esculentum* L. Mill.). Plant Sci 168:121–130

Verma AK, Chand L (2005) *Agrobacterium*-mediated transformation of pigeonpea (*Cajanus cajan* L.) with *uid*A and *cryIA(b)* genes. Physiol Mol Biol Plant 11:99–109

Wahab S (2009) Biotechnological approaches in the management of plant pests, diseases and weeds for sustainable agriculture. J Biopest 2:115–134

Wang XG, Zhang GH, Liu CX, Zhang YH, Xiao CZ, Fang RX (2001) Purified cholera toxin B subunit from transgenic tobacco plants possesses authentic antigenicity. Biotechnol Bioeng 72:490–494

Wang DJ, Brandsma M, Yin Z, Wang A, Jevnikar AM, Ma S (2008) A novel platform for biologically active recombinant human interleukin-13 production. Plant Biotechnol J 6:504–515

Weng LX, Deng HH, Xu JL, Li Q, Zhang YQ, Jiang ZD, Li QW, Chen JW, Zhang LH (2010) Transgenic sugarcane plants expressing high levels of modified cry1Ac provide effective control against stem borers in field trials. Transgenic Res 20(4):759–772

Whiteley RH, Schnepf HE (1986) The molecular biology of parasporal *cry*stal body formation in *Bacillus thuringiensis*. Ann Rev Microbiol 40:549–576

Wu C, Fan Y, Zhang C, Olica N, Datta SK (1997) Transgenic fertile japonica rice plants expressing a modified *cryIA(b)* gene resistant to yellow stem borer. Plant Cell Rep 17:129–132

Wu SJ, Koller CN, Miller DL, Bauer LS, Dean DH (2000) Enhanced toxicity of Bacillus thuringiensis Cry3A delta-endotoxin in coleopterans by mutagenesis in a receptor binding loop. FEBS Lett 473:227–232

Wunn J, Kloti A, Burkhardt PK, Biswas CG, Launis K, Iglesias VA, Potrykus I (1996) Transgenic indicia rice breeding line IR58 expressing a synthetic cryIAb gene from *Bacillus thuringiensis* provides effective insect pest control. Bio/Technology 14:171–176

Xie R, Zhuang M, Ross LS, Gomez I, Oltean DI, Bravo A, Soberon M, Gill SS (2005) Single amino acid mutations in the cadherin receptor from *Heliothis virescens* affect its toxin binding ability to Cry1A toxins. J Biol Chem 280:8416–8425

Ye GY, Yao HW, Shu QY, Cheng X, Hu C, Xia YW, Gao MW, Altosaar I (2003) High levels of stable resistance in transgenic rice with a *cry1Ab* gene from *Bacillus thuringiensis* Berliner to rice leaffolder, *Cnaphalocrocis medinalis* (Guenée) under field conditions. Crop Prot 22:171–178

Zaidi MA, Mohammadi M, Postel S, Masson L, Altosaar I (2005) The Bt gene *cry2Aa2* driven by a tissue specific ST-LS1 promoter from potato effectively controls *Heliothis virescens*. Trans Res 14(3):289–298

Zambryski P, Tempe J, Schell J (1989) Transfer and function of T-DNA genes from agrobacterium Ti and Ri plasmids in plants. Cell 56(2):193–201

Zhang X, Candas M, Griko NB, Taussig R, Bulla LA Jr (2006) A mechanism of cell death involving an adenylyl cyclase PKA signaling pathway is induced by the Cry1Ab toxin of *Bacillus thuringiensis*. Proc Natl Acad Sci U S A 103(26):9897–9902

Zhao JZ, Li YX, Collins HL, Cao J, Earle ED, Shelton AM (2001) Different cross-resistance patterns in the diamond back moth (Lepidoptera: Plutellidae) resistant to *Bacillus thuringiensis* toxin cry1C. J Econ Entomol 94(6):1547–1552

Zhao JZ, Cao J, Li Y, Collins HL, Roush RT, Earle ED, Shelton AM (2003) Transgenic plants expressing two *Bacillus thuringiensis* toxins delay insect resistance evolution. Nat Biotechnol 21(12):1493–1497

Zhao J-Z, Cao J, Collins HL, Bates SL, Roush RT, Earle ED, Shelton AM (2005) Concurrent use of transgenic plants expressing a single and two *Bacillus thuringiensis* genes speeds insect adaptation to pyramided plants. Proc Natl Acad Sci U S A 102:8426–8430

Check for updates

3 Characterization and Efficiency of Rhizobial Isolates Nodulating *Cytisus monspessulanus* in the Northwest of Morocco: In Relation to Environmental Stresses

Taoufik Belechheb, Mohammed Bakkali, Amin Laglaoui, and Abdelhay Arakrak

Abstract

Phenotypic characteristics of 69 rhizobial strains isolated from root nodules of *Cytisus monspessulanus* leguminous shrub growing in soils collected from the northwest of Morocco were studied. Tolerance to salinity, high temperatures, acid and alkaline pH, heavy metals, carbon and nitrogen source assimilation, and symbiotic and cultural characteristics allowed the description of a wide physiological diversity among tested isolates. Numerical analysis of the phenotypic characteristics showed that below the boundary level of 48, 91% of average similarity isolates fell into at least four distinct groups. A number of potential isolates have been identified for inoculation trials. They were efficient and able to form nodules with indigenous rhizobia in this region.

Keywords

Rhizobia · *Cytisus monspessulanus* · Biological nitrogen fixation (BNF)

3.1 Introduction

Cytisus monspessulanus is a legume shrub native to the Mediterranean region very promising for regeneration of degraded soils in semiarid regions. A method of rehabilitating a degraded land is the establishment of agroforestry systems (Le Houérou 1993), where shrub legumes play an important role (Lefroy et al. 1992; Douglas et al. 1996). One of its advantages is good forage quality during winter because it can

T. Belechheb · M. Bakkali · A. Laglaoui · A. Arakrak (✉)
Equipe de Recherche de Biotechnologies et Génie des Biomolécules (ERBGB), Faculté des Sciences et Techniques de Tanger, Tanger, Morocco

© Springer Nature Singapore Pte Ltd. 2020

M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_3

produce large amounts of biomass in the arid continental climate zones (González-Andrés and Ortiz 1996a). In addition, the quality of the grass produced by *Cytisus monspessulanus* is similar to that of alfalfa (González-Andrés and Ortiz 1996b). Also, it is characterized by its high seed germination and plant survival in the field, its high dry matter production potential, and its favorable and consistent chemical composition during the year. Moreover, *Cytisus monspessulanus* can invade a wide range of habitats including roadsides, fields, logged areas, bluffs, and coastal areas; it can adapt well to open, sunny, and well-drained sites; and it has been introduced for use as ornamentals or hedge plants (Parsons and Cuthbertson 2001).

This perennial leguminous shrub fixes atmospheric nitrogen (N2) via symbiotic bacteria (general term "rhizobia") in root nodules and converts it into ammonia (NH3) to provide nitrogen nutrients for both rhizobia and the host plant (Howard and Rees 1994; Dixon and Kahn 2004). This can give them an advantage under low soil nitrogen (N) conditions if other factors are favorable for growth (Raven 2010; Andrews et al. 2013). Furthermore, N2 fixation by legumes can be a major input of N into natural and agricultural ecosystems (Andrews et al. 2007; Vitousek et al. 2013), which plays a key role in the nitrogen cycle. Biological nitrogen fixation (BNF) accounted for 65% of global nitrogen resources (in term of mineral, not N2 gas), while chemical syntheses made about 30% (Dixon and Kahn 2004). Three-quarters of BNF are generated through symbiosis between rhizobia and legume plants. However, knowledge about rhizobia and *Cytisus monspessulanus* is still unknown that is needed to enhance this symbiosis within this shrub conservation plans, particularly in the northwest of Morocco.

The aim of this study is to assess the characterization, efficiency, and diversity of the rhizobia that nodulates *Cytisus monspessulanus* in the region of Tangier, northwest of Morocco.

First, plant samples were collected from different locations, and the bacteria were evaluated based on their response to various physiological conditions such as salinity stresses, extreme pH, high temperature, and heavy metal tolerance. Then, the bacteria were assessed for their infectivity, focusing only on rhizobial strains that are very competitive and can adapt well to extreme environmental conditions.

3.2 Materials and Methods

3.2.1 Location of Nodule Collection

The collection of root nodule was set up in three sites at Tangier City, northwest of Morocco. This place is characterized by the following geographic coordinates: latitude 35°46′02″ N, longitude 5°47′59″ W, and altitude of sites 150 m (Fig. 3.1).

3.2.1.1 Rhizobia Isolation and Purification

The bacterial strains used in this study were isolated from the root of *Cytisus monspessulanus* plant collected according to the method described by Vincent (1970) and Somasegaran and Hoben (1994). The nodules were sterilized with 0.1%

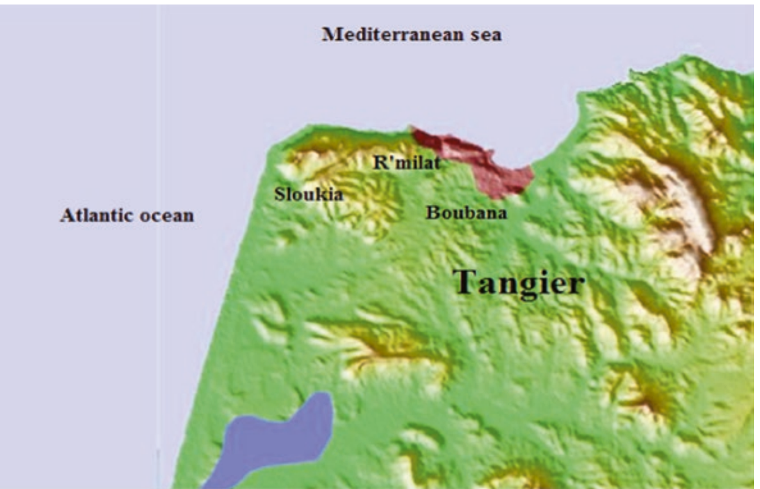


Fig. 3.1 Geographical location of sampling sites

acidified mercuric chloride and ethanol and then washed thoroughly in at least ten changes of sterile distilled water. The isolation of bacteria was achieved by the method of Vincent (1970). The colonies growing on three media, yeast extract mannitol + Congo red (YEM+CR), glucose peptone agar + bromocresol purple (GPA+BCP), and YEM + bromothymol blue (YEM+BTB), were ascertained to correspond to the phenotypic descriptions recommended by Vincent, depending on the shape and color of the colonies.

3.2.2 Response to Environmental Stress Factors

Isolates were examined for growth under different stress conditions of high temperature, high salinity, and extreme pH. In the case of temperature tolerance, isolates were kept at 28 (as a control), 38, 40, 42, and 45 °C on YEM plates for 4–5 days. The ability of the isolates to grow in different concentrations of salt was tested by streaking isolates on YEM medium containing 0.5%, 1%, 2%, and 5% (w/v) NaCl. Similarly, the growth of rhizobial strains was compared at different pH (3.2, 5.7, 9, and 10) on YEM medium.

3.2.3 Utilization of Carbon and Nitrogen Sources

Isolates were tested for their ability to utilize some carbohydrate as a sole carbon source. For the analysis of carbohydrate utilization, a modified YEM agar was used, where yeast extract was reduced to 0.05 g/L (Somasegaran and Hoben 1994) and 0.01% NH_4NO_3 was utilized as a source of nitrogen. Mannitol was replaced by

another carbohydrate to a final concentration of 1% w/v. Two control media were used for comparison: YEM containing mannitol as a positive control, and the medium without any carbon source as a negative control. A modified mannitol medium, in which yeast extract was replaced by 0.1% w/v of the tested amino acid and mineral salts, was used to investigate the utilization of nitrogen compounds. N-free modified mannitol medium (devoid of any nitrogen source) was used as a control. All the plates were incubated at 28 °C for 2–7 days.

3.2.4 Heavy Metal Tolerance

All isolates were tested for their sensitivity to five heavy metals salts, namely $CuCl_2$, $ZnCl_2$, $MnCl_2$, $AlCl_3$, and $PbCl_2$. Sensitivity pattern was studied on YEM agar plate containing graded concentration of heavy metals. The stock solution of heavy metals was prepared in distilled water, and solution was added to the YEM medium after filtration through Millipore membrane (0.2 μm porosity). In all experiments, growth was recorded after 3 days of incubation at 28 °C in triplicate.

3.2.5 Authentication of Isolates

All the rhizobial isolates were evaluated as pure cultures that could form nodules on their respective host plants. Seeds of the leguminous plants were pre-germinated in petri dish after scarification with H2SO4. The pre-germinated seeds were planted in growth pouches (Somasegaran and Hoben 1994) and inoculated with 1 ml YEM broth culture of each isolate with each treatment replicated three times. Seven days after planting, the growth pouches were inoculated a second time with 1 ml YEM broth culture of each isolate. Uninoculated pouches received weekly 0.5% (w/v) KNO3 as nitrogen source and served as a positive control. The pouches were placed in racks and kept in the greenhouse. Plants were harvested 14 weeks after planting, and a nodule scoring chart was applied to evaluate the infectivity of strains using the chart proposed by (Howieson and Dilworth 2016). Effectiveness of strains in nitrogen fixation was evaluated by scoring the total fresh/dry matter weight, plant height, and nodule number.

3.2.6 Numerical Analysis

The unweighted pair group method with arithmetic mean (UPGMA) (Sneath and Sokal 1973) was used for cluster analysis of phenotypic features. The similarity coefficient was computed, and the results are shown as a dendrogram using the XLSTAT software (2014). The data obtained will be subject to statistical analysis using the SAS software (2002) followed by mean comparison using Duncan's test. The values are means of three replicates.

3.3 Results and Discussion

3.3.1 Phenotypic Evaluation

A total of 69 bacteria were recovered from the root nodules of *Cytisus monspessulanus* collected from three sites (Boubana, R'milat, and Sloukia) in the region of Tangier and were examined for 50 phenotypic characteristics.

The majority of the isolates (85%) were creamy or white opaque with little to moderate exopolysaccharide (EPS) production and differed slightly in their absorption of Congo red dye similar to other bacteria hosted in the root nodules of three Mediterranean wild legume species *Hedysarum carnosum*, *Hedysarum spinosissimum* subsp. *capitatum*, and *Hedysarum pallidum* (Benhizia et al. 2004). Other interesting and useful characteristics of rhizobia are other growth reactions in the standard YEM medium containing bromothymol blue (BTB) as the pH indicator. In our study, all colonies produce an acid reaction on YEM+BTB plates and changed to yellow after 3 days of incubation at 28 °C. These rhizobia can be classified as fast-growing rhizobia according to Somasegaran and Hoben (1994). Unlike the earlier belief that rhizobia have no ability to grow on glucose peptone agar medium (Somasegaran and Hoben 1994; Vincent 1970), in this study, some isolates grew on this medium and turn the medium to yellow (Fig. 3.2). Finally, all retrieved strains were Gram negative. These characteristics are the first clues to the identification of rhizobia, according to Vincent (1970) and Somasegaran and Hoben (1994).

3.3.2 The Numbers Are the Number of Isolates Giving Positive Reaction

The isolated strains showed a large diversity among rhizobia and formed heterogeneous group based on physiological properties such as carbon/nitrogen substrate assimilation; tolerance to pH, salt, temperature, and heavy metal; and their geographic origin (Table 3.1). This geographic diversity in rhizobial species composition has been shown to be related to local environmental conditions (Yang et al. 2013; Li et al. 2012).

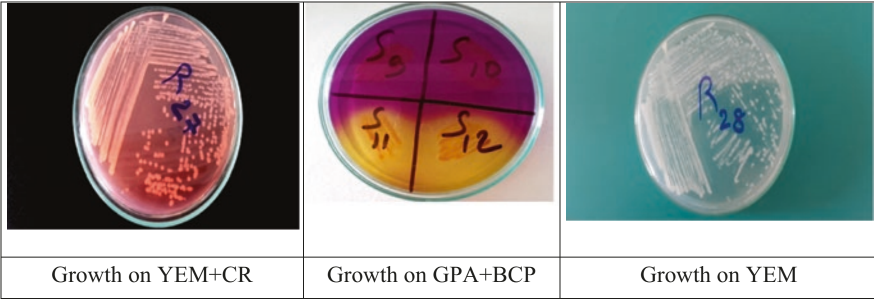


Fig. 3.2 Growth of isolates in different media

Table 3.1 Physiological characteristics of root-nodule isolates

Characteristic			Sites		
			Boubana	R'milat	Sloukia
			$n = 21$	$n = 32$	$n = 16$
Growth at temperature °C		28	21	32	16
		40	11	8	9
		45	0	1	2
Growth at pH		3.2	18	0	12
		9	21	29	16
		10	21	29	16
NaCl tolerance %		0.5	21	32	16
		2	21	11	16
		5	16	0	16
Carbohydrate assimilation		Sucrose	21	13	14
		Raffinose	21	6	16
		Rhamnose	18	18	14
		Maltose	21	10	16
		Galactose	21	9	16
		Glucose	18	18	14
Utilization of nitrogen source		His	21	3	16
		Pro	21	28	16
		Arg	21	30	16
		Asp	21	32	16
		KNO3	3	0	7
		NH4Cl	21	32	13
Resistance to heavy metals µg/ml	Aluminum	100	21	29	16
		200	21	26	13
		400	5	0	0
	Zinc	50	21	17	16
		100	21	14	11
		200	21	6	9
	Manganese	200	21	32	16
		300	21	28	16
		400	21	26	13
	Lead	400	21	28	16
		600	21	28	14
		1000	10	7	2
	Copper	100	12	4	3
		200	0	2	0
		400	0	0	0

n = number of isolates

Our isolates have a better assimilation which exceeds 80% of the nitrogen compounds tested, with the exception of ammonium chloride, which appears to be unusable as a source of nitrogen. On the other hand, it was noted that all isolates can use the various carbohydrates tested and do not require mannitol as a carbon source.

The osmotolerance was found to be very high, with up to 5% of NaCl for all isolates from the Sloukia site and the majority from Boubana site, while bacteria harvested from the R'milat site are inhibited by salinity. Sloukia isolates are also able to grow over a fairly wide range of pH from 3.2 to 10, while those of R'milat cannot withstand the acidity of the environment.

As for the temperature, the total tolerance ranges from 28 °C to 45 °C, except for the CmS4, CmS11, and CmR17 isolates, which have been shown to be resistant to high temperature.

In soil, salinity and pH changes are generally accompanied by mineral toxicity. We therefore assessed the degree of inhibition of the growth of the isolates using five different heavy metals. Our results showed the sensitivity of isolates to high concentrations of heavy metals in the order Cu > Al > Pb > Zn > Mg, with the exception of isolates from Boubana (CmB21, CmB22), Sloukia (CmS9), and R'milat (CmR21) which conform to the results of Parvaze and Mohammad (2012) and Luo et al. (2011), respectively. The RL9 strain isolated from the lentil nodules was able to withstand high lead concentrations of up to 1400 μg/ml, and the LRE07 strain isolated from the plant *Solanum nigrum* resisted toxic concentrations of heavy metals.

The selection of strains resistant to heavy metals is of great practical interest. Several studies are currently investigating the use of symbiosis between legumes and resistant rhizobia as an effective means of "bioremediation" against soil contamination by heavy metals (Gianfreda and Rao 2004a). In addition, the use of rhizobium as a preventive agent in contaminated soils has been reported by Abbas and Kamel (2004).

The analysis using UPGMA (unweighted pair group method with arithmetic mean) allowed us to highlight phenotypic groups that contain a wide diversity among the four clusters (Fig. 3.3) determined within each cluster. Indeed, the isolates of each cluster exhibit great variability in the phenotypic tests examined. Cluster I consists of heat-resistant isolates that have demonstrated basic pH tolerance and resistance to heavy metals (isolates belonging to the site of R'milat, CmR). Cluster II is the most heterogeneous with isolates belonging to the three different nodule-harvesting sites, with resistance to most of the factors tested. In addition, isolates belonging to the same geographical site can be grouped in different clusters, as is the case for the CmB and CmS isolates that are distributed to clusters II, III, and IV.

This phenotypic diversity allows us, however, to select the isolates which are the right candidates for any inoculation trial of *Cytisus monspessulanus*, depending on the different pedoclimatic conditions considered (salt stress, extreme temperatures, etc.)

3.3.3 Plant Assay

The capacity of the rhizobia to produce an infection on the roots of legumes and to induce the formation of the nodules is called infectivity. This property is limited to a specific group of rhizobium and host, where the infection is induced. It is important to test the ability of strains to produce nodules with the original plant from which they were isolated (Beck et al. 1993).

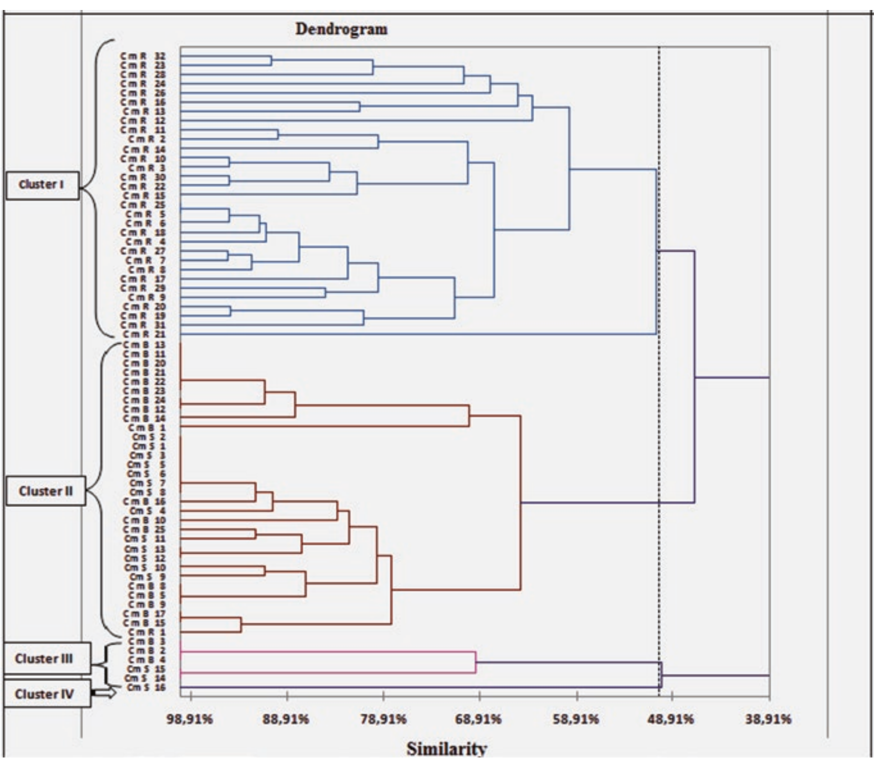


Fig. 3.3 Phenogram showing phenotypic relatedness among 69 isolates from *Cytisus monspessulanus* nodules based on average-linkage cluster analysis of 50 characteristics

In our study, the infectivity was tested with the isolates which have shown an ability to grow under extreme environmental conditions (CmR6, CmR31, CmB3, CmB12, CmS3, CmS9) and showed a large diversity in their capacity to infect the host plant and to fix atmospheric nitrogen. The mean comparison showed several overlapping groups with significant differences between the isolates of the three sites.

Among these isolates, it would appear that CmS9 and CmS3 isolates represented the most infectious and gave the best results with respect to stem height (59.25 cm/55.75 cm), fresh matter weight (12.47 g/10.18 g), dry matter weight (5.29 g/4.44 g), and nodules (33.5 g/27.5 g), compared to positive control and other isolates, while CmR31 was the least efficient with only 8.92 g of fresh matter weight, 2.31 g of dry matter weight, and 17.5 g of nodules (Table 3.2). Also, we noted that the stem height and the weight of fresh and dry matter increase with the number of nodules. This is confirmed by the study of Rupela and Dart (1980) that showed a significant correlation between the increase of dry matter and the number or the dry weight of nodules. And in another study, it has been shown that the dry matter yield was rather correlated with the nodule leghemoglobin concentration than with the number or the dry weight of nodules (Dudeja et al. 1981).

Table 3.2 Nodulation and efficiency of *Cytisus monspessulanus* evaluated at three sites in Tangier, Morocco

Isolate	Stem height (cm)	Weight of fresh matter (g)	Weight of dry matter (g)	Nodule number
CmS9	$59.25^{a} \pm 2.86$	$12.47^{a} \pm 0.53$	$5.29\ ^{a} \pm 0.33$	$33.5^{a} \pm 1.92$
CmS3	$55.75^{a,b} \pm 1.58$	$10.18^{b} \pm 0.53$	$4.44^{b} \pm 0.31$	$27.75^{b} \pm 3.65$
Positive control	$54.50^{a-c} \pm 1.77$	$9.25^{b,c} \pm 0.59$	$3.57^{c} \pm 0.43$	0
CmR31	$48.5^{b-d} \pm 2.87$	$8.92^{c} \pm 0.87$	$2.41^{d} \pm 0.16$	$17.5^{d} \pm 2.44$
CmR6	$47.5^{a} \pm 8.05$	$9.96^{b,c} \pm 0.36$	$3.27^{c} \pm 0.21$	$22.5^{c} \pm 2.32$
CmB3	$47^{b-d} \pm 2.13$	$9.31^{b,c} \pm 0.74$	$3.2^{c} \pm 0.40$	$21.25^{c,d} \pm 3.15$
CmB12	$44^{d} \pm 6.50$	$9.33^{b,c} \pm 0.60$	$2.79^{c,d} \pm 0.48$	$22.5^{c} \pm 2.4$
SEM	1.267	0.243	0.191	1.053
Sig.	**	***	***	***

SEM standard error of the mean
Values with different letters (a–d) in the same column are significantly different ($P < 0.0001$)
Sig.: Level of significance: (**): $p < 0.01$; (***): $p < 0.001$

3.4 Conclusion

To conclude, this study is the first work that phenotypically characterizes root-nodule microsymbionts of *Cytisus monspessulanus* in Morocco.

Combined analysis of all phenotypic data allowed us to identify high-performing isolates. These were justified by the differences between the clusters defined here, based on the behavior of the isolates in relation to the extreme environmental conditions (carbon/nitrogen source utilization, heavy metal tolerance, salinity, pH, and maximum temperature). Moreover, our results show that among the six isolates, two showed their effectiveness and infectivity by the high intensity of nodule formation on the roots of the host plant and consequently the fixation of nitrogen.

References

Abbas SM, Kamel EA (2004) *Rhizobium* as a biological agent for preventing heavy metal stress. Asian J Plant Sci 3(4):416–424

Andrews M, Scholefield D, Abberton MT, McKenzie BA, Hodge S, Raven JA (2007) Use of white clover ASAN alternative to nitrogen fertilizer for dairy pastures in nitrate vulnerable zones in the UK: productivity, environmental impact and economic considerations. Ann Appl Biol 151:11–23

Andrews M, Raven JA, Lea PJ (2013) Do plants need nitrate ? The mechanisms by which nitrogen form affects plants. Ann Appl Biol 163:174–199

Beck DP, Materon LA, Afandi F (1993) Practical *Rhizobium-Legume* technology manual. Technical manual 19. International Center for Agricultural Research in the Dry Areas, Aleppo, Syria, p 389

Benhizia Y, Benhizia H, Benguedouar A, Muresu R, Giacomini A, Squartini A (2004) Gamma proteobacteria can nodulate legumes of the genus Hedysarum. Syst Appl Microbiol 27:462–468

Dixon R, Kahn D (2004) Genetic regulation of biological nitrogen fixation. Nat Rev Microbiol 2:621–631

Douglas GB, Bulloch BT, Foote AG (1996) Cutting management of willows (Salix spp.) and leguminous Shrubs for drilling during summer. N Z J Agric Res 39:175–144

Dudeja SS, Khurana AL, Kundu BS (1981) Effect of *Rhizobium* and phospho microorganisms on yield and nutrient uptake in chickpea. Curr Sci 50:503–505

Gianfreda L, Rao MA (2004a) Potential of extra cellular enzymes in remediation of polluted soils: a review. Enzym Microbial Technol 35:339–354

Howard JB, Rees DC (1994) Nitrogenase: a nucleotide-dependent molecular switch. Annu Rev Biochem 63:235–264

Howieson JG, Dilworth MJ (2016) Australian Centre for International Agricultural Research. Workingwithrhizobia

Gianfreda L, Rao MA (2004b) Potential of extra cellular enzymes in remediation of polluted soils: a review. Enzym Microbial Technol 35:339–354

González-Andrés F, Ortiz JM (1996a) Potential of *Cytisus* and *allied* genera (*Genisteae*: Fabaceae) as forage shrubs. N Z J Agric Res 39:195–204

González-Andrés F, Ortiz JM (1996b) Potential of *Cytisus*and *allied* genera (*Genisteae*: Fabaceae) as drilling shrubs. Chemical composition of the drilling and conclusions. N Z J Agric Res 39:205–213

Lefroy EC, Dann PR, Wildin JH, Wesley-Smith RN, McGowan AA (1992) Trees and shrubs as sources of fodder in Australia. Agrofor Sys 20:117–139

Le Houérou HN (1993) Land degradation in Mediterranean Europe: agroforestry can be a part of the solution? A prospective review. Agrofor Sys 21:43–61

Li M, Li Y, Chen WF, Sui XH, Li Y, Li Y, Wang ET, Chen WX (2012) Genetic diversity, community structure and distribution of rhizobia in the root nodules of Caragana spp. rom arid and semi-arid alkaline deserts, in the north of China. Syst Appl Microbiol 35:239–245

Luo SY, Wan X, Xiao H, Guo H, Chen L, Xi Q, Zeng G, Liu C, Chen J (2011) Isolation and Characterization of endophytic bacterium LREo7 from cadmium hyperaccumulator *Solanum nigrum* Land its potential for remediation. Appl Microbial Biotechnol 89:1637–1644

Parvaze AW, Mohammad SK (2012) Bioremediation of lead by a plant growth promoting rhizobium species RL9. Bacteriol J 2(4):66–78

Parsons WT, Cuthbertson EG (2001) Noxious weeds of Australia. CSIRO Publishing, Melbourne, pp 478–480, 692p

Raven JA (2010) Why are mycorrhizal fungi and symbiotic nitrogen-fixing bacteria not genetically integrated into plants? Ann Appl Biol 157:381–391

Rupela OP, Dart PJ (1980) Research on symbiotic nitrogen fixation by chickpea at ICRISAT. In: Proceedings of the International Workshop on Chickpea Impovement, 28 Feb–2 Mar, Hyderabad, India

Sneath PHA, Sokal RB (1973) Numerical taxonomy. The principles and practice of numerical classification. WH Freeman and Co., San Francisco, CA

Somasegaran P, Hoben HJ (1994) Handbook for Rhizobia: methods in Legume- Rhizobium Technology. Springer, New York, p 450

Vincent JM (1970) A manual for the practical study of root nodule bacteria. In: IBP handbook, no 15. Blackwell Scientific Publications Ltd., Oxford

Vitousek PM, Menge DNL, Reed SC, Cleveland CC (2013) Biological nitrogen fixation: rates, patterns and ecological controls in terrestrial ecosystems. Philos Trans R Soc B 368:20130119

Yang W, Kong Z, Chen W, Wei G (2013) Genetic diversity and symbiotic evolution of rhizobia from root nodules of *Coronillavaria*. Syst Appl Microbiol 36:49–55

Check for updates

4 Isolation and Characterization of the Roots and Soil Endomycorrhizae of *Hedysarum pallidum* Desf. in the Northeast of Morocco

Rachid M'saouar, Mohammed Bakkali, Amin Laglaoui, and Abdelhay Arakrak

Abstract

This study aims to describe the endomycorrhizal status of *Hedysarum pallidum* Desf. in the northeast of Morocco. A detailed description of the mycorrhizal associations in this species soil and roots is reported for the first time in this study. To achieve this goal, some tests were run on soil and root sample collected from the rhizosphere of *Hedysarum pallidum* Desf. The microscopic examination of the roots revealed the presence of typical endomycorrhizal structures. The mycorrhizal frequency was greater than 98%, the mycorrhizal intensity was high (50.9%), and the arbuscular intensity reached 34.76%. The spore number of the arbuscular mycorrhizal fungi (AMF) isolated from the soil was 1200 spores/100 g of soil. These spores presented three AMF genera: *Glomus*, *Scutellospora*, and *Septoglomus*. The diversity of arbuscular mycorrhizal fungi present in the rhizosphere can be selected and used in improving the production of vigorous plants.

Keywords

Arbuscular mycorrhizal fungi · Diversity · *Hedysarum pallidum* Desf. · Morocco

4.1 Introduction

Hedysarum pallidum Desf. (Fig. 4.1), a Mediterranean herbaceous legume, participates in the soil conservation and has the ability to fix its own nitrogen. However, its potential use as a forage plant was emphasized.

R. M'saouar · M. Bakkali · A. Laglaoui · A. Arakrak (✉)
Research Team of Biotechnology and Biomolecular Engineering (ERBGB) Faculty of Sciences and Techniques, Tangier, Morocco

© Springer Nature Singapore Pte Ltd. 2020
M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_4

Fig. 4.1 *Hedysarum pallidum* Desf. from the site of Touissit (northeast of Morocco)

This wild steppic plant was able to survive on the most polluted soils in the abandoned antimony mining area of DjebelHamimat (Algeria) (Rached-Mosbah 1997); it also tolerates the salty soils (Foury 1954).

Mycorrhizal is a symbiotic association between plant root and specialized soil fungi, with evidence that it helps plants in the acquisition of immobile nutrients such as P, N, Zn, and Cu in deficient soils (Ibiremo and Fagbola 2008). Arbuscular mycorrhizal fungi (AMF) provide plants with a higher protection against pathogens and toxic elements in the soil (Herrman et al. 2004). The host plants are strongly affected by AMF, and this association has been classified as vital in the structuring among plant species (Wardle 2002).

Presently, the mycorrhizal association and its beneficial role toward plants are accepted as a universal phenomenon. Mycorrhizal associations are so prevalent that the non-mycorrhizal plant is more of an exception than the rule. In addition, plants growing under natural conditions differ in their ability to enrich the soil by the mycorrhizal propagules; the effectiveness of AMF depends on the native host species (Azcon-Aguilar et al. 2003) (Caravaca et al. 2005).

However, there are no studies on the potential of *Hedysarum pallidum* Desf. to produce arbuscular mycorrhizal inoculum to be used in revegetation strategies. So, the present work was undertaken to study the arbuscular mycorrhizal fungi (AMF) of *Hedysarum pallidum* Desf. in the northeast of Morocco. It provides the basis for any project aiming the improvement of this plant growth through the AMF as these symbiotic fungi are the most favorable in this context. For this purpose, specific tests were carried out: extraction, counting, and identification of endomycorrhizal spores of soils collected from the northeast of Morocco and measurement of the root's mycorrhization rate.

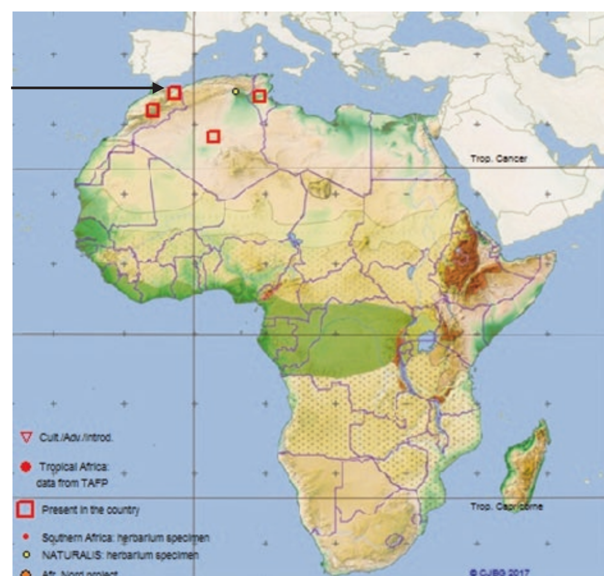


Fig. 4.2 Geographic location of the sample site

4.2 Materials and Methods

4.2.1 Soil Samples and Plant Material

Sampling of soil is performed in the first 20 cm deep on the site of Touissit (Cardinal direction: northeast/latitude: 34.470020°N/longitude: 1.773897°W) in the northeast of Morocco (Fig. 4.2). Three individual plants of *Hedysarum pallidum* Desf. were randomly chosen, and three soil samples were taken. The soil was air-dried, sieved on 2-mm mesh sieves, and placed in favorable conditions throughout the duration of the study.

4.2.2 Methods

4.2.2.1 AMF Spore Extraction

Spores were extracted following the wet sieving method described by Gerdemann and Nicolson (1963). In a 1-L beaker, 100 g of each composite sample of soil is submerged in 0.5 L of tap water and stirred for 1 minute with a spatula. After 10 to 30 s of settling, the supernatant is passed through a sieve of four bunks with decreasing mesh size (500, 200, 80, and 50 μm). This operation was repeated twice. The content retained by the sieves of 200, 80, and 50 μm was divided into two tubes and centrifuged for 5 min at 2000 rev/min. The supernatant was discarded, and a viscosity gradient is created by adding 20 ml of sucrose solution at 60% in each centrifuge tube (Walker 1982). The mixture is rapidly stirred, and the tube provided is centrifuged again for 1 min at

3000 rpm/min. Unlike the first centrifuging, the supernatant is poured onto the sieve of 50 µm; the resulting substrate was rinsed with distilled water to remove sucrose. The spores were then recovered with a little distilled water in an Erlenmeyer flask and counted, and their density (spore number per 100 g dry soil) was determined.

4.2.2.2 Measuring of the Root Mycorrhization Rate

As described by Phillips and Hayman (1970), roots were first washed with water, and the finest ones were cut into a length of 1 cm, immersed in a solution of 10% potassium hydroxide (KOH), and placed in a water bath at 90 °C for 2 h. Fragments were rinsed with distilled water and stained with a solution of cresyl blue for 15 min at 90 °C in water bath. They were finally rinsed with distilled water and observed under a microscope, each fragment being carefully checked along its entire length, at magnifications of 100 and 400 to record mycorrhizal structures, such as arbuscules, hyphal walls, vesicles, and intra- and intercellular hyphae. The AMF arbuscule and vesicle frequency and levels inside the root bark were measured by the method of Trouvelot et al. (1986) and expressed as frequency of AMF colonization (F%), intensity of AMF colonization (M%), and arbuscule abundance (A).

Parameters of mycorrhization were calculated with the MYCOCALC software (available at: http://www.dijon.inra.fr/mychintec/Mycocalc-prg/download.html.)

4.3 Results and Discussion

4.3.1 Richness, Diversity, and Identification of AMF Spores

Concerning the estimation of the spore density in the soil, the average recorded was 1200 spores/100 g of soil, and extracted spores had generally a spherical shape. A detailed analysis of the morphological characteristics of the spore's community revealed the presence of three genera (Fig. 4.3) in the order of Glomales: *Scutellospora*, which was the most abundant, *Glomus*, and *Septoglomus*.

The largest proportion belongs to the family of Gigasporaceae, when it comes to *Scutellospora* sp. The family of Glomeraceae is represented by *Glomus* sp. and *Septoglomus* (Fig. 4.4).

Analysis of AMF spores found in the soil of *H. pallidum* Desf. showed that on average their number was high and reached 1200 spores/100 g soil, which was more important than some results in the literature. It was reported that about 170 spores/100 g dry soil were found in the association *Quercus ilex-Tetraclinis articulata* (Bakkali Yakhlef et al. 2009), about 63–98 spores/100 g soil in the coastal dunes of the Souss-Massa (Hatimi and Tahrouch 2007), and about 2–22 spores/100 g soil in the rhizosphere of *Casuarina* sp. (Tellal et al. 2008).

However, about 5384 spores were obtained in 100 g of soil cultivated previously by peanut (Bouhraoua et al. 2015).

Our study has shown a high density of spores of mycorrhizal fungi in soil under *H. pallidum* Desf., indicating a significant mycorrhizal potential infection; counting AMF spores from soils showed that the plants are capable to enrich the soil with mycorrhizal propagules. The result reflects the biological soil fertility.

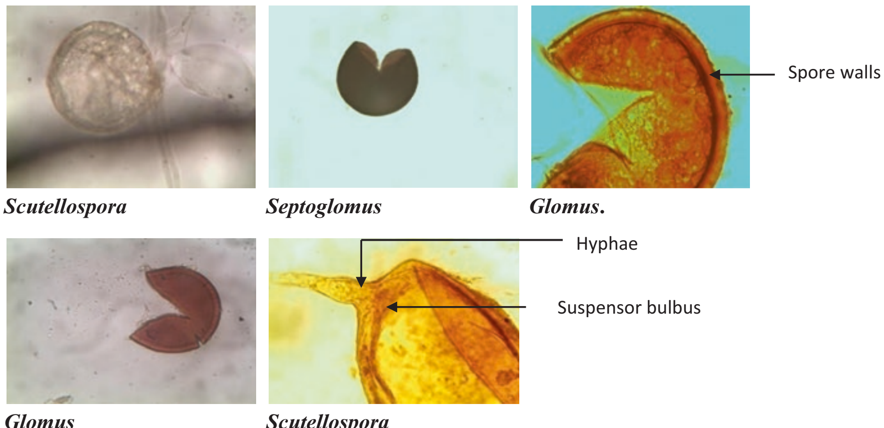


Fig. 4.3 Showing genera of Glomales: *Scutellospora* that was the most abundant, *Glomus*, and *Septoglomus*

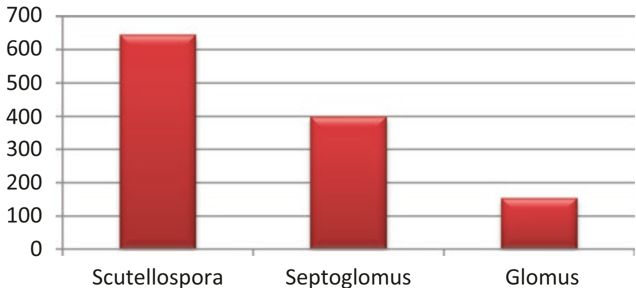


Fig. 4.4 Abundance of the endomycorrhizal genera spores isolated from the rhizosphere of *Hedysarum pallidum* Desf.

4.3.2 Evaluation of Root Mycorrhization

The examination of the roots of *Hedysarum pallidum* Desf. showed that all of them were mycorrhized and densely colonized, and the mycorrhizal frequency of the roots measured in the studied site was important reaching 98.89% (Fig. 4.5). Also, the mycorrhization intensity attained 50.9%, and the arbuscular intensity was 34.76%.

The observation of *Hedysarum pallidum* Desf. root fragments collected from the studied site helped to demonstrate the presence of mycorrhizal structures: intra- and extraradical hyphae, vesicles, and arbuscules (Fig. 4.6).

The mycorrhizal roots rate of *H. pallidum* Desf. observed in this study appeared higher compared with those of *Tetraclinis articulata* (between 27 and 57%) and *Ceratonia siliqua* (40%) recorded in Morocco by Abbas et al. (2006).

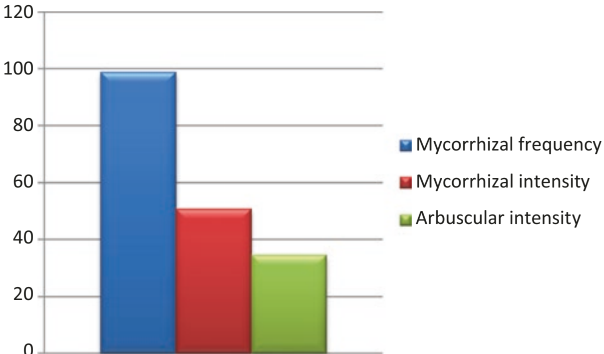


Fig. 4.5 Mycorrhizal parameters of *Hedysarum pallidum* Desf. on the soil of the studied site

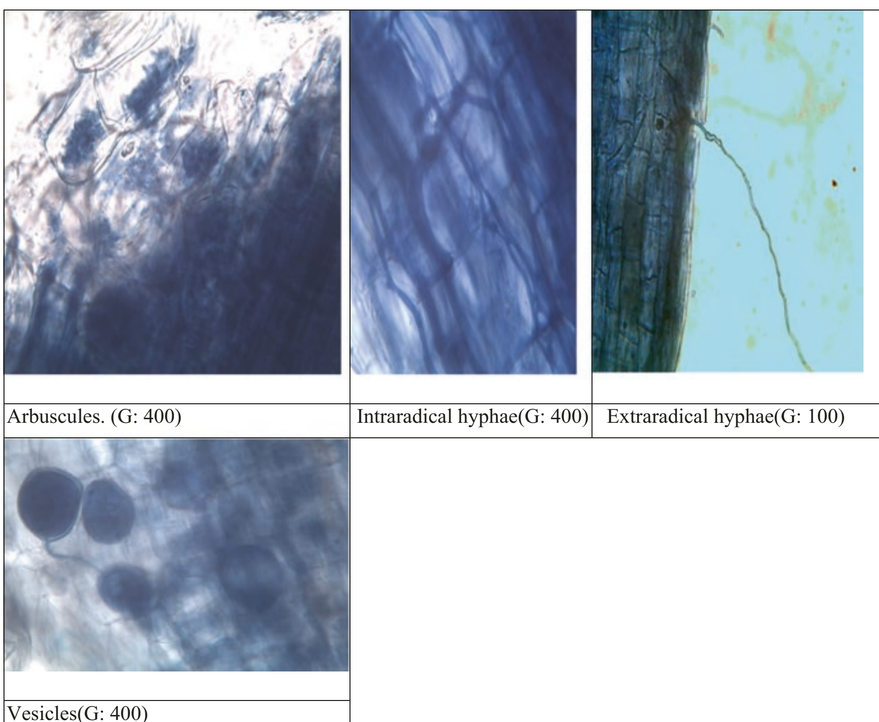


Fig. 4.6 Six roots with mycorrhizal infection of *Hedysarum pallidum* Desf.

The mycorrhizal infection observed high soil propagule pressure on the roots of *Hedysarum pallidum* Desf., also indicating a relatively large abundance of arbuscules and vesicles in the roots. These parameters indicate the ability of fungi to spread into the root system of the plant and to establish exchanges through the fine arbuscular ramifications. *Hedysarum pallidum* Desf. was able to provide a high

number of infective propagules per unit of soil weight. Previous reports have already described that many plants from the Mediterranean area form arbuscular mycorrhizae association and have been classified as "obligatory mycorrhizal" or as "highly dependent on mycorrhiza (Caravaca et al. 2002).

4.4 Conclusion

By integrating particular parameters (richness and diversity of AMF spores, high mycorrhizal frequency, and different mycorrhizal structures in the roots), *Hedysarum pallidum* Desf. is regarded as a mycotrophic legume establishing a close symbiosis between the endomycorrhizae of the rhizosphere. This species promotes the growth of the propagules in the soil which is biologically fertile. For this reason, autochthonous plant species are widely used for reclaiming degraded lands in Mediterranean areas (Caravaca et al. 2002). Therefore, the diversity of arbuscular mycorrhizal fungi naturally present in the soils can be selected and used in (a) the restoration of degraded ecosystems, (b) the improvement of the production of vigorous forage plants, and (c) the valuation of the fallows and their enrichment in organic nitrogen.

References

Abbas Y, Ducousso M, Abourough M, Azcon R, Duponnois R (2006) Diversity of arbuscular mycorrhizal fungi in Tetraclinisarticulata (Vahl) masters woodlands in Morocco. Ann For Sci 63:285–291

Azcon-Aguilar C et al (2003) Analysis of the mycorrhizal potential in the rhizosphere of representative plant from desertifcation-threatened Mediterranean shrublands. Appl Soil Ecol 22:29–37

Bakkali Yakhlef S Abbas Y, Abourouh M, Hafidi M, Duponnois R 2009 Diversité phénotypique et moléculaire des champignons mycorhiziens de l'association Quercus ilex-Tetraclinisarticulata. Actes du congrès international "Biotechnologie microbienne au service du développement" (MICROBIOD) Marrakech, MAROC 02–05 Novembre 2009. www.ucam.ac.ma/micro

Bouhraoua D, Aarab S, Laglaoui A, Bakkali M, Arakrak A (2015) Effect of PGPR on growth and mycorrhization of KT22's peanut variety (Arachishypogaea L.) grown in the northwest of Morocco. Am J Res Commun 3(2):12–24. www.usa-journals.com, ISSN: 2325-4076

Caravaca F, Barea JM, Figueroa D, Roldán A (2002) Assessing the effectiveness of mycorrhizal inoculation and soil compost addition for enhancing reafforestation with Olea europaea subsp. sylvestris through changes in soil biological and physical parameters. Appl Soil Ecol 20:107–118

Caravaca F, Alguacil MM, Barea JM, Roldán A (2005) Survival of inocula and native AM fungi species associated with shrubs in a degraded Mediterranean ecosystem. Soil Biol Biochem 37:227–233

Foury A (1954) Les légumineuses fourragères au Maroc (deuxiéme partie). Cah Rech Agron 5:287–658. INRA, Rabat, Maroc

Gerdemann JW, Nicolson TH (1963) Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. Trans Br Mycol Soc 46:235

Hatimi A, Tahrouch S (2007) Caractérisation chimique, botanique et microbiologique du sol des dunes littoral du Souss Massa. Biomatec Echo 2(5):85–97

Herrman S, Oelmmuller R, Buscot F (2004) Manipulation of the onset of ectomycorrhiza formation by indole-3-acetic acid, activated charcoal or relative humidity in the association between

oak micronuttines and Pilodermacroceum influence on plant development and photosynthesis. J Plant Physiol 161(5):509–517
Ibiremo OS, Fagbola O (2008) Effect of phosphate fertilizers and arbuscular mycorrhizal fungi inoculation on the growth of cashew seedlings in two soils in Nigeria. Niger J Soil Sci 18:138–146.4
Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular–arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55:158–161
Rached-Mosbah O (1997) Les exploitations minières et leurs nuisances, exemple d'une ancienne mine d'antimoine dans le Djebel Hamimat. IIème journée de l'environnement de Constantine
Tellal M, Qarro M, Arahou M, Abourouh M, Tellal R, Ghazi L, Douira A (2008) Effet de l'Actinomycète Frankia sur la croissance et la fixation de l'azote de Cusuarinaglauca et C.cunnunghamiana. Revue sécheresse 19(3):211–216
Trouvelot A, Kouch J, Gianinazzi-Pearson V (1986) Mesure du taux de mycorhization VA d'un systèmeradiculaire: recherche de méthodes d'estimation ayant une signifcationfonctionelle. In: Actes du 1er Séminaire sur les mycorhize: physiologie et génétique, 1–5 juillet 1985. Inra, Dijon, France, Paris, pp 217–221
Walker C (1982) Systematics and taxonomy of arbuscular endomycorhizal fungi (Glomales) a possible way forward. Agronomie 12:887–897
Wardle DA (2002) Communities and ecosystems: linking above ground and belowground components. Princeton University Press, Princeton, NJ

Friends and Foes: Phyto-Microbial Interactions in Molecular Perspective

5

Shyam Solanki, Gazala Ameen, Debankur Sanyal, Shalu Jain, Ammar Elakhdar, Shwetank Lall, Kishore Chittem, Leah Brueggeman, Ajay Kumar, and Robert Brueggeman

Abstract

Soil acts as an natural abode for plants as well as for diverse micro/macroflora and fauna, and thus provides the rhizosphere environment, the fraction of soil surrounding the root system, where plants interact with their root microbiomes. Plants maintain a continuum of interactions with associated microbes that have effects on their cellular physiology resulting in changes in development and function, which can have both positive and negative outcomes. Plant-microbial or microbial-microbial interactions that occur at the plant root-soil interface can also have dynamic effects on the rhizosphere microbiome that greatly affects the overall health and vigor of plants, a key metric in agricultural productivity. This chapter reviews the current understanding of the range of interactions happening in the rhizosphere and the recent advancements that next generation sequencing technologies have had on the ability to identify and classify the rhizosphere microbiome.

S. Solanki (✉) · G. Ameen · L. Brueggeman · R. Brueggeman
Department of Crop and Soil Sciences, Washington State University, Pullman, Washington, DC, USA
e-mail: shyam.solanki@wsu.edu

D. Sanyal
Department of Soil Science, North Dakota State University, Fargo, ND, USA

S. Jain · K. Chittem
Department of Plant Pathology, North Dakota State University, Fargo, USA

A. Elakhdar
Institute of Plant Genetic Resources, Kyushu University, Fukuoka, Japan

S. Lall
ICAR-Indian Agricultural Statistics Research Institute, New Delhi, India

A. Kumar
Department of Plant Sciences, North Dakota State University, Fargo, ND, USA

© Springer Nature Singapore Pte Ltd. 2020
M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_5

Keywords
Rhizosphere · Microbiome · Next generation sequencing (NGS) · PhyloChip · 16S rRNA · Shotgun metagenomics

5.1 Introduction

"We know more about the movement of celestial bodies than about the soil underfoot."

This remark from Leonardo da Vinci can still be used to describe the current knowledge gaps in the understanding of soil microbiomes. Although scientific perspectives and approaches have accelerated our understanding in recent generations, we have just begun to break the shackles in this field of science as characterizing and understanding the diversity underlying the biology and ecology of the soil microbiome are still one of the great challenges in science. Soils act as the natural biological support with a complex group of micro- and macrofauna that represents one of the most important and richest microbial communities on earth. This complex ecosystem that harbors vast genetic diversity, which has only just begun to be detected, let alone harnessed, is hypothesized to contain new forms of antibiotics, catalysts, and metabolites that could be utilized for yet to be imagined purposes in human medicine, agriculture, and beyond (Gans et al. 2005; Wagg et al. 2014).

As the home of many forms of life, mediating diverse and multiphyletic interactions, the soil acts as the *factory of life*. Soil organisms consist of harmful, beneficial, and neutral living components in the perspective of plant health and productivity. The rhizosphere, defined as *all soil under plant root influence*, acts as a hotspot between soil inhabiting organisms and plant roots, thus providing the temporal and special cues that drive the dynamic interactions that occur in it (Hinsinger and Marschner 2006; Raaijmakers et al. 2009). The rhizosphere communities are largely made up of organisms such as archaea, bacteria, oomycetes, fungi, algae, arthropods, and their associated viruses as well as other classes of living organisms (Buée et al. 2009; Mendes et al. 2013).

Many biotic and abiotic factors including but not limited to soil type, soil properties, prevailing environmental conditions, inhabiting flora and fauna, and agricultural management practices have strong influence on the structural and functional diversity of rhizosphere communities (Berg and Smalla 2009). Also, the importance of plant species and how their root properties play a large role in the function of soil-phyto-microbial interactions cannot be understated. Plants in their natural environment are surrounded by a remarkable diversity of plant associated microbes, termed as "the plant microbiota." Establishment of microbiota in and around the plant depends on multiple factors that facilitate niche colonization, a term used for establishment of a population of species in a community, taking account of totality of biological and environmental factors affecting the species and utilization of these factors in the species establishment (Bulgarelli et al. 2013; Vandermeer 1972). Clearly, the majority of bacterial diversity in the soil is dominated by a few

bacterial phyla such as Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, and Proteobacteria (Fierer et al. 2009). However, it has been shown that the presence of the host plant drives the diversity in the rhizosphere microbiome filtering few classes of bacteria and selecting for Actinobacteria and Proteobacteria classes. Understanding this complex biophysicochemical interplay and its components is imperative to use soil biodiversity for protective agriculture. Next generation omics such as metagenomics and metabolomics approaches provides a remarkable tool to understand the complex plant microbiota and their intertwined complex interactions.

To understand microbial population, the use of next generation sequencing (NGS) provides an excellent tool for detection of genetic diversity represented by 16S rRNA coding gene sequencing, shotgun metagenome (whole genome) or transcriptome profiling. Classification of microbial groups can be achieved by analyzing similarity of sequenced reads to a known reference which has predetermined taxonomical units for phylotyping, or by similarity of sequenced reads within a microbial community and assigning operational taxonomic units (OTUs) (Chen et al. 2013; Schloss and Westcott 2011). Here we will attempt to sketch the rhizosphere microbial interaction in detail and the genomics approaches utilized to classify the microbial community involved in such interactions.

5.1.1 Friends and Foes: A Side to Pick

Rhizosphere acts as a battlefield between plants and microbes, full of microbial warriors, few acting as foe, few as friends, and the rest not directly taking part in this battle but just being part of the dynamic microbiome interactions with microbiome structure and community, deciding the fate of such interactions around the plant roots. Many bacterial and fungal communities help plants to derive micro- and macronutrients essential for plant growth and development. Plant reciprocates the favor by providing habitat and carbon sources to the bacterial and fungal communities in its rhizosphere. Such mutualistic relationships benefit both interacting partners and are also utilized in agricultural practices. However, pathogenic microbes within the microbiome are greedy and seek out opportunities to utilize the plant carbon sources without reciprocating any energy. This invasion and infection of the plant system to use its nutrient source for its establishment, colonization, and reproduction are the foundation of a host-pathogen relationship and once established as a specialized pathogen begin a molecular arms race where both constantly evolve to gain the upper hand in the interaction.

5.1.2 Microbiome Communities as a Friend for Plant

Root microbiomes provide a remarkable pool of friends for plants, helping them in the nutrient cycling and maintenance of rhizosphere activity essential for proper plant health and vigor. Many bacterial communities majorly belonging to Actinobacteria,

Bacteroidetes, Firmicutes, and Proteobacteria inhabit a soil zone that is in close proximity to the plant root interface, making up an active site of the soil biome. Few of these bacterial species help in N_2 fixation symbiotically or nonsymbiotically. Legumes fix atmospheric N_2 symbiotically with a specific group of bacteria in the genus *Rhizobium* (Phillips 1980). Rhizobia, a group of gram-positive, spore-forming bacteria, belong to the α-subclass of Proteobacteria (Rhizobiaceae) and nodulate legumes (Hong et al. 2012; Peters et al. 1995). The Proteobacteria have been classified into six different genera, namely, *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Allorhizobium*, *Azorhizobium*, and *Sinorhizobium*, which are phylogenetically separated from each other (De Lajudie et al. 1998). The Rhizobia reduce atmospheric N_2 inside specialized organs, "nodules," which are formed as an outcome of a multistep process initiated with infection of plant roots by Rhizobia due to positive chemotaxis to root exudates (Caetano-Anollés et al. 1992). The Rhizobia bacteria are attracted to amino acids, the dicarboxylic acids present in the root exudates, and even to very low concentrations of excreted flavonoids, which are exudates from the secretory part of *rhizodeposition*, and the bacteria once in contact subsequently attach to the plant root surface. The infection proceeds with specific attachment of bacterial polysaccharides (lipochitooligosaccharides) to specific lectin proteins of host plants, followed by root hair curling, deforming, and branching (Bohlool and Schmidt 1974; Van Rhijn and Vanderleyden 1995; Yuan et al. 2017). Infection threads are formed, cortical cells divide to form nodules, and Rhizobia are released in the plant cell cytoplasm through the infection thread (Wood and Newcomb 1989) and colonize and initiate biological nitrogen fixation, a symbiotic interaction between the plant and the transformed bacteriod. Biological nitrogen fixation (BNF) is an important input of nitrogen (N) in the global agricultural systems. BNF is catalyzed by a complex metalloenzyme, nitrogenase, which is composed of two main components: a heterotetrameric core and a homodimeric dinitrogenase reductase subunit (Kim and Rees 1994). The dinitrogenase reductase is an iron (Fe) protein containing 4 Fe atoms (Fe_4S_4), and the heterotetramer is a molybdenum-iron (MoFe) protein containing 2 Mo and 30 Fe atoms (Peters et al. 1995). This enzyme system helps the reduction of dinitrogen (N_2) that involves reduction of Fe binding protein by different electron carriers (like ferredoxin), followed by transfer of one electron from Fe protein to Mo-Fe protein, and subsequently from Mo-Fe protein to the N_2 (the substrate) in the last step of reduction (Kim and Rees 1994). This conversion (reduction) of atmospheric N_2 to ammonia (NH_3) can only be carried out by a small group of microorganisms like Rhizobia, *Frankia* and *Azospirillum*, known as "diazotrophs" (Burns and Hardy 2012).

The first discovered *Rhizobium* genes related to nitrogen fixation were *nif* (fixation) and *nod* (nodulation) genes, and it was found that the *nod* genes and genes related to host specificity were tightly clustered with the *nif* genes(Long 2001). Dinitrogenase reductase subunit of the nitrogenase complex is encoded by the *nif*H gene, while the heterotetrameric core is encoded by *nif*D and *nif*K (Yates et al. 1992). The *nif*H gene is a universally accepted biomarker for BNF as it is found to be highly conserved among N_2-fixing organisms in different natural environments (such as marine, terrestrial, and hydrothermal sites) and is widely used to study the ecological and evolutionary aspects of N_2-fixing bacteria

(Izquierdo and Nüsslein 2006; Mehta et al. 2003; Wartiainen et al. 2008). A previous study by Hennecke et al. (1985) reported that the phylogeny of the *nif*H genes is closely related to the 16S rRNA gene and the *nif*H genes and might have evolved with the bacterial evolution. This finding is in contrast with the convention of "lateral gene transfer" among microorganisms. However, a comprehensive study focused on sequence analysis of a large number of *nif*H genes was carried out and found that the phylogenetic trees of *nif*H genes were inconsistent with the phylogenetic tree of 16S rRNA; instead, a phylogenetic similarity was found with *nod*A genes (Haukka et al. 1998). The *nif*H genes were mostly studied by culture-independent approaches because these provide better understanding of the bacterial community than the culture-dependent method microbiome community studies (Poly et al. 2001). Along with PCR amplification followed by denaturing gradient gel electrophoresis (DGGE), other approaches like DNA hybridization analysis, restriction fragment analysis, and cloning and sequencing approaches were used to study the diversity and abundance of *nif*H genes (Freitag and Prosser 2003; Hamelin et al. 2002; Neufeld et al. 2001; Stres et al. 2004). Composition of *nif*H gene pools were also studied in various environments using various techniques, such as polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP), PCR cloning, DGGE, and fluorescently labeled terminal (FLT)-RFLP (Chelius and Lepo 1999; Piceno and Lovell 2000; Zehr et al. 1995).

Different soil physicochemical properties like texture, total carbon (C), and total N influence the variation in *nif*H gene pools, and the *nif*H genetic structures are the outcomes of the adaptation of the changing or constant soil environmental conditions (Poly et al. 2001). In a gene expression study on *Azotobacter vinelandii*, it was revealed that the *nif*H gene expression is positively correlated with N_2-fixation (Bürgmann et al. 2003). Again, different dry bean cultivars showed differential *nif*H gene expressions, depending on their symbiotic efficiencies with the rhizobia strains, correlated with their N_2-fixation potentials (Sanyal et al. 2020, Akter et al. 2013).

The diversity and abundance of *nif*H genes have been associated with N_2-fixation rates and thus depend on the structure and dynamics of bacterial communities (Hsu and Buckley 2009; Reed et al. 2010). PCR and qPCR are considered as powerful tools to study specific gene expression and soil microbiome diversity (Bürgmann et al. 2003; Zehr and McReynolds 1989). Zehr and McReynolds (1989) designed degenerate universal PCR primers to target a wide range of variable *nif*H gene sequences in diverse bacterial communities and for the first time were able to study community composition. Later, many universal primers were designed to target the *nif*H gene in the most conserved portions of amino acid sequences (Bürgmann et al. 2004). In an "in silico" analysis, 51 universal and 35 group specific *nif*H primers were assayed using more than 20,000 *nif*H gene sequences, and 15 universal markers were identified that successfully targeted more than 90% of the N_2-fixers (Gaby and Buckley 2017). However, this study also revealed that many of these primers targeted genes that are not related to N_2-fixation, and were often found to miss significant variants of *nif*H genes (Gaby and Buckley 2017).

5.1.3 Microbial Foes: A Concern for Plant Health

Soil-borne pathogens are major yield limiting factor in most agriculture crops worldwide. Soil-borne pathogens can survive in the bulk soil as a part of the soil microbiome for several years. When a suitable host plant is available, the pathogens identify the host plant by perceiving cues from the rhizosphere and initiate infection to cause disease. The rhizosphere acts as a playground to the complex microbial community, which can also affect the host pathogen interaction influencing the outcome of the pathogen infection process, resulting in compatible (susceptibility and disease) or incompatible (resistant) interactions. Bacterial pathogens such as *Erwinia* and *Pectobacterium* species cause disease in plants and reduce their health and vigor and are important soilborne pathogens. Fungi and oomycetes make up one of the most important groups of plant pathogens accounting for more than 70% of all the major crop diseases (Agrios 2005). Fungi are eukaryotic, filamentous, multicellular, and heterotrophic organisms. They produce a network of hyphae called the mycelium and absorb nutrients from their host (Alexopoulos et al. 1996). Most of host-pathogen associations are very specific with a specialized host range. The host-limiting fungi are termed as biotrophs (feed from living host cells without killing them) or necrotrophs (kill the host tissues to derive nutrients as part of the colonization process). Some of the fungal pathogens overwinter in soil and may survive for years in soil even in the absence of their host. Nevertheless, controlling root fungal pathogens is a major challenge compared to foliar pathogens that attack the aboveground portions of the plant (Bruehl 1987). *Fusarium* root rot is a prominent root disease and is caused by a complex of several *Fusarium* species. For example, four species of the *Fusarium* (*F. graminearum*, *F. culmorum*, *F. poae*, and *F. sporotrichioides*) are mainly associated with the foot and crown rot disease in wheat (Kuzdraliński et al. 2014). Another devastating disease of wheat and barley is Fusarium head blight (FHB), which also causes grain contamination by secreting harmful mycotoxins, leaving them useless for consumption as well as planting material (McMullen et al. 2012). To date, at least 15 known species of the *F. graminearum* species complex have been reported as the causal agent of FHB (O'Donnell et al. 2008; Wang and Cheng 2017). Specific species were more prevalent than others in FHB disease complex to wheat, and such variations are related to the ecological preference of the pathogens. One of the most common species, *F. oxysporum*, causes vascular wilt disease in a wide variety of economically important crops (Beckman 1987). Sudden death syndrome, a major yield-limiting soybean disease, is caused by *F. virguliforme* (previously named *Fusaium solani* f. sp. *glycines*).

Signaling processes are the first and most critical step in defining either success or failure in establishing disease. Fungal and oomycete pathogens depend on several different signaling molecules from the host plant to germinate, grow, and persist in the rhizosphere. Many cues such as amino acids, organic acids, flavonols, glucosinolates, indole compounds, fatty acids, and polysaccharides are secreted from microbial community and plant roots in the rhizosphere to establish communication. This wide range of secreted compounds is collectively known as

rhizodeposition (Dakora and Phillips 2002; De-la-Peña and Loyola-Vargas 2014). The type and composition of root secretion are dependent on plant species and cultivars, growth stage, and stress factors, which all greatly influence the rhizosphere microbiome communities. Root exudates can affect the microbial community in the soil favoring beneficial microbes while preventing the growth of harmful microbes (Huang et al. 2014; Szoboszlay et al. 2016). Chemical analysis of vegetable root exudates revealed the presence of major organic acids such as citric, succinic, and malic acid and major sugars such as fructose and glucose (Badri et al. 2012; Kamilova et al. 2006). Low concentrations of phenolic compounds like p-hydroxybenzoic, gallic, coumaric, cinnamic, ferulic, salicylic, and sinamic acids in root exudates stimulated conidial germination of *F. oxysporum* f. sp. *niveum*, while inhibitory effects were observed at higher concentrations (Wu et al. 2008a, b) indicating roles of rhizodeposition in the species dynamics of rhizosphere microbiota. A study by Wasmann and VanEtten (1996) showed that transformation-mediated chromosome loss and pisatin demethylase gene (*PDA1*) disruption can decrease the virulence of the pea pathogen *Nectria haematococca* (anamorph *F. solani* f. sp. *pisi*). Pisatin (a phytoalexin) can be detoxified by cytochrome P-450-mediated demethylation. Recently, a cluster of five pea pathogenicity (PEP) genes, which are expressed during *N. haematococca* infection, were identified in close proximity to *PDA1* on a supernumerary chromosome (Han et al. 2001). The PEP gene cluster have differences in GC-content and codon bias compared to genes on essential chromosomes indicating that horizontal transfer occurred for these pathogenicity gene clusters.

5.2 Metagenomics Approaches for Taxonomical and Functional Classification of Microbiomes

To understand and unravel the components of microbiomes, it is required to adopt a comprehensive approach rather than studying each component separately. Use of molecular techniques such as PCR, qPCR, and DGGE provides valuable tools to study individual elements of the microbiome, but they are insufficient to undertake large-scale analyses to understand the system as a whole (Sharpton 2014). The advent of next generation sequencing techniques and recent high-throughput sequencing technologies (HTST) coupled with state-of-the-art bioinformatics pipelines to assemble sequencing reads provided a boom for soil microbiome studies to explore the diverse taxonomical inhabitants in the rhizosphere. Reduced sequencing cost of per mega base of DNA sequence (Fig. 5.1) further made technology available to all, and genome sequencing became more common specially for small genomes such as fungal, bacterial, and viral whole genome sequencing and pan-genome sequencing (Wetterstrand 2016). Today, many approaches are available for microbiome species identification and classification in conjunction with NGS platforms.

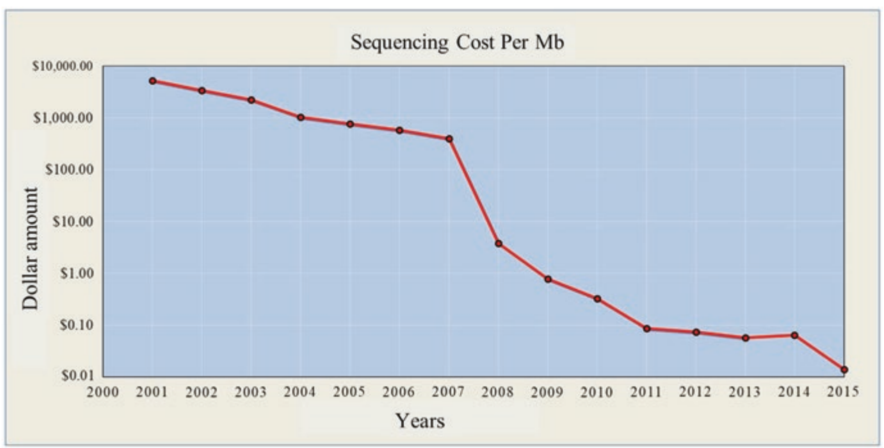


Fig. 5.1 An estimation of raw cost of per megabase (Mb) of DNA sequence over a period of time (reproduced from the National Institutes of Health (Wetterstrand 2016))

5.2.1 PhyloChip and Amplicon-Based Classification of Microbiomes

Identification and phylogenetic classification of fungal species largely rely on internal transcribed spacer 1 (ITS1) and ITS2 region sequencing of the ribosomal RNA (rRNA) cistron as a universal DNA barcode (Schoch et al. 2012; Stielow et al. 2015). However, ITS2 is considered as a region of choice for fungal classification due to better representation in databases and less variability in length (Nilsson et al. 2009; Op De Beeck et al. 2014). For fungal and oomycete genomics and functional data analysis, FungiDB provides an excellent database for the design of experimental primers to carry out phylogenetic analysis (Stajich et al. 2012). Nonetheless, the major portion of the soil microbiome is encompassed by prokaryotes such as bacterial species; thus, we will limit our discussion to the perspective of bacterial microbiome. Nuclear 16S rRNA (ribosomal RNA) gene sequencing has been successfully utilized to identify prokaryotic microbial diversity in natural and agricultural ecosystems (Fierer et al. 2012; Pace et al. 1986). The ubiquitous prokaryotic 16S rRNA cistron is comprised of nine V1-V9 hypervariable regions flanked by conserved DNA sequences suitable for universal primer binding and amplification. Thus, the diversity in variable region is utilized to classify the organisms based on sequence dissimilarities (Chakravorty et al. 2007; Ree et al. 1989). The 16S rRNA gene sequences for bacterial microbiome studies are available from the National Center for Biotechnology Information (NCBI) or other secondary datasets such as EzBioCloud (Yoon et al. 2017). The recent interest of the scientific community to study the soil microbiome in agricultural and non-agricultural ecosystems has led to the development and utilization of the high-density 16S rRNA gene oligonucleotide microarray popularly known as the *PhyloChip* (Brodie et al. 2007). In the study by Weinert et al. (2011), a high-density 16S rRNA probe-based PhyloChip

array capable of detecting 8741 known OTUs was utilized to study and characterize the bacterial microbiota of two different field sites with different soil properties from the rhizosphere of three different potato cultivars. Some 2432 OTUs were detected and classified into 43 phyla in which only one phyla comprised of less than 10 OTUs. Interestingly, differences in soil between two sites influence root bacterial microbiota describing 28% divergence in detected bacterial OTUs, whereas only 9% of the OTUs showed cultivar dependency. Approximately 4% of the OTUs were dependent on both cultivar and site, belonging to Proteobacteria, Bacteroidetes, Firmicutes, Chloroflexi, and Streptomycetaceae phyla. Thus, these phyla dominated the bacterial community in each site irrespective of cultivars grown. This study concludes that historical profiling of soil plays a major role in determining the potato rhizosphere bacterial microbiota composition rather than the effect of the cultivar genotype itself. However, such probe-based studies relied on dominant ribotypes in the sample profile and were limited to the number of known probe OTUs on the PhyloChip. The latest generation of PhyloChip arrays includes 60,000 OTUs capable of classifying147 phyla and 1123 classes of bacterial and archaeal domains, thus enormously increasing its capacity (Hazen et al. 2010). Using the high density PhyloChip, Mendes et al. (2011) identified 33,346 bacterial and archaeal OTUs in the rhizosphere of sugar beet plants grown in disease suppressive soil for *Rhizoctonia solani*, a fungal pathogen (Mendes et al. 2011). Interestingly, Proteobacteria, Firmicutes, and Actinobacteria were found associated with disease suppression in the study samples (Mendes et al. 2011). Thus, this study implicates a role of natural modification in microbial communities to provide disease suppression, a remarkable finding useful for agricultural crop settings. A study carried out on wild and domesticated barley (Bulgarelli et al. 2015) to understand associated bacterial root microbiota indicated a large share of microbiota is comprised of Actinobacteria, Bacteroidetes, and Proteobacteria using the 16S rRNA survey with an NGS approach. Interesting inferences were made upon annotation and functional classification of sample sequences acquired from bulk soil and host inhabited rhizosphere and root samples. Root and rhizosphere bacterial taxas were significantly enriched for the 12 functional categories of proteins considered to be important for adaptation of microbes to interact with the host root along with protein families for sugar transport and iron mobilization. Thus, the presence of the host barley plants drives the diversity of the rhizosphere for protein functional categories considered to be important for host-microbe interactions either pathogenic or mutualistic (Bulgarelli et al. 2015).

5.2.2 Shotgun Metagenomics for Microbiome Studies

Despite the majority of studies utilizing DNA barcodes and 16S rRNA amplicon-based pyrosequencing, shotgun sequencing approaches for metagenome analyses provide a remarkable upgrade to classify and understand the structure and function of rhizosphere microbiome interactions. PCR-based sequencing approaches can introduce amplification bias (Hong et al. 2009) and require availability of known

taxonomically informative genetic markers and thus make analysis difficult for novel or highly diversified bacterial taxa. Further, horizontal gene transfer between distantly related taxons, especially for 16S rRNA genetic locus, can result in over-representation of diversity (Acinas et al. 2004). Thus, shotgun approaches enables researchers to study uncultured microbiota and also overcome the majority of the aforementioned concerns for PCR-based sequencing approaches. The shotgun sequencing approach was successfully utilized in a study conducted by Castañeda and Barbosa (2017) to investigate the taxonomic diversity of bacterial and fungal species and their metabolic function in the forest and vineyard soils in Chile. They found bacterial OTU abundance in both habitats, i.e., forest and vineyard, and Proteobacteria accounted for the majority of bacterial phyla and most of the fungal communities were assigned to the Ascomycota. Thus, using shotgun approaches enabled researchers to study the soil from both the bacterial and fungal perspective using a single DNA library, whereas in amplicon-based sequencing, it is required to make fungal and bacterial amplicon libraries separately based on ITS and 16S rRNA markers, respectively.

5.2.3 Approaches to Acquire and Analyze Metagenomics Data

Various large-scale coordinated efforts were initiated to investigate and characterize the soil microbial taxonomy and functional diversity such as the Earth Microbiome Project (Gilbert et al. 2014), ECOFINDERS (http://projects.au.dk/ecofinders/), and TerraGenome (Vogel et al. 2009). These initiatives provide a good resource of information for any interested novice laboratories that decide to make the leap into soil microbiome studies. However, many practical roadblocks still prevail before carrying out a microbiome analyses. Since the majority of environmental microbes are unculturable, this is a major hurdle to acquiring and studying representative natural environment samples. For example, using soil samples from a single experimental site, Delmont et al. found that the DNA extraction methods can produce larger variations in the metagenomic composition of the microbiome than either the season of sampling or the soil depth (Delmont et al. 2012). Thus, just DNA extraction methods used can have a profound effect on confounding the data for analysis of metagenomic variation. Since the requirement of a high-quality DNA sample is the first step and depending on the sequencing platform, DNA amplification steps may be required for low DNA yielding soil samples (permafrost soil), these PCR steps further add bias to the samples (Mackelprang et al. 2011). Thus, to adopt a common efficient method although unlikely to develop is required for a true representation of sample.

One of the important steps to exploring the complexity of soil microbiomes is the analysis of large amount of sequencing data after the NGS run. Many bioinformatics pipelines and methods have been described. The recent availability of many online analysis tools such as Galaxy and KBase that can perform sequence quality analysis and assembly has made this task a bit less complex and has provided an excellent alternative for setting up a computing facility and expertise in “big data”

analysis. Open-source pipelines such as Quantitative Insights Into Microbial Ecology (QIIME2) (Caporaso et al. 2010) or MEGAN6 (Huson et al. 2007, 2016) are very useful tools, which can be installed locally to analyze data offline. EBI metagenomics provides a cloud-based automated pipeline for analysis and archiving of metagenomic data with tools to determine the phylogenetic diversity as well as functional and metabolic analysis of submitted datasets (Mitchell et al. 2016) (Fig. 5.2). Many informative web resources and literature are also available to walk through the process of data analysis, yet researchers must pave their own paths when utilizing these publicly available tools (Table 5.1) (Clooney et al. 2016; Kunin et al. 2008; Oulas et al. 2015; Quince et al. 2017; Thomas et al. 2012).

5.3 Concluding Remarks

Biodiversity loss is a major concern affecting ecosystem homeostasis and stability among its inhabitants. Soil biodiversity comprised of micro- and macrofauna is still largely understudied; thus, we have insufficient information on the effects that climate change and environmental pollution are having on the soil ecosystems worldwide. The majority of these soil inhabitants or the microbiome components play a crucial role in soil health, nutrient cycle, rhizosphere interactions, and ecosystem function; thus, mass extinction and loss of ecosystem homeostasis could be happening under foot, and we may never be aware of it or its consequences until it is too late.

On the rhizosphere frontline between plant and microbes, many microbial friends, foes, and spectators stand tall in the arena presenting numerous possibilities for interaction. The microbiome structure and community decide the fate of such interactions in the rhizosphere. Many bacterial and fungal pathogen communities prevail as a part of soil microbiome, yet introduction of the agricultural systems sufficiently modify the microbiome structure of particular soils leading to the establishment of a unique microbial community. In a study conducted by Costa et al. (2007) comparing uncultured *Pseudomonas* in bulk and rhizosphere soil, it was observed that strawberries infected with *Verticillium dahliae* attract fluorescent pseudomonads having close relatedness to *P. fluorescens* strain F113, a biocontrol agent. This strain produces DAPG (2,4-diacetylphloroglucinol), a phenolic compound having antiphytopathogenic properties (Bangera and Thomashow 1999; Costa et al. 2007). Thus, such examples indicate the summoning of beneficial bacteria from microbial pools by plant for their own benefits. On the other hand, pathogenic microbes act as foes continuously invading plants as a host to gain nutrients required for their pathogenic lifestyle. Many fungal pathogens such as *Sclerotinia sclerotiorum*, *Fusarium oxysporum*, and *Pythium* spp. cause root disease in a wide plant host range adversely affecting the plant health as previously discussed. Symbiotic microbes live in a mutualistic relationship with plants and thus work as a friend in the soil microbiome fraction. The use of *Bacillus* and *Trichoderma* strains (Benítez et al. 2004; Chowdhury et al. 2015) is common as a biocontrol agent to augment the soil microbial population to keep check on pathogenic fungal strains,

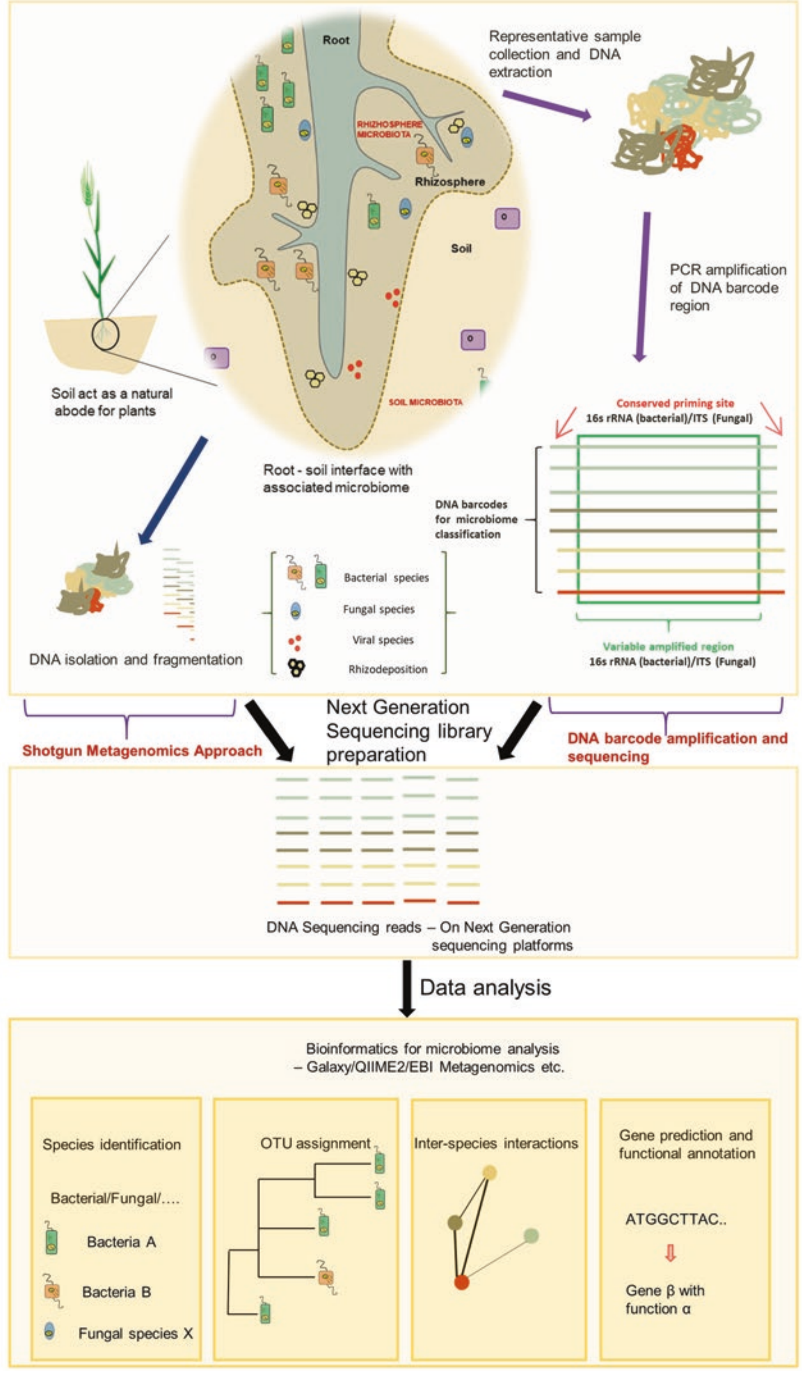


Fig. 5.2 An illustration and schematic overview describing the rhizosphere microbiome and its interactions. PCR amplicon-based or shotgun metagenomics approaches can be adopted using next generation sequencing (NGS) approaches. Various bioinformatics pipelines can be adopted for sequence assembly and alignment for microbiome identification, taxonomical classification, and functional annotation

Table 5.1 Few of the Web-based graphical user interface (GUI) and command line interface (CLI) bioinformatics pipelines for microbiome metagenomics data analysis and visualization

Resource	Type	Link
EBI metagenomics	Web	https://www.ebi.ac.uk/metagenomics/
IMG/M	Web	https://img.jgi.doe.gov/cgi-bin/m/main.cgi
MG-RAST	Web	http://metagenomics.anl.gov/
Metavir	Web	http://metavir-meb.univ-bpclermont.fr/
LEfSe (GALAXY)	Web	https://bitbucket.org/biobakery/biobakery/wiki/lefse
KBase	Web	https://kbase.us/
MicrobesOnline	Web	http://www.microbesonline.org/
MEGAN6	GUI	https://ab.inf.uni-tuebingen.de/software/megan6/welcome
QIIME2	CLI	http://qiime.org/index.html
PATRIC	Web	https://www.patricbrc.org/

and they are extensively studied. *Pseudomonas*-based formulations are also popular for soil augmentation and biocontrol (Weller 2007). Interaction among microbial communities, plant roots, and rhizodeposition to establish rhizosphere microbiota is a complex mechanism and needed to be understood for use for improved agricultural practices and soil health.

The integration of modern genetic tools is allowing for the dissection of the complex structure and makeup of the rhizosphere microbiome in relation to plant-microbe and microbe-microbe interactions and for the study of their intertwined molecular signaling pathways. Next generation sequencing approaches for metagenomics and metabolomics analysis coupled with conventional phenotype-dependent approaches to study the structure and function of microbial life in the plant rhizosphere illuminated and enhanced our limited understanding of these interactions and its components. This information will help us to make better use of the currently underutilized and vastly untapped resources of the soil microbiome. Shotgun-based NGS approaches will not only enable us to classify different known soil microbes but will also help to identify rare species prevailing in certain ecosystem important for its maintenance. It is important to establish a larger comprehensive catalog of microbial communities in different soil types for future comparative studies and taxonomical and functional classification. Characterizing the specific inhabitants of soil microbiome communities will allow us to pinpoint their contributions and cooperation in natural ecosystems and bring them under the purview of biotechnological manipulations to work as microbial factories. The microbiome acts as a dynamic entity and requires its largest to smallest community fractions to work properly as a system of ‘fellowship or disunity’ for its maintenance. Individual microbial contribution to its microbiome entity can be sketched by the famous remark quoted by the fictional character Galadriel in J.R.R Tolkien’s novel Lord of the Rings—“Even the smallest person can change the course of the future.”

References

Acinas SG, Marcelino LA, Klepac-Ceraj V, Polz MF (2004) Divergence and redundancy of 16S rRNA sequences in genomes with multiple rrn operons. J Bacteriol 186:2629–2635
Agrios G (2005) Plant pathology, 5th edn. Academic Press, San Diego, CA
Akter Z, Pageni B, Lupwayi N, Balasubramanian P (2013) Biological nitrogen fixation and nif H gene expression in dry beans (Phaseolus vulgaris L.). Can J Plant Sci 94:203–212
Alexopoulos CJ, Mims C, Blackwell M (1996) Introductory mycology. John Wiley and Sons, New York, NY
Badri DV, Chaparro JM, Manter DK, Martinoia E, Vivanco JM (2012) Influence of ATP-binding cassette transporters in root exudation of Phytoalexins, signals, and in disease resistance. Front Plant Sci 3:149
Bangera MG, Thomashow LS (1999) Identification and characterization of a gene cluster for synthesis of the polyketide antibiotic 2,4-diacetylphloroglucinol from Pseudomonas fluorescens Q2–87. J Bacteriol 181:3155–3163
Beckman, C. (1987). The nature of wilt diseases of plants (APS press)
Benítez T, Rincón AM, Limón MC, Codón AC (2004) Biocontrol mechanisms of Trichoderma strains. Int Microbiol 7:249–260
Berg G, Smalla K (2009) Plant species and soil type cooperatively shape the structure and function of microbial communities in the rhizosphere. FEMS Microbiol Ecol 68:1–13
Bohlool BB, Schmidt EL (1974) Lectins: a possible basis for specificity in the rhizobium–legume root nodule symbiosis. Science 185:269–271
Brodie EL, DeSantis TZ, Parker JPM, Zubietta IX, Piceno YM, Andersen GL (2007) Urban aerosols harbor diverse and dynamic bacterial populations. Proc Natl Acad Sci U S A 104:299–304
Bruehl, G. (1987). Soilborne plant pathogens (Macmillan publishing company)
Buée M, De Boer W, Martin F, van Overbeek L, Jurkevitch E (2009) The rhizosphere zoo: an overview of plant-associated communities of microorganisms, including phages, bacteria, archaea, and fungi, and of some of their structuring factors. Plant Soil 321:189–212
Bulgarelli D, Schlaeppi K, Spaepen S, Ver Loren van Themaat E, Schulze-Lefert P (2013) Structure and functions of the bacterial microbiota of plants. Annu Rev Plant Biol 64:807–838
Bulgarelli D, Garrido-Oter R, Münch PC, Weiman A, Dröge J, Pan Y, McHardy AC, Schulze-Lefert P (2015) Structure and function of the bacterial root microbiota in wild and domesticated barley. Cell Host Microbe 17:392–403
Bürgmann H, Widmer F, Sigler WV, Zeyer J (2003) mRNA extraction and reverse transcription-PCR protocol for detection of nifH gene expression by Azotobacter vinelandii in soil. Appl Environ Microbiol 69:1928–1935
Bürgmann H, Widmer F, Von Sigler W, Zeyer J (2004) New molecular screening tools for analysis of free-living diazotrophs in soil. Appl Environ Microbiol 70:240–247
Burns R, Hardy R (2012) Nitrogen fixation in bacteria and higher plants. Springer, Berlin
Caetano-Anollés G, Wrobel-Boerner E, Bauer WD (1992) Growth and movement of spot inoculated rhizobium meliloti on the root surface of alfalfa. Plant Physiol 98:1181–1189
Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI et al (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336
Castañeda LE, Barbosa O (2017) Metagenomic analysis exploring taxonomic and functional diversity of soil microbial communities in Chilean vineyards and surrounding native forests. Peer J 5:e3098
Chakravorty S, Helb D, Burday M, Connell N, Alland D (2007) A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. J Microbiol Methods 69:330–339
Chelius M, Lepo J (1999) Restriction fragment length polymorphism analysis of PCR-amplified nifH sequences from wetland plant rhizosphere communities. Environ Technol 20:883–889

Chen W, Zhang CK, Cheng Y, Zhang S, Zhao H (2013) A comparison of methods for clustering 16S rRNA sequences into OTUs. PLoS One 8:e70837

Chowdhury SP, Hartmann A, Gao X, Borriss R (2015) Biocontrol mechanism by root-associated Bacillus amyloliquefaciens FZB42 - a review. Front Microbiol 6:780

Clooney AG, Fouhy F, Sleator RD, O' Driscoll A, Stanton C, Cotter PD, Claesson MJ (2016) Comparing apples and oranges?: next generation sequencing and its impact on microbiome analysis. PLoS One e0148028:11

Costa R, Gomes NCM, Krögerrecklenfort E, Opelt K, Berg G, Smalla K (2007) Pseudomonas community structure and antagonistic potential in the rhizosphere: insights gained by combining phylogenetic and functional gene-based analyses. Environ Microbiol 9:2260–2273

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

De Lajudie P, Willems A, Nick G, Moreira F, Molouba F, Hoste B, Torck U, Neyra M, Collins MD, Lindström K et al (1998) Characterization of tropical tree rhizobia and description of Mesorhizobium plurifarium sp. nov. Int J Syst Bacteriol 48(Pt 2):369–382

De-la-Peña C, Loyola-Vargas VM (2014) Biotic interactions in the rhizosphere: a diverse cooperative enterprise for plant productivity. Plant Physiol 166:701–719

Delmont TO, Prestat E, Keegan KP, Faubladier M, Robe P, Clark IM, Pelletier E, Hirsch PR, Meyer F, Gilbert JA et al (2012) Structure, fluctuation and magnitude of a natural grassland soil metagenome. ISME J 6:1677–1687

Fierer N, Strickland MS, Liptzin D, Bradford MA, Cleveland CC (2009) Global patterns in belowground communities. Ecol Lett 12:1238–1249

Fierer N, Lauber CL, Ramirez KS, Zaneveld J, Bradford MA, Knight R (2012) Comparative metagenomic, phylogenetic and physiological analyses of soil microbial communities across nitrogen gradients. ISME J 6:1007–1017

Freitag TE, Prosser JI (2003) Community structure of ammonia-oxidizing bacteria within anoxic marine sediments. Appl Environ Microbiol 69:1359–1371

Gaby JC, Buckley DH (2017) The use of degenerate primers in qPCR analysis of functional genes can cause dramatic quantification bias as revealed by investigation of nifH primer performance. Microb Ecol 74:701

Gans J, Wolinsky M, Dunbar J (2005) Computational improvements reveal great bacterial diversity and high metal toxicity in soil. Science 309:1387–1390

Gilbert JA, Jansson JK, Knight R (2014) The earth microbiome project: successes and aspirations. BMC Biol 12:69

Hamelin J, Fromin N, Tarnawski S, Teyssier-Cuvelle S, Aragno M (2002) nifH gene diversity in the bacterial community associated with the rhizosphere of Molinia coerulea, an oligonitrophilic perennial grass. Environ Microbiol 4:477–481

Han Y, Liu X, Benny U, Kistler HC, VanEtten HD (2001) Genes determining pathogenicity to pea are clustered on a supernumerary chromosome in the fungal plant pathogen Nectria haematococca. Plant J 25:305–314

Haukka K, Lindström K, Young JP (1998) Three phylogenetic groups of nodA and nifH genes in Sinorhizobium and Mesorhizobium isolates from leguminous trees growing in Africa and Latin America. Appl Environ Microbiol 64:419–426

Hazen TC, Dubinsky EA, DeSantis TZ, Andersen GL, Piceno YM, Singh N, Jansson JK, Probst A, Borglin SE, Fortney JL et al (2010) Deep-sea oil plume enriches indigenous oil-degrading bacteria. Science 330:204–208

Hennecke H, Kaluza K, Thöny B, Fuhrmann M, Ludwig W, Stackebrandt E (1985) Concurrent evolution of nitrogenase genes and 16S rRNA in rhizobium species and other nitrogen fixing bacteria. Arch Microbiol 142:342–348

Hinsinger P, Marschner P (2006) Rhizosphere—perspectives and challenges—a tribute to lorenz hiltner 12–17 September 2004—Munich, Germany. Plant Soil 283:vii–viii

Hong S, Bunge J, Leslin C, Jeon S, Epstein SS (2009) Polymerase chain reaction primers miss half of rRNA microbial diversity. ISME J 3:1365–1373

Hong Y, Ma Y, Wu L, Maki M, Qin W, Chen S (2012) Characterization and analysis of nifH genes from Paenibacillus sabinae T27. Microbiol Res 167:596–601
Hsu S-F, Buckley DH (2009) Evidence for the functional significance of diazotroph community structure in soil. ISME J 3:124–136
Huang X, Chaparro J, Reardon K, Zhang R, Shen Q, Vivanco J (2014) Rhizosphere interactions: root exudates, microbes, and microbial communities. Botany 92:267–275
Huson DH, Auch AF, Qi J, Schuster SC (2007) MEGAN analysis of metagenomic data. Genome Res 17:377–386
Huson DH, Beier S, Flade I, Górska A, El-Hadidi M, Mitra S, Ruscheweyh H-J, Tappu R (2016) MEGAN community edition - interactive exploration and analysis of large-scale microbiome sequencing data. PLoS Comput Biol 12:e1004957
Izquierdo JA, Nüsslein K (2006) Distribution of extensive nifH gene diversity across physical soil microenvironments. Microb Ecol 51:441–452
Kamilova F, Kravchenko LV, Shaposhnikov AI, Azarova T, Makarova N, Lugtenberg B (2006) Organic acids, sugars, and L-tryptophane in exudates of vegetables growing on stonewool and their effects on activities of rhizosphere bacteria. Mol Plant-Microbe Interact 19:250–256
Kim J, Rees DC (1994) Nitrogenase and biological nitrogen fixation. Biochemistry 33:389–397
Kunin V, Copeland A, Lapidus A, Mavromatis K, Hugenholtz P (2008) A bioinformatician's guide to metagenomics. Microbiol Mol Biol Rev 72:557–578, Table of Contents
Kuzdraliński A, Szczerba H, Tofil K, Filipiak A, Garbarczyk E, Dziadko P, Muszyńska M, Solarska E (2014) Early PCR-based detection of Fusarium culmorum, F. graminearum, F. sporotrichioides and F. poae on stem bases of winter wheat throughout Poland. Eur J Plant Pathol 140:491–502
Long SR (2001) Genes and signals in the rhizobium-legume symbiosis. Plant Physiol 125:69–72
Mackelprang R, Waldrop MP, DeAngelis KM, David MM, Chavarria KL, Blazewicz SJ, Rubin EM, Jansson JK (2011) Metagenomic analysis of a permafrost microbial community reveals a rapid response to thaw. Nature 480:368–371
McMullen M, Bergstrom G, Wolf E, Dill-Macky R, Hershman D, Shaner G, Sanford D (2012) A unified effort to fight an enemy of wheat and barley: Fusarium head blight. Plant Dis 96:1712–1728
Mehta MP, Butterfield DA, Baross JA (2003) Phylogenetic diversity of nitrogenase (nifH) genes in deep-sea and hydrothermal vent environments of the Juan de Fuca ridge. Appl Environ Microbiol 69:960–970
Mendes R, Kruijt M, de Bruijn I, Dekkers E, van der Voort M, Schneider JHM, Piceno YM, DeSantis TZ, Andersen GL, Bakker PAHM et al (2011) Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science 332:1097–1100
Mendes R, Garbeva P, Raaijmakers JM (2013) The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiol Rev 37:634–663
Mitchell A, Bucchini F, Cochrane G, Denise H, ten Hoopen P, Fraser M, Pesseat S, Potter S, Scheremetjew M, Sterk P et al (2016) EBI metagenomics in 2016—an expanding and evolving resource for the analysis and archiving of metagenomic data. Nucleic Acids Res 44:D595–D603
Neufeld JD, Driscoll BT, Knowles R, Archibald FS (2001) Quantifying functional gene populations: comparing gene abundance and corresponding enzymatic activity using denitrification and nitrogen fixation in pulp and paper mill effluent treatment systems. Can J Microbiol 47:925–934
Nilsson RH, Ryberg M, Abarenkov K, Sjökvist E, Kristiansson E (2009) The ITS region as a target for characterization of fungal communities using emerging sequencing technologies. FEMS Microbiol Lett 296:97–101
O'Donnell K, Ward TJ, Aberra D, Kistler HC, Aoki T, Orwig N, Kimura M, Bjørnstad S, Klemsdal SS (2008) Multilocus genotyping and molecular phylogenetics resolve a novel head blight pathogen within the Fusarium graminearum species complex from Ethiopia. Fungal Genet Biol 45:1514–1522

Op De Beeck M, Lievens B, Busschaert P, Declerck S, Vangronsveld J, Colpaert JV (2014) Comparison and validation of some ITS primer pairs useful for fungal metabarcoding studies. PLoS One 9:e97629

Oulas A, Pavloudi C, Polymenakou P, Pavlopoulos GA, Papanikolaou N, Kotoulas G, Arvanitidis C, Iliopoulos I (2015) Metagenomics: tools and insights for analyzing next-generation sequencing data derived from biodiversity studies. Bioinform Biol Insights 9:75–88

Pace NR, Stahl DA, Lane DJ, Olsen GJ (1986) The analysis of natural microbial populations by ribosomal RNA sequences. In: Marshall KC (ed) Advances in microbial ecology. Springer, Boston, MA, pp 1–55

Peters JW, Fisher K, Dean DR (1995) Nitrogenase structure and function: a biochemical-genetic perspective. Annu Rev Microbiol 49:335–366

Phillips D (1980) Efficiency of symbiotic nitrogen fixation in legumes. Annu Rev Plant Physiol 31:29–49

Piceno YM, Lovell CR (2000) Stability in natural bacterial communities: I. nutrient addition effects on rhizosphere diazotroph assemblage composition. Microb Ecol 39:32–40

Poly F, Ranjard L, Nazaret S, Gourbière F, Monrozier LJ (2001) Comparison of nifH gene pools in soils and soil microenvironments with contrasting properties. Appl Environ Microbiol 67:2255–2262

Quince C, Walker AW, Simpson JT, Loman NJ, Segata N (2017) Shotgun metagenomics, from sampling to analysis. Nat Biotechnol 35:833–844

Raaijmakers JM, Paulitz TC, Steinberg C, Alabouvette C, Moënne-Loccoz Y (2009) The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant Soil 321:341–361

Ree HK, Cao KM, Thurlow DL, Zimmermann RA (1989) The structure and organization of the 16S ribosomal RNA gene from the archaebacterium Thermoplasma acidophilum. Can J Microbiol 35:124–133

Reed SC, Townsend AR, Cleveland CC, Nemergut DR (2010) Microbial community shifts influence patterns in tropical forest nitrogen fixation. Oecologia 164:521–531

Sanyal D, Solanki S, Ameen G, Brueggeman RS, Chatterjee A (2020) Understanding the expression dynamics of symbiont rhizobial nifH and nitrogen assimilatory NR and GS genes in dry bean (Phaseolus vulgaris L.) genotypes at various growth stages. Legum Sci. https://doi.org/10.1002/leg3.26

Schloss PD, Westcott SL (2011) Assessing and improving methods used in operational taxonomic unit-based approaches for 16S rRNA gene sequence analysis. Appl Environ Microbiol 77:3219–3226

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Fungal Barcoding Consortium, and Fungal Barcoding Consortium Author List (2012) Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proc Natl Acad Sci U S A 109:6241–6246

Sharpton TJ (2014) An introduction to the analysis of shotgun metagenomic data. Front Plant Sci 5:209

Stajich JE, Harris T, Brunk BP, Brestelli J, Fischer S, Harb OS, Kissinger JC, Li W, Nayak V, Pinney DF et al (2012) FungiDB: an integrated functional genomics database for fungi. Nucleic Acids Res 40:D675–D681

Stielow JB, Lévesque CA, Seifert KA, Meyer W, Iriny L, Smits D, Renfurm R, Verkley GJM, Groenewald M, Chaduli D et al (2015) One fungus, which genes? Development and assessment of universal primers for potential secondary fungal DNA barcodes. Persoonia 35:242–263

Stres B, Mahne I, Avgustin G, Tiedje JM (2004) Nitrous oxide reductase (nosZ) gene fragments differ between native and cultivated Michigan soils. Appl Environ Microbiol 70:301–309

Szoboszlay M, White-Monsant A, Moe LA (2016) The effect of root exudate 7,4′-dihydroxyflavone and Naringenin on soil bacterial community structure. PLoS One 11:e0146555

Thomas T, Gilbert J, Meyer F (2012) Metagenomics - a guide from sampling to data analysis. Microb Inform Exp 2:3

Van Rhijn P, Vanderleyden J (1995) The rhizobium-plant symbiosis. Microbiol Rev 59:124–142

Vandermeer JH (1972) Niche theory. Annu Rev Ecol Syst 3:107–132
Vogel T, Simonet P, Nalin R, Philippot L (2009) TerraGenome: a consortium for the sequencing of a soil metagenome. Nat Rev Microbiol 7:252
Wagg C, Bender SF, Widmer F, van der Heijden MGA (2014) Soil biodiversity and soil community composition determine ecosystem multifunctionality. Proc Natl Acad Sci U S A 111:5266–5270
Wang C-L, Cheng Y-H (2017) Identification and trichothecene genotypes of Fusarium graminearum species complex from wheat in Taiwan. Bot Stud (Taipei, Taiwan) 58:4
Wartiainen I, Eriksson T, Zheng W, Rasmussen U (2008) Variation in the active diazotrophic community in rice paddy—nifH PCR-DGGE analysis of rhizosphere and bulk soil. Appl Soil Ecol 39:65–75
Wasmann C, VanEtten H (1996) Transformation-mediated chromosome loss and disruption of a gene for pisatin demethylase decrease the virulence of Nectria haematococca on pea. Molecular Plant-microbe Interactions: MPMI (USA)
Weinert N, Piceno Y, Ding G-C, Meincke R, Heuer H, Berg G, Schloter M, Andersen G, Smalla K (2011) PhyloChip hybridization uncovered an enormous bacterial diversity in the rhizosphere of different potato cultivars: many common and few cultivar-dependent taxa. FEMS Microbiol Ecol 75:497–506
Weller DM (2007) Pseudomonas biocontrol agents of soilborne pathogens: looking back over 30 years. Phytopathology 97:250–256
Wetterstrand K (2016) DNA sequencing costs: data from the NHGRI genome sequencing program (GSP)
Wood S, Newcomb W (1989) Nodule morphogenesis: the early infection of alfalfa (Medicago sativa) root hairs by rhizobium meliloti. Can J Bot 67:3108–3122
Wu H, Liu D, Ning-Ling, Bao W, Ying R, Ou Y, Huo Z, Li Y, Shen Q (2008a) Effects of vanillic acid on the growth and development of Fusarium oxysporum f. sp. niveum. Allelopath J 22:111–121
Wu H-S, Raza W, Fan J-Q, Sun Y-G, Bao W, Liu D-Y, Huang Q-W, Mao Z-S, Shen Q-R, Miao W-G (2008b) Antibiotic effect of exogenously applied salicylic acid on in vitro soilborne pathogen, Fusarium oxysporum f.sp.niveum. Chemosphere 74:45–50
Yates M, Stacey G, Burris R, Evans H (1992) Biological nitrogen fixation. 685–735
Yoon S-H, Ha S-M, Kwon S, Lim J, Kim Y, Seo H, Chun J (2017) Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int J Syst Evol Microbiol 67:1613–1617
Yuan Z, Zhang Z, Wang X, Li L, Cai K, Han H (2017) Novel impacts of functionalized multiwalled carbon nanotubes in plants: promotion of nodulation and nitrogenase activity in the rhizobium-legume system. Nanoscale 9:9921–9937
Zehr JP, McReynolds LA (1989) Use of degenerate oligonucleotides for amplification of the nifH gene from the marine cyanobacterium Trichodesmium thiebautii. Appl Environ Microbiol 55:2522–2526
Zehr JP, Mellon M, Braun S, Litaker W, Steppe T, Paerl HW (1995) Diversity of heterotrophic nitrogen fixation genes in a marine cyanobacterial mat. Appl Environ Microbiol 61:2527–2532

6 Isolation and Screening of Inorganic Phosphate Solubilizing *Pseudomonas* Strains from the *Lotus creticus* Rhizosphere Soil from the Northwest of Morocco

Imane Achkouk, Saida Aarab, Amin Laglaoui, Mohammed Bakkali, and Abdelhay Arakrak

Abstract

In vitro screening of plant growth-promoting (PGP) traits was carried out using 63 *Pseudomonas*, isolated from the rhizosphere of *Lotus creticus* collected from the northwest region of Morocco. The isolates were tested for their capacity of solubilizing tricalcium phosphate (TCP) on Pikovskaya (PVK) solid medium, and 20 *Pseudomonas* could solubilize TCP. We then selected five phosphate solubilizing bacteria (PSB) based on their halo diameters to undergo more tests. As a result, all five isolates were positive for hydrogen cyanide (HCN) production, and the amount of indole acetic acid (IAA) produced by them fluctuated between 5.72 ± 0.09 mg/L and 195.16 ± 0.38 mg/L. All the selected strains could produce siderophores. Except urease activity, all isolates possessed at least two enzyme activities such as cellulase, chitinase, protease, and amylase activities. In addition, all these PSB were able to produce ammonia. Thus, the strains were evaluated by TCP solubilizing quantitative assay in PVK liquid medium. The concentrations of solubilized P were between 20.80 mg/L and 159.55 mg/L. This solubilization was accompanied by a pH decrease of the medium from 7 to 4.16. Furthermore, the five PSB were tested in vitro for antagonism against phytopathogenic fungus *Fusarium oxysporum*, and all the PSB except LCP18 strain were capable of inhibiting its growth.

I. Achkouk · A. Laglaoui · M. Bakkali · A. Arakrak (✉)
Equipe de Recherche de Biotechnologies et Génie des Biomolécules (ERBGB), Faculté des Sciences et Techniques de Tanger, Tanger, Morocco

S. Aarab
Equipe de Recherche de Biotechnologies et Génie des Biomolécules (ERBGB), Faculté des Sciences et Techniques de Tanger, Tanger, Morocco

Faculté des Sciences et Techniques d'Al Hoceima, Ajdir Al Hoceima, Morocco

© Springer Nature Singapore Pte Ltd. 2020

M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_6

Keywords
Plant growth-promoting (PGP) · Phosphate solubilizing bacteria (PSB) · *Fusarium oxysporum*

6.1 Introduction

One of the most frequently encountered problems is the low availability of phosphorus (P) for plants, which is one of the essential components for the plants. This low availability is due to the fact that the vast majority of P in the soil is present in insoluble forms, while the plants absorb it only in two soluble forms: the monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{2-}) ions (Glass 1989). In calcareous soils, which is the case of the majority of Moroccan soils (FAO 2006), the P-Ca forms represent 80–90% of total mineral phosphorus (Arakrak et al. 2006).

Therefore, there is a growing awareness on the use of environment-friendly, sustainable nutrient management practices that lay emphasis on restoration and maintenance of soil fertility both in the short- and long-term. Thus, effective biological technologies like the use of plant growth-promoting rhizobacteria (PGPR) are being exploited for enhancing crop yields. Among PGPR, *Pseudomonas* are considered to be the most promising group of PGPR involved in growth promotion and biocontrol of plant pathogens (Singh et al. 2010). In previous studies, *Pseudomonas* spp. have shown increased production of indole acetic acid (IAA) (Patten and Glick 2002), the production of siderophores (Dey et al. 2004), phosphate solubilization (Wu et al. 2005), ACC deaminase, root elongation, degradation of toxic compound (Bano and Musarrat 2003), and as biological control agent against phytophatogens (Dey et al. 2004).

According to the remarkable PGPR characters of *Pseudomonas* sp., we isolated bacteria from the rhizosphere of *Lotus creticus* belonging to this genus. The isolates were evaluated for phytostimulator and biocontrol capabilities in order to gain potential inoculants to enhance crop yields.

6.2 Materials and Methods

6.2.1 Isolation of *Pseudomonas* from *Lotus creticus* Rhizosphere

Soil samples were collected from the rhizosphere soil of *Lotus creticus* growing in Achakar and Tahadartregion (Tangier). The rhizosphere was dug out with intact root system. The samples were placed in plastic bags and stored immediately at 4 °C. The rhizospheric soil was suspended in 9 mL of physiological saline solution, centrifuged for 1 h (200 rpm, 28 °C), and 1 ml sample was diluted from 10^{-1} to 10^{-7}. Aliquots of 100 μL of each dilution were plated on a King's B solid medium. The plates were incubated at 28 °C for 3 days (King et al. 1954). After incubation, morphologically variable colonies were picked up and purified on the same medium, King's B plates.

6.2.2 Phosphate Solubilization

The isolates were screened for phosphate solubilization; the colonies were transferred to the Pikovskya medium and then incubated at 28 °C. The plates were examined after 7 days of incubation, and data were recorded; visual detection of phosphate solubilizing ability of bacteria is possible by plate screening methods that show clear zone around the colonies in media containing insoluble mineral phosphate (tricalcium phosphate) as P source. The diameter of solubilization was calculated by subtracting the total diameter (diameter halo + colony diameter) of the diameter of colony. Isolates with solubilizing diameters $\geq$0.4 cm were conserved.

6.2.3 Production of Hydrogen Cyanide (HCN)

To estimate HCN production, 100 μL of bacterial culture were streaked on TSA supplemented with 4.4 g/L glycine. Filter paper discs (9 cm diameter) were soaked in 2% sodium carbonate in 0.5% picric acid solution and were placed in the lid of each Petri dish (Bakker and Schippers 1987). The plates were sealed with parafilm and incubated at 28 °C. Change in color from yellow to orange or brown indicated the synthesis of HCN production.

6.2.4 Determination of Indole Acetic Acid (IAA) Production

The tested bacterial strains were cultured for 2 days in sucrose-minimal salts (SMS) medium (sucrose 1%; $(NH_4)_2SO_4$ 0.1%; K_2HPO_4 0.2%; $MgSO_4$ 0.05%; NaCl 0.01%; yeast extract 0.05%; $CaCO_3$ 0.05%; pH 7.2) supplemented with 0.05% of L-tryptophan. After incubation, 1 mL of supernatant was mixed with 2 mL of Salkowski reagent, and the development of a pinkish color indicated the production of IAA (Gordon and Weber 1951). The absorbance of pink color developed after 25 min of incubation at room temperature was read at 535 nm.

6.2.5 Production of Siderophores

The bacteria were spot inoculated on King’s B medium, and the plates were incubated for 3 days at 28 °C. A layer of chrome azurol S (CAS) medium (Schwyn and Neilands 1997) was poured on the surface of these plates. After 24 h in the dark, change in color of CAS medium from blue to orange indicated the production of siderophores.

6.2.6 Production of Hydrolytic Enzymes

6.2.6.1 Cellulase

The strains were grown on carboxyl methyl cellulose (CMC) agar containing (g/L) KH_2PO_4 1, $MgSO_4.7H_2O$ 0.5, NaCl 0.5, $FeSO_4.7H_2O$ 0.01, $MnSO_4.H_2O$ 0.01, NH_4NO_3 0.3, CMC 10, and Agar 15. The pH was adjusted to 7 with 1 M NaOH. The

plates were incubated at 30 °C for 5 days. At the end of incubation, agar medium was flooded with an aqueous solution of Congo red (0.1%) and then washed with NaCl solution (1 M). Formation of clear zone indicated cellulose degradation (Miller 1959).

6.2.6.2 Chitinase

In order to demonstrate the production of the chitinase enzyme that degrades chitin, colloidal chitin was used as carbon source. The selective medium was prepared using (g/L) colloidal chitin 0.8, K_2HPO_4 2.7, KH_2PO_4 0.3, $MgSO_4.7H_2O$, NaCl 0.5, KCl 0.5, yeast extract 0.13, and agar 20. The plates were incubated, and the production of chitinase was indicated with the formation of transparent halos around the colonies.

6.2.6.3 Protease

The protease production is tested using an agar medium prepared by mixing two fractions A and B. The fraction A contains (g/L) dried milk 50 and ionized water 500 mL, and it is autoclaved at 115 °C for 10 min. The fraction B contains (g/L) agar 20 and ionized water 500 mL and is autoclaved at 120 °C for 20 min. After sterilization, and at a temperature of 55–60 °C, the two fractions were mixed aseptically and poured on the Petri dishes. Then, 100 μL of fresh culture of each isolate was then deposited separately on the dishes which were incubated at 28 °C for 48 h. The formation of a clear halo around the colony indicates the degradation of the protein.

6.2.6.4 Amylase

Amylase production was evaluated on starch agar plates containing (g/L) peptone 5, beef extract 3, soluble starch 2, and agar 15. The isolates were streaked on starch agar plates and incubated for 48 h at 30 °C. Amylase production was detected by flooding the plates with iodine solution (Cappuccino and Sherman 1992).

6.2.6.5 Urease

Testing the production of urease consists in the alkalinization of the medium containing urea as the sole nitrogen source, and phenol red is used as a pH indicator. The medium is prepared using (g/L) glucose 1, peptone 1, NaCl 0.5, KH_2PO_4 2, Congo red 0.012, and agar 20. The pH was adjusted to 7 with 1 M NaOH. After sterilization of the medium, and at a temperature of 45 °C, 50 mL of 40% urea sterilized by filtration was added. The plates were inoculated with bacteria to assess. After incubation, the production of urease is indicated by the development of a purplish pink color around the colonies on a yellow background.

6.2.7 Production of Ammonia

All the bacterial isolates were tested for the production of ammonia as described by Cappuccino and Sherman (1992). Twelve-hour-old bacterial cultures were inoculated in peptone water (10 mL) and incubated for 48–72 h at 36 ± 2 °C. Development

of brown to yellow color after addition of Nesseler's reagent indicates a positive test for ammonia; no color change indicates negative test.

6.2.8 Qualitative Phosphate Solubilization Assay

Isolates were tested for their ability to solubilize the phosphorus in the liquid medium; this is realized by inoculating 50 mL of PVK liquid medium by 500 µL of bacterial culture. Autoclaved and not inoculated media were used as controls. The inoculated media and controls were incubated for 7 days at 28 °C on shaker (180 rpm). The media were centrifuged at 13.000 rpm for 20 min. The soluble P of the supernatant was determined by the colorimetric method of Ames (1966), and the pH of the medium was also determined.

6.2.9 Antifungal Activity

The antifungal activity was tested using potato dextrose agar (PDA) (Rabindran and Vidyasekaran 1996). Bacterial isolates were tested for their ability to inhibit the growth of the plant pathogenic fungus *Fusarium oxysporum* isolated and characterized by El Aaraj et al. (2015). A 5-mm agar disk of the fungus was deposited in the center of the PDA Petri plates. A volume of 20 µL of each bacterial culture was seeded in 3 cm spot of the fungal strain. A negative control of the fungal strain is tested in the absence of bacteria. Plates were incubated for 7 days at 25 °C and examined for evidence of fungal growth inhibition. The zone of inhibition was determined using the following formula:

$$\% \text{ Inhibition of radial growth} = 100 \times (r1 - r2/r1)$$

where r1 is the radial growth of the mycelium in control and r2 is the radial growth of the mycelium in treatment. The results represent the average of three replicates.

6.2.10 Statistical Analysis

The data are reported as means ± SD (standard deviation) for three replicates. The results were compared by analysis of variance (ANOVA) according to Fisher's protected LSD test ($p < 0.05$). Data analysis was made on the rate of produced IAA, solubilized P, and percentage of inhibition.

6.3 Results and Discussion

The rhizosphere of plants is known to be an environmentally preferred area by soil microorganisms due to its rich nutrient availability. PGPR colonize plant roots and improve its growth by a variety of mechanisms. The exact mechanism by which PGPR stimulate the plant growth is not clearly known, although several activities such as phosphate solubilization production of hormones and inhibiting the growth of phytopathogens are generally thought to be involved in promoting plant growth.

6.3.1 Isolation and Selection of Phosphate Solubilizing Bacteria (PSB)

A total of 63 isolates were tested for their inorganic phosphate solubilizing activity on solid medium. The solubilization of TCP is indicated by the formation of a clear halo around the bacterial colony on solid PVK medium. A total of 20 isolates out of 63 tested (31.74%) in PVK solid medium were able to form a transparent halo indicating the phosphate solubilization, and halo diameters were between 0.2 and 1.2 cm. Zaidi et al. (2009) explained that the solubilization of inorganic phosphorus usually occurs following the action of low molecular weight organic acids which are synthesized by various soil bacteria. These organic acids can react as ion-mineral chelating agents or can cause a decrease in the pH to acidify the medium. This acidification leads to the release of the phosphate ions from the mineral P by substituting the $Ca^{2+}$with H^{+}. So the TCP solubilization in solid medium depends primarily on the concentration and the type of organic substances produced by the rhizobacteria. At the end of this test, five best isolates that formed halo diameters ⩾0.4 cm were selected for further tests of PGP activities.

6.3.2 Screening of PGP Activities of Isolated *Pseudomonas*

The PSB retained, namely, five isolates, underwent the qualitative test of HCN production, in which the gas production is indicated by a color change of filter paper from yellow to orange/brown. Indeed, all of the PSB were able to produce this gas, which is involved in the biocontrol mechanism of plants (Table 6.1). In fact, Voisard

Table 6.1 PGP activities of tested *Pseudomonas*

Isolates	Concentration of IAA (mg/L)	Halo diameters (cm)	HCN	Siderophores
LCP18	5.72 ± 0.09[a]	1.2	+++	+
LCP25	6.04 ± 0.88[a]	0.8	++	+
LCP79	9.01 ± 0.93[a]	0.7	++	+
LCP88	195.16 ± 0.38[b]	0.4	+++	++
LCP90	5.83 ± 0.12[a]	0.5	++	+

The letters on the bars of the same parameter indicate significant differences according to Fisher's protected LSD test ($p < 0.05$)

et al. (1989) showed that the production of HCN by *Pseudomonas* strains suppresses infectious diseases of plants while the mutant strain defective in the synthesis of HCN loses the ability to protect plants against diseases. This shows the crucial antagonist role played by this compound in the biocontrol mechanism. However, it was shown by Bakker and Schippers in 1987 that this compound may also cause negative effects on plant growth by inhibiting growth of the roots. Blumer and Haas (2000) reported that the HCN is formed from glycine by the action of HCN synthase enzyme, which is associated with the plasma membrane of certain rhizobacteria. We can say then that the presence of this enzyme is essential for the formation of HCN in which its quantity controls the concentration.

The results showed that LCP88 isolate produced significantly the highest concentration (195.16 ± 0.38 mg/L) (Table 6.1). The amounts produced by LCP18, LCP25, LCP79, and LCP90 were not significantly different. Patten and Glick (1996) explained that IAA is the most common plant hormone and one of the most characterized hormones. In addition, Mohite (2013) showed in a study of isolation and characterization of IAA producing bacteria from the rhizosphere soils of several plants, banana, corn, cotton, and wheat, that five retained strains br1, br2, br3, wr2, and mr2 produced more IAA when incubated in the medium provided with the tryptophan than when incubated in medium without L-tryptophan. The production of auxin by the PGPR may cause positive and negative effects on plant growth (Vacheron et al. 2013). The effectiveness of the auxin depends on its concentration; at a low concentration, it improves plant growth (Patten and Glick 2002), while at a high concentration, it inhibits the root growth (Xie et al. 1996).

Iron plays an important role in nutrition and plant protection. Therefore, the ability to synthesize siderophores was evaluated for the retained PSB that were all positive by forming an orange halo around the colony (Table 6.1). Thus, our results are in agreement with those obtained by Castagno et al. (2011) in which all the PSB isolated from the rhizosphere of *Lotus tenuis* were positive for the production of siderophores. Similarly, Vansuyt et al. (2007) showed that the iron-pyoverdine synthesized by *P. fluorescens* strain C7 and tested on *Arabidopsis thaliana* plants increased iron levels in the plant and improved their growth.

6.3.3 Production of Lytic Enzymes

The PSB were tested for their capacity of producing some lytic enzymes, and Table 6.2 shows the results: two of the five isolates showed positive cellulase activity and were able to form a halo around the bacterial colony. Hence, four of the isolates produced chitinase during this qualitative test, and the highest chitinolytic activity was observed for the LCP90 isolate. In addition, the evaluation of the capacity of the isolates to hydrolyze starch allowed us to identify three isolates with amylase activity. The protease production test showed that four PSB were positive. These three enzymes have the ability to degrade component of the fungal cell wall, which is an important mechanism of fungal inhibition (Reetha et al. 2014).

Table 6.2 The results of the qualitative tests for cellulase, chitinase, protease, amylase, and urease and ammonia production of the five isolates selected

Isolates	Cellulase	Chitinase	Protease	Amylase	Urease	Ammonia
LCP18	–	+	+	–	–	++
LCP25	+	+	–	–	–	+
LCP79	++	++	+++	++	–	+
LCP88	–	–	+	+	–	+++
LCP90	–	++++	++	++	–	+++

(−) absence of production; (+) low production; (++) average production; (+++) strong production

Based on the change in color of the culture medium by means of the pH indicator (phenol red), none of the PSB were able to hydrolyze the urea. Indeed, Someya et al. (2007) demonstrated that bacteria producing a single lytic enzyme may well be used in combination with other biocontrol agents, thus leading to a synergistic inhibitory effect against phytopathogens.

6.3.4 Ammonia Production

The addition of the Nessler's reagent during the qualitative test of the production of ammonia causes a change in the color of the medium from yellow to orange/brown. This allowed us to identify the capacity of the five strains to produce this compound. It has been demonstrated that volatile substances are involved in the biocontrol mechanism against various phytopathogens.

6.3.5 Quantitative Test of Phosphate Solubilization in Liquid Medium

The solubilized P assay was carried out according to Ames method (Ames 1966). The P concentrations obtained during this test were between 20.80 mg/L and 159.55 mg/L (Fig. 6.1). Similar results were obtained in a study of tricalcium phosphate solubilization by *Rhizobium* isolated from the nodules of three legumes (*Hydesarum coronarium*, *Vicia sativa*, and *Lupinus angustifolius*) carried out by Aarab et al. (2009). They tested six best isolates for their ability to solubilize TCP in liquid medium. The results obtained in this study ranged from 9.62 mg/L to 158.64 mg/L. Besides, another study by Park et al. (2010) who isolated PSB from different rhizospheric samples showed that six isolates tested solubilized quantities higher than 250 mg/L.

Indeed, the concentrations of solubilized P by the isolates LCP79 and LCP90 were significantly higher than the other isolates. It should be noted that the isolates LCP18 and LCP25 formed important solubilization diameters in PVK solid medium: 1.2 cm and 0.8 cm, respectively, while they solubilized less P in liquid medium 71.91 mg/L and 59.38 mg/L. Conversely, the isolate LCP90 and LCP79 were less efficient in the qualitative test than the quantitative one; they formed

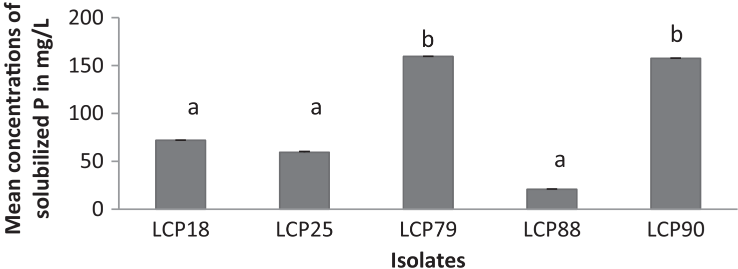


Fig. 6.1 Concentrations of solubilized P by the five PSB retained after 7 days of incubation. The letters on the bars of the same parameter indicate significant differences according to Fisher's protected LSD test ($p < 0.05$)

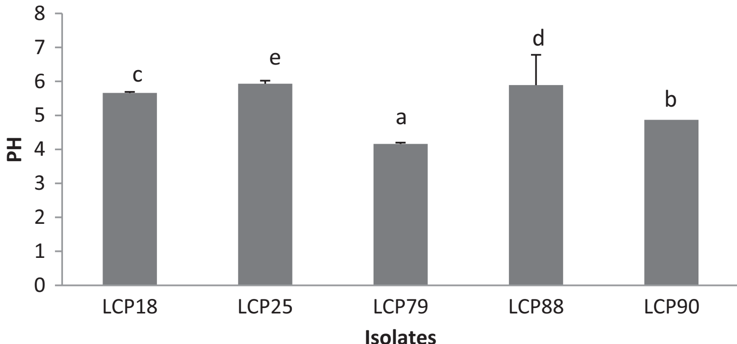


Fig. 6.2 Final pH of the five PSB incubation media after 7 days of incubation. The letters on the bars of the same parameter indicate significant differences according to Fisher's protected LSD test ($p < 0.05$)

modest diameters in the solid PVK medium (0.5 and 0.7 cm, respectively); however, they solubilized higher concentrations of P (175.46 and 159.55 mg/L, respectively). These results are in agreement with those obtained by Cherif (2014) who showed that the analysis of the correlation between P solubilization on solid and liquid PVK medium is not significant. Also, Alam et al. (2002) obtained a significantly negative correlation between the concentration of solubilized P from the tricalcium phosphate and the solubilization halo diameter. It should be noted that the solubilization of the TCP is accompanied by a decrease in pH value after 7 days of incubation of the five isolates (Fig. 6.2). The initial pH of the medium (7) decreased to a value of 4.16. The decrease of the pH of the medium was significantly different between the five isolates.

The analysis of the dependence of the amount of solubilized P and the final pH of the medium revealed the existence of a significantly negative correlation ($r = -0.93$) (Fig. 6.3). This shows that the more the pH of the medium is acid, the more the phosphate is solubilized.

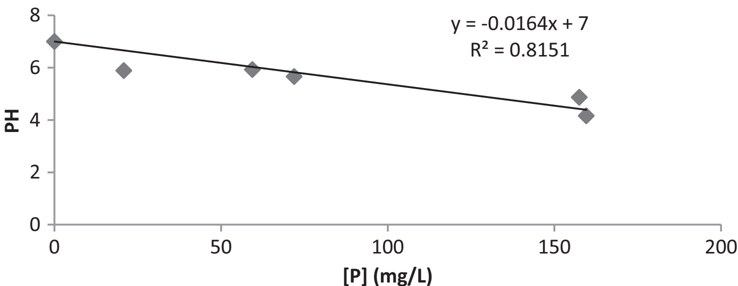


Fig. 6.3 Correlation between solubilized P and pH

This is in agreement with Vinodrai and Vyas (2014) who tested the ability of four isolates to solubilize P in liquid medium, in which the correlation between these two parameters was negative. Moreover, Wani et al. (2008) reported that the P solubilization is very often associated with a decrease in the pH of the medium, and they showed the existence of a significant negative correlation between the pH values and the inorganic P concentrations, in particular calcium phosphate. According to the literature, several mechanisms can be adapted by the PSB to solubilize inorganic P; Hwangbo et al. (2003) have shown that the production of organic acids by PSB plays an important role in the acidification of the medium facilitating the P solubilization. Further, Vassilev and Medina (2006) showed that there are other mechanisms by which PSB can solubilize inorganic P than the secretion of organic acids, for example, via the production of siderophores. Patel et al. (2008) demonstrated that this bio-solubilization can even occur following the secretion of phenolic compounds and humic substances.

6.3.6 Antagonism Test

The antifungal activity of the strains was tested on PDA medium toward the fungus *Fusarium oxysporum*, which is known for its phytopathogenicity. As a result, all the isolates, except LCP18, were able to inhibit the growth of the fungus by contact inhibition in the Petri plate after 7 days of incubation. The inhibition percentages of the isolate LCP79 and LCP90 were significantly the highest, while the inhibition percentages of the isolate LCP25 were significantly the lowest. Similarly, Tarun et al. (2012) tested ten PGPR isolated from the rice rhizosphere in the Tarai region of India for their inhibitory potency, and they found only three isolates (PGB5, PGB6, and PGB10) of these PGPR tested that were able to inhibit the growth of *Fusarium oxysporum*. They later suggested that the siderophores produced by these three bacteria act as suppressors of the growth of phytopathogenic fungi, which is consistent with our results since all the isolates were positive for siderophore production except the isolate LCP18 (Fig. 6.4).

According to Geetha et al. (2014), several mechanisms may explain the inhibition of phytopathogenic fungi by *Bacillus* and *Pseudomonas* spp., such as the production of antibiotics, synthesis of hydrolytic enzymes, competition for nutrients, or

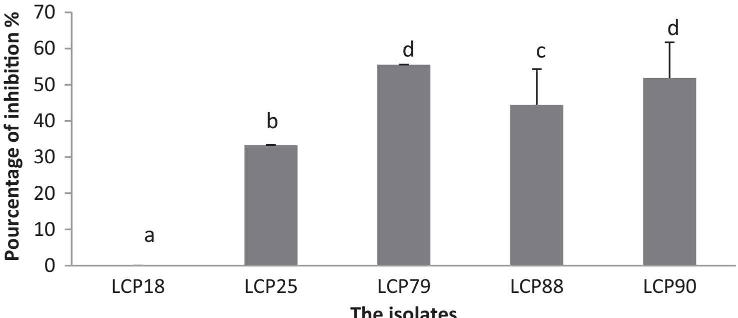


Fig. 6.4 Antagonist effect of the isolates retained against the phytopathogenic fungus *Fusarium oxysporum*. The letters on the bars of the same parameter indicate significant differences according to Fisher's protected LSD test ($p < 0.05$)

a combination of these mechanisms in synergy. Besides, Toyoda and Utsumi (1991) reported that some PGPR degrade fusaric acid produced by *Fusarium* sp., which is the causative agent of wilting, and therefore prevent pathogenesis. In addition, Prashar et al. (2013) observed that the production of compounds such as ammonia and HCN is the main source of inhibition of the fungus tested. It was reported that HCN is produced in various quantities by different species of *Pseudomonas*, as well as by other bacteria such as *Chromobacterium violaceum* (Blom et al. 2011).

Finally, it can be said that the inhibition of fungal growth by the isolates is probably associated with HCN production, ammonia production, siderophore production, lytic enzymes, or even other activities that were not researched in our case. These evaluated activities can act either separately or in synergy.

6.4 Conclusion

The use of PGPR falls within the context of the fertilization of saline and arid soils and stimulates the growth and natural defenses of plants, of which the purpose is to reduce the application of phytosanitary products and to mitigate the inhibitory effects of poor soils. In this context, this study confirms the importance of some phosphate solubilizing *Pseudomonas* isolated from the rhizosphere of *Lotus creticus*. The selected isolates have different phytobenificial properties and activities in vitro. All isolates produced IAA, and this makes it possible to classify the isolates as phytostimulators. The production of lytic enzymes, HCN, and ammonia demonstrates the biocontrol activity that these isolates may possess against other types of fungi and phytopathogenic bacteria. The results obtained are extremely encouraging in view of the use of phosphate solubilizing bacteria in agriculture as biofertilizers for the improvement growth and development of plants.

References

Aarab S, Laglaoui A, Bakkali M, Arakarak A (2009) Sélection des isolats rhizobiens solubilisant le phosphate tricalcique: inoculation des graines du Triticale (variéteMomtaz). COVAPHOS III 5:147–152

Alam S, Khalil S, Ayub N, Rashid M (2002) In vitro Solubilization of inorganic phosphate by phosphate solubilizing microorganisms (PSM) from maize rhizosphere. Int J Agric Biol 4:454–458

Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol 8:115–118

Arakrak A, Frossard E, Jacquin F (2006) Adsorption du phosphore dans les sols calcaires. COVAPHOS II 3:159–169

Bakker AW, Schippers B (1987) Microbial cyanide production in the rhizosphere in relation to potato yield reduction and Pseudomonas spp. mediated plant growth stimulation. Soil Biol Biochem 19:451–457

Bano N, Musarrat J (2003) Isolation and characterization of phorate degrading soil bacteria of environmental and agronomic. Lett Appl Microbiol 36:349–353. https://doi.org/10.1046/J.1472-.2003.01329.X

Blom D, Fabbri C, Eberl L, Weisskopf L (2011) Volatile-mediated killing of Arabidopsis thaliana by bacteria is mainly due to hydrogen cyanide. Appl Environ Microbiol 77(3):1000–1008

Blumer C, Haas D (2000) Mechanism, regulation, and ecological role of bacterial cyanide biosynthesis. Arch Microbiol 173:170–177

Cappuccino JC, Sherman N (1992) A laboratory manual, 3rd edn. Benjamin/Cummings Pub. Co., New York, pp 125–179

Castagno LN, Estrella MJ, Sannazzaro AI, Grassano AE, Ruiz OA (2011) Phosphate-solubilization mechanism and in vitro plant growth promotion activity mediated by Pantoea eucalypti isolated from Lotus tenuis rhizosphere in the Salado River Basin (Argentina). J Appl Microbiol. ISSN 1364–507

Cherif H (2014) Amélioration de la croissance du blé dur en milieu salin par inoculation avec Bacillus sp. etPantoeaagglomerans isolées de sols arides. Thése doctorat, Université Ferhat Abbas Sétif 1, Faculté des Sciences de la Nature et de la Vie, P1–177

Dey R, Pal KK, Bhatt DM, Chauhan SM (2004) Growth promotion and yield enhancement of peanut (Arachishypogaea L.) by application of plant growth-promoting rhizobacteria. Microbiol Res 159:371–394. https://doi.org/10.1016/J.MICRES.2004.08.004

El Aaraj C, Bakkali M, Infantino A, Arakrak A, Laglaoui A (2015) Mycotoxigenic fungi in cereals grains and coffee from the North of Morocco. Am J Res Commun 3(2). www.usa-journals.com, ISSN: 2325–4076

FAO (2006) Utilisation des engrais par cultureauMaroc 1ere édition. Rome

Geetha K, Venkatesham E, Hindumathi A, Bhadraiah B (2014) Isolation, screening and characterization of plant growth promoting bacteria and their effect on VignaRadita (L.) R.Wilczek. Int J Curr Microbiol Appl Sci 3(6):799–809

Glass ADM (1989) Plant nutrition: an introduction to current concepts. Jones and Bartlett Publishers, Boston, MA

Gordon, Weber RP (1951) Colorimetric estimation of indole-acetic acid. Plant Physiol 26:192–195

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

King EO, Ward MK, Randey DE (1954) Two simple media for the demonstration of pyocyanin and fluorescein. J Lab Clin Med 44:301–307

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428

Mohite B (2013) Isolation and characterization of indole acetic acid (IAA) producing bacteria from rhizospheric soil and its effect on plant growth. J Soil Sci Plant Nutr 13(3):638–649. https://doi.org/10.4067/S0718-95162013005000051

Park J, Bolan N, Mallavarapu M and Naidu R (2010) Enhancing the solubility of insoluble phosphorus compounds by phosphate solubilizing bacteria. In: 19th World Congress of Soil Science, Soil Solutions for a Changing World, 1–6 August, Brisbane, Australia, pp. 65–68

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

Patten CL, Glick BR (1996) Bacterial biosynthesis of indole-3-acetic acid. Can J Microbiol 42(3):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(8):3795–3801

Prashar P, Kapoor N, Sachdeva S (2013) Isolation and characterization of Bacillus sp with in vitro antaonistic activity against Fusarium oxysporum from rhizosphere of tomato. J Agric Sci Technol 15:1501–1512

Rabindran R, Vidyasekaran P (1996) Crop Prot 15:715–721

Reetha S, Selvakumar G, Bhuvaneswari G, Thamizhiniyan P, Ravimycin T (2014) Screening of cellulase and pectinase by using Pseudomonas fluorescence and Bacillus subtilis. Int Lett Nat Sci 8(2):75–80

Schwyn B, Neilands JB (1997) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

Singh N, Kumar S, Bajpai VK (2010) Biocontrol of Macrophominaphaseolina by chemotactic fluorescent Pseudomonas aeruginosa PN1 and its plant growth promontory activity in chirpine. Crop Prot 29:1142–1147

Someya N, Tsuchiya K, Yoshida T, Noguchi MT, Akutsu K, Sawada H (2007) Co-inoculation of an antibiotic-producing bacterium and a lytic enzyme-producing bacterium for the biocontrol of tomato wilt caused by Fusarium oxysporum f. sp. lycopersici. Biocontrol Sci 12:1–6

Tarun S, Navin K, Nishant R (2012) Isolation, screening and characterization of PGPR isolates from rhizosphere of rice plants in Kashipur region (Tarai region). Biotechnol Int 5(3):69–84

Toyoda H and Utsumi R (1991) Method for the prevention of Fusarium diseases and microorganisms used for the same. US Patent No. 4, 988, p. 586

Vacheron J, Desbrosses G, Bouffaud M, Touraine B, Moenne-Loccoz Y, Muller D (2013) Plant growth-promoting rhizobacteria and root system functioning. Front Plant Sci 4:1–19

Vansuyt G, Robin A, Briat JF, Curie C, Lemanceau P (2007) Iron acquisition from Fe pyoverdine by Arabidopsis thaliana. Mol Plant Microbe Interact 20:441–447

Vassilev N, Medina A, Azco´n R, Vassileva R (2006) Microbial solubilization of rock phosphate on media containing agro-industrial wastes and effect of the resulting products on plant growth and P uptake. Plant Soil 287:77–84

Vinodrai BP, Vyas RMB (2014) Screening and characterization of plant growth and health promoting rhizobacteria. Int J Curr Microbiol Appl Sci 3(6):139–155

Voisard C, Keel C, Haas D, Dèfago G (1989) Cyanide production by Pseudomonas fluorescens helps suppress black root rot of tobacco under gnotobiotic conditions. EMBO J 8(2):351

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

Wu SC, Cao ZH, Li ZG, Cheung KC, Wonga MH (2005) Effects of biofertilizer containing Nfixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. Geoderma 125:155–166. https://doi.org/10.1016/J.GEODERMA.2004.07.003

Xie H, Pasternak J, Glick B (1996) Isolation and characterization of mutants of the plant growth-promoting rhizobacterium Pseudomonas putida GR12-2 that overproduce indoleacetic acid. Curr Microbiol 32:67–71. https://doi.org/10.1007/s002849900012

Zaidi A, Khan MS, Musarrat J (2009) Microbial strategies for crop improvement. Springer, Berlin

Check for updates

Screening and Characterization of Phosphate-Solubilizing Rhizobia Isolated from *Hedysarum pallidum* in the Northeast of Morocco

7

Samia Hamane, Mounir Hassani Zerrouk, Anass E. Lyemlahi, Saida Aarab, Amin Laglaoui, Mohammed Bakkali, and Abdelhay Arakrak

Abstract

Due to high cost of chemical fertilizers and negative environmental effects, the use of rhizobia as plant growth-promoting rhizobacteria (PGPR) has shown potentials to be a promising technique to assure a sustainable agriculture. Our goal was to select rhizobia strains isolated from nodules of *Hydesarum pallidum* plants present in Touissit, northeast of Morocco, exhibiting different activities that can stimulate directly and/or indirectly plant growth. A total of 37 bacteria were isolated, of which 19 were capable of solubilizing tricalcium phosphate (TCP). Based on the diameter of solubilization halos (diameter ≥0.4 cm), 15 strains were selected and evaluated for more PGP activities *in vitro* for selected isolates. As a result, 11 bacteria were proved to be able to synthesize hydrogen cyanide (HCN). Amounts of indole acetic acid (IAA) produced by these bacteria ranged between 1.04 and 3.43 mg L^{-1}. Their ability to secrete siderophores was also evaluated; 80% of the strains were positive for these compounds' production. We also looked for extracellular enzymes such as cellulase, amylase, protease, chitinase, and urease. The percentages of bacteria that were positive for the production of these hydrolytic enzymes were, respectively, 73.3%, 93.3%, 40%, 26.67%, and 33.3%. Eight of selected bacteria were checked for

S. Hamane · A. E. Lyemlahi · A. Laglaoui · M. Bakkali · A. Arakrak (✉)
Equipe de Recherche de Biotechnologies et Génie des Biomolécules (ERBGB), Faculté des Sciences et Techniques de Tanger, Tanger, Morocco

M. H. Zerrouk
Faculté polydisciplinaire de Larache, Larache, Morocco

S. Aarab
Equipe de Recherche de Biotechnologies et Génie des Biomolécules (ERBGB), Faculté des Sciences et Techniques de Tanger, Tanger, Morocco

Faculté des Sciences et Techniquesd'Alhoceima, Ctre Ait Youssef Ou Ali, Morocco

© Springer Nature Singapore Pte Ltd. 2020
M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_7

quantitative assay of TCP solubilization, and soluble P concentrations were between 2 and 137 mg/L, accompanied by a drop in media pH from 5.67 to 3.87. This study reveals the potential of some rhizobia to be used as efficient biofertilizers.

Keywords
Hydesarum pallidum · *Siderophores* · Plant growth-promoting rhizobacteria (PGPR)

7.1 Introduction

Agriculture today faces challenges, such as imbalance in nutritional status, biological and physical properties of soil, and fluctuating climatic factors. All these issues are the interlinked contributing factors for reduced agricultural productivity. Sustainability and environmental safety of agricultural production rely on eco-friendly approaches like biofertilizers. Microbes influencing the plant growth positively by any mechanism are referred as plant growth-promoting rhizobacteria (PGPR) (Kloepper et al. 1986). PGPR are important for sustainable agriculture in order to promote the growth and yield of plants and circulation of plant nutrients. Also they deem to be the best alternative to chemical fertilizers due to the fact that application of PGPR as biofertilizers reduces the cost of crop production. Phosphorus (P), the most limiting nutrient for plant growth, is required by the plants for fundamental functions. Consequently, acquisition of sufficient concentration of P enhances the growth and development of plants in different production systems (Hayat et al. 2010; Ahemad et al. 2009; Vikram and Hamzehzarghani 2008). The use of phosphate-solubilizing bacteria as inoculants simultaneously increases P uptake by the plant and crop yield. Several species of phosphate-solubilizing bacteria have been identified so far including *Pseudomonas*, *Bacillus*, *Rhizobium*, *Bradyrhizobium*, and *Berkholderia* (Rodriguez et al. 1999) having both wide and limited host ranges. These bacteria are usually associated with rhizosphere, rhizoplane, or root tissues (Khan et al. 2009b). Besides phosphate solubilizing, they can also improve plant growth by utilizing diverse mechanisms, phytohormone production, nitrogen fixation, and biocontrol of plant pathogens (Vessey 2003; Bhattacharyya and Jha 2011). A single PGPR may possess one or more than one of these plant beneficial traits (Vessey 2003). Keeping in view the importance of *Rhizobium* as PGPR, the present study was undertaken to characterize and study different strains of *Rhizobium* isolated from nodules of *Hydesarum pallidum* plants for optimum biofertilizer production.

7.2 Materials and Methods

7.2.1 Bacterial Isolates

The method of isolating root-nodulating bacteria from nodules was described by Vincent (1970). After incubation for 3 days at 28 °C, single colonies were picked and checked for purity by repeated streaking on to YEM plate containing Congo red (25 mg/mL) and Gram stain reaction. The pure isolates were stored in 25% (v/v) glycerol at −20 °C.

7.2.2 Inorganic Phosphate Solubilization

A method of spotting of isolates on Pikovskaya (1948)) agar plates was applied to confirm their ability to dissolve inorganic phosphate. The inoculated plates were incubated at 28 °C for 7 days and then observed on solid plates for halo formation. The diameter of the halo of solubilization was calculated by subtracting the colony diameter from the total diameter (diameter of the halo + colony diameter).

7.2.3 Hydrogen Cyanide (HCN) Production

To estimate HCN production, a spot of bacterial cultures was streaked on YEM plate amended with 4.4 g/L glycine. A Whatman filter paper no. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed on the top of each plate (Bakker and Schippers 1987). Plates were sealed with parafilm and incubated at 28 ± 2 °C. Development of orange to red color indicated HCN production.

7.2.4 Siderophores Production

The plates of YEM were spot-inoculated with test bacteria and incubated at 28 ± 2 °C for 3 days. A layer of chrome azurol S (CAS) medium (Schwyn and Neilands 1997) was poured on the surface of each plate. After 24 h in the dark, development of orange halo around the bacteria was considered as positive for siderophore production.

7.2.5 Quantitative Assay of Indole Acetic Acid (IAA) Production

Bacterial cultures grown in SMS medium were used to inoculate other tubes containing the same SMS medium supplemented with 0.05% tryptophan and incubated at 28 °C for 2 days.

All media were centrifuged at 13.000 rpm for 3 min. The supernatant (1 mL) was mixed with 2 mL of Salkowski reagent, and the development of a pinkish color indicated the production of IAA (Gordon and Weber 1951). The optical density was

taken at 535 nm after 20 min of incubation at room temperature. Concentrations of produced IAA were measured with the help of its standard curve.

7.2.6 Cellulase Production

Carboxymethyl cellulose (CMC) agar plates were prepared by screening for cellulose enzyme production according to the method by Miller (1959). After incubation, the plates were flooded with Congo red solution for 15 min, followed by destaining with the salt solution (1 M) for 15 min. The colonies developing a yellow halo reflect the production of the cellulase enzyme.

7.2.7 Urease Production

Urease test was performed by inoculating the isolates on urease medium. The plates were inoculated with the selected strains and incubated for 5 days at 30 °C.

7.2.8 Amylase Production

For the qualitative determination of amylase production, starch agar medium (SAM) was used. About 10 μL of fresh bacterial culture of each isolate was deposited on the SAM medium. After 48 h of incubated at 37 °C, the reading is carried out by impregnating a 1% iodine solution for 5 min. The clear zone surrounding the bacterial colony indicates the production of amylase (Marx et al. 2004).

7.2.9 Protease Production

The production of protease is carried out using a skimmed milk agar. The formation of a transparent halo around the colony indicates the degradation of the protein.

7.2.10 Chitinase Production

The method of Renwick et al. (1991) was followed for the qualitative determination of chitinase production. The cultures of bacterial isolates were spot-inoculated on minimal medium agar plates containing chitin as a sole source of carbon, followed by incubation for 7 days at 30 ± 2 °C. The plates were observed for the development of clear halo zones around the developed colonies, confirming chitinase production.

7.2.11 Quantitative Assay of P Solubilization

The test isolates were inoculated in 50 mL PVK broth and negative control consisted of uninoculated broth. All flasks were incubated at 28 ± 2 °C with shaking for 7 days. The cultures were centrifuged at 13,000 rpm for 20 min, and the P of supernatant was determined by the colorimetric method as described by Ames (1966)). The amount of soluble P was detected from the standard curve of KH_2PO_4. The pH was determined using a pH meter.

7.2.12 Statistical Analysis

The data are reported as means ± SD (standard deviation) for three replicates or more. The results were subjected to analysis of variance (ANOVA) according to Fisher's protected LSD test ($p < 0.05$) using the Statgraphics Plus version 4.0.

7.3 Results and Discussion

7.3.1 Selection of PSB

In the present study, the selected strains were confirmed as phosphate-solubilizing bacteria (PSB) by observing halo zone around the bacterial colony (Fig. 7.1). A total of 37 isolates were obtained from *H. pallidum* nodules. Based on halo's solubilization (diameter ≥0.4 cm), 15 PSB were selected for identification and characterization for plant growth-promoting (PGP) activities. Solubilization index (SI) of these bacteria on solid media ranged between 2.4 and 3 (Table 7.1). The highest SI was observed for HPT10. These results are consistent with literature studying phosphate-solubilizing

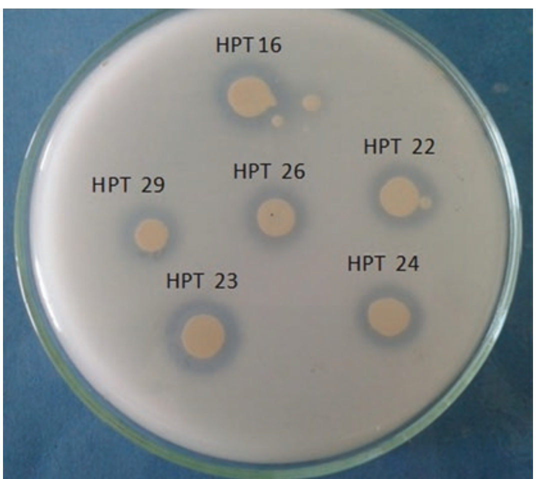


Fig. 7.1 Solubilization of TCP on Pikovskaya agar medium by rhizobium isolates

Table 7.1 Solubilization of TCP by selected rhizobia

Isolates	SI	D.H (cm)
HPT 24	2.56	0.50
HPT 23	2.88	0.70
HPT 22	2.50	0.40
HPT 26	2.50	0.40
HPT 29	2.63	0.50
HPT 16	2.56	0.50
HPT 11	2.75	0.60
HPT 35	2.75	0.60
HPT 2	2.50	0.40
HPT 33	2.67	0.60
HPT 10	3.00	0.70
HPT 15	2.44	0.40
HPT 9	2.67	0.60
HPT 8	2.40	0.40
HPT 7	2.63	0.50

Solubilization index (SI) = diameter of the colony + halo zone / diameter of the colony

bacteria by similar tests of clear halo formation around colonies on agar medium containing insoluble phosphate (Alikhani et al. 2006; Khan et al. 2007; Babana et al. 2013). These halos could be due to the production of organic acids or due to the activity of phosphatase enzymes of phosphate-solubilizing bacterial strains (Khan et al. 2009a).

7.3.2 In Vitro Screening of Isolates for Multiple PGP Activities

7.3.2.1 Hydrogen Cyanide (HCN) Production

In the present study, 15 isolates of rhizobia were screened in vitro for HCN production. Eleven isolates produced HCN in varied amounts based on color intensity of the reaction. Hydrogen cyanide is a secondary metabolite produced during the early stationary growth phase (Knowles and Bunch 1986) by several PGPR, notably *Pseudomonas* spp. and *Bacillus* (Wani et al. 2007) and *Rhizobium* spp. (Wani et al. 2008a, b). It is released by microbial communities in solution, acts as a secondary metabolite, confers a selective advantage onto the producer strains (Vining 1990), and provides protection to plants against weeds and various pathogens, therefore serving as a biocontrol agent (Sahin et al. 2004).

7.3.2.2 Siderophore Production

Another important trait of PGPR, which may indirectly influence the plant growth, is the production of siderophores. They bind to the available form of iron Fe3+ in the rhizosphere, thus making it unavailable to the phytopathogens and protecting the plant health. In this present investigation, 12 rhizobia strains were siderophore producers. The current results are dissimilar with Abdul et al. (2014) were they

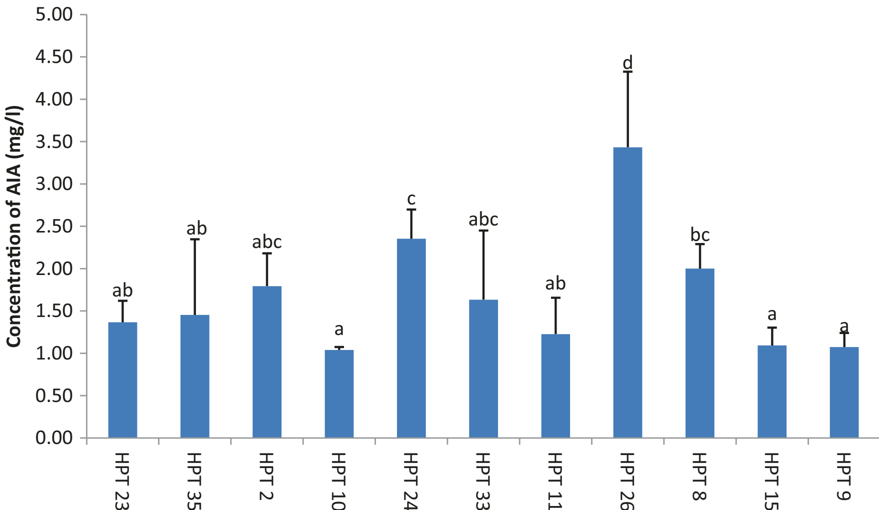


Fig. 7.2 Amount of AIA released by the PSB selected. Values are means of three replications. Means followed by the same letter are not significantly different at $P < 0.05$ (Fisher's least significant difference (LSD) test); ± values indicate standard errors of the means.

found that out of eight rhizobia isolated from nodules of *Swabi pea*, only four were able to produce siderophores. The antifungal activity of the test isolates indicated a close relationship between production of HCN and siderophores.

7.3.2.3 Quantification of Phytohormone (IAA)

All of the 15 *Rhizobium* strains were subjected to IAA production test. IAA was measured in the presence of L-tryptophan (Asghar et al. 2002). Eleven of the strains produced low concentrations of IAA ranging from 3.43 mg/L to 1.04 mg/L. Among these isolates, HPT 26 and HPT 24 produced maximum amounts of IAA (3.43–2.35 mg/L). The lowest amount was detected in the presence of HPT 10 (1.04 mg/L) (Fig. 7.2).

The current results are different to Ahmad et al. (2008) who isolated 72 rhizobacteria, which produced IAA at significant concentrations. The ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant. Growth promotion may be attributed to other mechanisms such as production of plant growth-promoting hormones in the rhizosphere and other PGP activities (Glick 1995).

7.3.3 Characterization for Extracellular Hydrolytic Enzyme Production

In the present work, we investigated in vitro the production of extracellular hydrolytic enzymes by the 15 rhizobia selected. All the PSB chosen were found positive for amylase and cellulase production. Only four rhizobia showed chitinase activity

and five strains for urease activity developing a red halo around the colonies, while six strains were protease positive (Table 7.2). These bacterial traits not only confer significant advantage in the presence of phytopathogens, since their cell walls will be degraded by the extracellular enzymes and their deleterious effects suppressed but also help in the process of P mineralization in soils. It was reported that PSB with cellulolytic activity enhanced the mineralization and decomposition of crop residues (Hameeda et al. 2006). Production of hydrolytic enzymes by PGPR is an important mechanism against phytopathogens for sustainable plant disease management. Bacterial strains with the ability of hydrolytic enzyme production could able to lysis the cell wall of pathogenic fungi and protect the host from pathogens (Moataza 2006). In another study, Naher et al. (2009) reported that some diazotrophs isolated from rice can produce hydrolytic enzymes.

7.3.4 Quantitative Assay of P Solubilization

After evaluating the phosphate-solubilizing ability on solid medium, the quantitative assay of this activity was carried out in PVK liquid medium. The results show

Table 7.2 Biochemical tests of selected isolates

Isolates	HCN	Siderophore	Urease	Cellulase	Protease	Chitinase	Amylase
HPT 24	–	+	–	–	–	–	+
HPT 23	+	+	+	+	+	–	+
HPT 22	–	–	–	–	–	+	+
HPT 26	+	++	–	+	+	–	+
HPT 29	++	+	–	+	–	–	+
HPT 16	++	+	–	+	–	–	+
HPT 11	+	–	–	+	–	++	–
HPT 35	+	+	+	+	+	+	+
HPT 2	+	–	+	+	++	–	+
HPT 33	+	++	–	+	–	–	+
HPT 10	++	+	–	–	+	–	+
HPT 15	++	+	+	+	+	–	+
HPT 9	++	++	+	+	–	–	+
HPT 8	–	++	–	–	–	++	+
HPT 7	–	+	–	+	–	–	+

that the solubilization ability varied among the eight PSB (Table 7.2). The highest P solubilized from 0.5% TCP was found in HPT 33 (137.39 mg/L), HPT 10 (119.39 mg/L), and HPT 24 (100.49 mg/L) compared to all the other PSB strains, while HPT 11 (2.99 mg/L) released low amount of P. The highest among of P soluble released by *Bacillus* and *Pseudomonas* with 373.07 mg/L and 368.58 mg/L, respectively, isolated from repeatedly chemical pesticide used agriculture soil from Dhanbad area (Tripti et al. 2012). In another study, Sridevi and Mallaiah (2007) shows that maximum solubilization was recorded with *Rhizobium* isolated from *Cassia absus* (620 mg/L) followed by *Rhizobium* sp. strain 19 (391 mg/L), and least in *Rhizobium* sp. strain 26 (156 mg/L) from *Sesbania sesban*.

The final pH of the culture filtrate ranged from 5.6 to 3.8 starting at initial pH of 6.8–7.0 after 7 days of incubation, indicating acidic nature. The maximum drop of pH was observed in HPT 33 (pH = 3.8) followed by HPT 10 (pH = 4.1). In Table 7.3, both the strains showed almost similar trend in increase in P solubilization as the pH decreases. The relationship between final culture pH and concentrations of solubilized P in the culture released by PSB was proved (Fig. 7.3). Significant negative correlation between final culture pH and concentrations of solubilized P was found ($r = -0.95$). If the final culture pH decreased, concentrations of solubilized P increased. Our findings are very well supported by the work done by Collavino et al. (2010) that showed that acidification of the broth medium coincided with phosphorus solubilization. Another study supports the previous reports. Meanwhile, a group of researchers from China has also shown that P released from insoluble form of phosphate was negatively correlated to the solution pH (Liu et al. 2012). Furthermore,

Table 7.3 Solubilization of 0.5% TCP by selected rhizobia

Isolates	[P] (mg/L)	pH_f
HPT 24	100.49(±15.42)[c]	4.26(±0.11)[b]
HPT 23	20.99 (±2.95)[b]	5.78 (±0.04)[d]
HPT 29	21.89 (±2.51)[b]	5.79 (±0.14)[d]
HPT 11	2.99 (±2.34)[a]	5.67 (±0.11)[d]
HPT 33	137.39 (±4.98)[e]	3.87 (±0.08)[a]
HPT 10	119.39 (±8.57)[d]	4.11 (±0.19)[ab]
HPT 15	18.89 (±1.45)[b]	5.77 (±0.04)[d]
HPT 7	95.74 (±5.01)[c]	3.81 (±0.10)[a]

Values are means of three replications. Means in the same column followed by the same letter are not significantly different at $P < 0.05$ (Fisher's least significant difference (LSD) test; ± values indicate standard errors of the means)

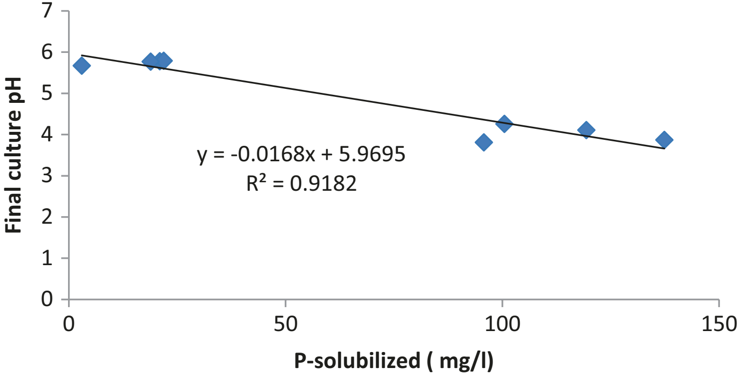


Fig. 7.3 Correlation analysis between final culture pH and soluble P

Pérez et al. (2007) also suggested that acidification of culture supernatants can be the main mechanism for P solubilization. It is well known in the literature that PSB solubilize insoluble phosphate in soil by secreting acid; this may indicate that our isolates might have used the same mechanism to solubilize TCP, which ultimately caused decline in the pH of final culture.

7.4 Conclusion

In our study, the characterization and screening of rhizobia from nodules of *H. pallidum* that helped in the selection of various phosphate-solubilizing bacteria have the potential to increase the available phosphate in the soil, which in turn will help to minimize the chemical fertilizer and promote sustainable agriculture. Some of the above-tested isolates could exhibit more than two or three PGP traits, which may promote plant growth directly or indirectly or synergistically.

References

Abdul W, Afzal A, Sultan T et al (2014) Isolation and biochemical characterization of rhizobium from *pea crop* at Swabi. Int J Biosci:231–240

Ahemad M, Zaidi A, Khan MS et al (2009) Biological importance of phosphorus and phosphate solubilizing microbes. In: Khan MS, Zaidi A (eds) Phosphate solubilizing microbes for crop improvement. Nova Science Publishers Inc., New York, pp 1–14

Ahmad F, Ahmad I, Khan MS (2008) Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. Microbiol Res 163:173–118

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

Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol 8:115–118

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 Soil 35:231–237

Babana AH, Dicko AH, Maïga K, Traoré D (2013) Characterization of rock phosphate-solubilizing microorganisms isolated from wheat (*Triticumaestivum*L.) rhizosphere in Mali. J Microbiol Microbial Res 1:1–6

Bakker AW, Schippers B (1987) Microbial cyanide production in the rhizosphere in relation to potato yield reduction and *Pseudomonas* spp. mediated plant growth stimulation. Soil Biol Biochem 19:451–457

Bhattacharyya P, Jha D (2011) Plant growth-promoting rhizobacteria (PGPsR): emergence in agriculture. World J Microbiol Biotechnol 28:1327–1350

Collavino MM, Sansberro PA, Mroginski LA, Aguilar OM (2010) Comparison of in vitro solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth. Biol Fertil Soils 46:727–738

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

Gordon, Weber RP (1951) Colorimetric estimation of indole-acetic acid. Plant Physiol 26:192–195

Hameeda B, Reddy YHK, Rupela OP, Kumar GN, Reddy G (2006) Effect of carbon substrates on rock phosphate solubilization by bacteria from composts and macrofauna. Curr Microbiol 53:298–302

Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60:579–598

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

Khan AA, Jilani G, Akhtar MS, Naqvi SMS, Rasheed M (2009a) Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. J Agric Biol Sci 1:48–58

Khan MS, Zaidi A, Wani PA, Ahemad M, Oves M (2009b) Functional diversity among plant growth-promoting rhizobacteria. In: Khan MS, Zaidi A, Musarrat J (eds) Microbial strategies for crop improvement. Springer, Berlin, pp 105–132

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

Knowles CJ, Bunch AW (1986) Microbial cyanide metabolism. Adv Microb Physiol 27:73–111

Liu Y, Feng L, H H, Jiang G, Cai Z et al (2012) Phosphorus release from low-grade phosphate rocks by low molecular weight organic acids. J Food Agric Environ 10:1001–1007

Marx JC, Blaise V, Collins T, D'Amico S, Delille D, Gratia E et al (2004) A perspective on cold enzymes: current knowledge and frequently asked questions. Cell Mol Biol (Noisy le-Grand, France) 50(5):643–655

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31:426–428

Moataza MS (2006) Destruction of *Rhizoctoniasolani* and *Phytophthoracapsici* causing tomato root-rot by *Pseudomonas fluorescences* lytic enzymes. Res J Agric Biol Sci 2:274–281

Naher UA, Radziah O, Shamsuddin ZH, Halimi MS, MRazi I (2009) Isolation of diazotrophs from different soils of tanjongkarang rice growing area in Malaysia. Int J Agric Biol 11:547–552

Pérez E, Sulbaràn M, Ball MM, Yarzàbal LA (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

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

Renwick A, Campbell R, Coc S (1991) Assesment of in vivo screening systems for potential biocontrol agents of *Gaeumannomycesgraminis*. Plant Pathol 40:524–532

Rodriguez R, Vassilev N, Azcon R (1999) Increases in growth and nutrient uptake of alfalfa grown in soil amended with microbially- treated sugar beet waste. Appl Soil Ecol 11:9–15

Sahin F, Cakmakci R, Kantar F (2004) Sugar beet and barley yields in relation to inoculation with N2-fixing and phosphate solubilizing bacteria. Plant Soil 265:123–129

Schwyn B, Neilands JB (1997) Universal chemical assay for the detection and determination of siderophores. Anal Biochem 160:47–56

Sridevi M, Mallaiah KV (2007) Phosphate solubilization by *Rhizobium* strains. Indian J Microbiol 49:98–102

Tripti, Kumar V, Anshumali (2012) Phosphate solubilizing activity of some bacterial strains isolated from chemical pesticide exposed agricultural soil. Int J Eng Res Dev 3(9):01–06

Vessey JK (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255:571–586

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

Vincent JM (1970) A manual for the practical study of root nodule bacteria. In: IBP Handbook, No 15. Blackwell Scientific Publications Ltd., Oxford

Vining LC (1990) Functions of secondary metabolites. Annu Rev Microbiol 44:395–427

Wani PA, Khan MS, Zaidi A (2007) Co-inoculation of nitrogen-fixing and phosphatesolubilizing 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 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) 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

Check for updates

8 Development of Abiotic Stress Tolerance in Crops by Plant Growth-Promoting Rhizobacteria (PGPR)

Sivakumar Subiramani, Sathishkumar Ramalingam, Thiruvengadam Muthu, Shivraj Hariram Nile, and Baskar Venkidasamy

Abstract

Agriculture production was effectively decreased by abiotic and biotic stresses, which affect the plant growth by ion toxicity, hormonal and nutritional imbalance, and physiological and metabolic changes. Plant growth-promoting rhizobacteria (PGPR) are the root-colonizing non-pathogenic bacterium, which helps in plant growth promotion and alleviation of the stress-induced changes to result in the maintenance of agricultural productivity. Plants inoculated with the PGPR provide resistance to various abiotic stresses such as salt, drought, and heavy metal toxicity. Some PGPR strains protect both the biotic and abiotic stresses. In addition, several PGPR contribute to multiple abiotic stress tolerance in plants. PGPR produce phytohormones, siderophores, organic acids, and stress-induced metabolites such as osmotic solutes, prolines, and antioxidant enzymes and up- and downregulates the expression of various stress-responsive genes that provide resistance to the plants under stressful conditions. The use of PGPR is a simple and effective alternative approach to genetic engineering and breeding methods for crop improvement, since breeding and genetic engineering are time-consuming, expensive, and laborious procedures. In this chapter, we described the potential role of PGPR in the abiotic stress tolerance in plants. Moreover, the mechanism of PGPR in drought, salt, and heavy metal stress alleviation was described briefly.

S. Subiramani · S. Ramalingam · B. Venkidasamy (✉)
Plant Genetic Engineering Laboratory, Department of Biotechnology, Bharathiar University, Coimbatore, Tamil Nadu, India

T. Muthu
Department of Applied Bioscience, College of Life and Environmental Sciences, Konkuk University, Seoul, Republic of Korea

S. H. Nile
Department of Bioresources and Food Science, College of Life and Environmental Sciences, Konkuk University, Seoul, Republic of Korea

© Springer Nature Singapore Pte Ltd. 2020
M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_8

Keywords
PGPR · Biotic stress · Abiotic stress · Salt · Drought · Heavy metal · Alleviation

8.1 Introduction

The soil zone surrounding the plant roots is called rhizosphere, where the interactions between the soil, root, and other biotic factors can occur, which is influenced by the plant roots. The organic carbon in the form of root exudates released from the plant roots provides nutrition for microbial growth, and therefore the rhizosphere soil exhibits maximal microbial population (Burdman et al. 2000). These microbes can interact with the plant roots, and these interactions might serve as favorable, deleterious, and neutral, therefore effectively influencing the plant growth and development (Ahmad et al. 2011a; Lau and Lennon 2011). Various types of microbes include bacteria, actinomycetes, fungi, algae, and protozoa were often colonized with the plant roots. The growth and development of the plants were enhanced by the application of these microbes are well documented. Among the different microbiota, bacteria constitute the major and dominant in rhizosphere soil (Kaymak et al. 2009). The microbial interaction specificity is influenced by the amount and constitution of the root exudates as well as the soil condition. The exudates released from the plant roots act as a signal for rhizobacteria to reach the root surface and interaction though a process called chemotaxis. Several studies hypothesized the role of PGPR in plant growth promotion, whereas their precise mechanism was still largely unrevealed. This growth-promoting activity differed between the bacterial strains and the compounds secreted by the microorganisms.

A diverse group of bacterial populations dwelling in the zonal root area referred as plant growth-promoting rhizobacteria (PGPR). PGPR include several genera such as *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Serratia*, and *Streptomyces* spp. (Ma et al. 2011a, b). On the basis of relationship to plants, PGPR are divided into symbiotic (intracellular) and a free-living (extracellular) bacterium. Rhizobia are the well-known group belonging to the intracellular PGPR, and they produce nodules in legume plants.

PGPR are associated with several plants and offer various favorable functions including enhanced plant growth and decreased sensitivity to diseases caused by plant pathogens (Shahzadi et al. 2012). The enhanced root hair branches, efficient seed germination, more leaf area per plant, liberation of phytohormones, enhanced availability of nutrients to plants, and enhanced plant biomass are the mechanisms of PGPR in plant growth promotion (Podile and Kishore 2006). Some studies showed that the production of the plant hormone, biocontrol agents, and enhanced uptake of nutrients by the plants are facilitated by the PGPR. Several investigations described the mechanisms of PGPR in plant growth promotion. Plant growth-promoting activity of PGPR was achieved through the production of siderophores and organic acids for nutrient mobilization and phytohormones (auxin, cytokinin, abscisic acid, gibberellic acid, and ethylene) for plant growth regulation (Dimkpa

et al. 2009). Modern agriculture faces several stresses including biotic (living) and abiotic (nonliving) stresses. Plant pathogens (virus, bacteria, and fungi), herbivores (insects, nematodes), and parasitic weeds are the major biotic stressors imposed a severe reduction in crop production. Similarly, the abiotic stress factors include excess water, drought, heat, and chilling also cause a major reduction in agriculture productivity, and this contributes >50% reduction in the yield (Tardieu and Tuberosa 2010). The oxidative stress induced by drought and salinity stresses causes metabolic and biochemical changes which in turn affects the crop yield (Shafi et al. 2010). Several studies reported the enhanced plant tolerance to abiotic stresses by PGPR treatment, and it can generate the "induced systemic tolerance" in plants to salt and drought stresses (Yang et al. 2009; Grichko and Glick 2001; Yuwono et al. 2005; Egamberdiyeva 2007; Sziderics et al. 2007; Belimov et al. 2009; Dimkpa et al. 2009a). In addition, PGPR facilitates the uptake of nutrients from the soil, which leads to decreased accumulation of fertilizers (nitrates and phosphates) in the cultivation field. Figure 8.1 showed the potential role of PGPR in the alleviation of abiotic stress tolerance in plants. It was reported that microbes isolated from one host plant root grown in desert farming environment improved the growth of another distinct host plant species cultivated under drought condition (Marasco et al. 2013). PGPR can be applied to plants for growth promotion through various methods. Biopriming is a common method of seed treatment with the bacterial cells for improving plant growth. The foliar spray also employed the application of PGPR in

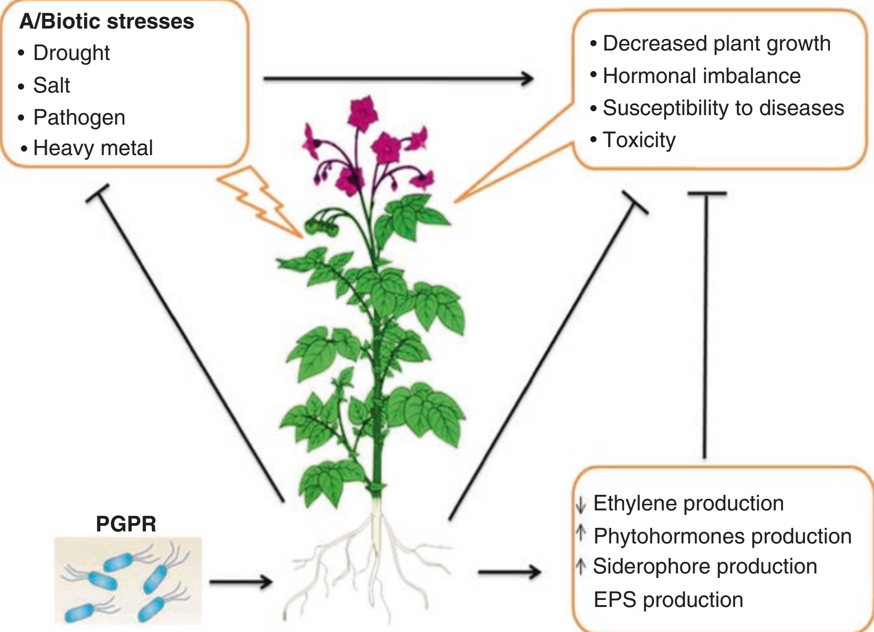


Fig. 8.1 Potential role of plant growth-promoting rhizobacteria (PGPR) in abiotic stress tolerance in plants. *EPS* exopolysaccharide

plants. Thus, the application of PGPR reduced the inclusion of fertilizer and decreased the environmental pollution which comes through the fertilizer runoff and contaminated the aquatic ecosystem.

8.2 Tolerance to Drought Stress

Similar to salt stress, drought is the growing risk factor that decreased the worldwide agricultural productivity. Decreased assimilation of photosynthetically active radiation, reduced use of radiation capability, and diminished harvest index are the main factors of crop yield loss due to drought stress (Farooq et al. 2009). Plants needed a various adaptation and alleviation strategies to survive under drought conditions. PGPR are effectively involved in the alleviation of drought stresses in plants. The plant rhizosphere/endorhizosphere-residing PGPR involved in the production of phytohormones, exopolysaccharides (EPS), volatile compounds, and ACC deaminase stimulates the production of osmolytes, antioxidants, regulation of stress-responsive genes, and alteration of root morphology, which results in drought tolerance in plants. The PGPR application enhanced shoot growth of the plants. Therefore, the PGPR maintain the shoot growth rates under drought stress, which leads to enhanced agricultural productivity. Improved shoot growth was observed in the PGPR (*Bacillus* spp.)-treated corn plants (Vardharajula et al. 2011). Stimulation of osmotic and stress-responsive genes by PGPR is essential for the plants to grow under drought stress. PGPR plays an important role in providing resistance and adaptation of plants to drought stresses and has the significant role in deciphering the future food security problems. In addition to the metabolic and osmotic changes induced in the plants by the PGPR treatment, it also alters the soil properties to facilitate the plant growth. Although the PGPR stimulates the plant growth and yield, the type of inoculation affects their activity. For instance, in species of Brassica oilseed rape, the application of either *Pseudomonas fluorescens* or *Pseudomonas putida* results in the better growth, yield, and alleviation of drought stress (Arvin et al. 2012), whereas the co-inoculation of *Pseudomonas fluorescens or Pseudomonas putida* leads to antagonistic effects which indicates that the inoculation methods also determine the mitigation of stress. List of studies was carried out for the drought stress resistance induced by the PGPR treatment in various plants was shown in Table 8.1**.** Another major advantage of PGPR application is the inclusion of resistant to multiple types (more than one stress) of stresses (abiotic/biotic). In the arid and semiarid lands, crops often faced multiple stresses, and the application of PGPR would provide resistant to multiple stresses (Mayak et al. 2004; Rodriguez et al. 2008). For example, the enhanced resistance to drought and pathogen attack was observed in the *Arabidopsis* plant symbiosis with the *Paenibacillus polymyxa* bacterium (Timmusk and Wagner 1999). Genetic engineering and plant breeding are commonly employed for the development of drought-resistant crop varieties, whereas it is an expensive and time-consuming process. However, the PGPR application could effectively alleviate the drought stress in

Table. 8.1 List of studies that used the PGPR strains for abiotic stress tolerance in plants

Crop	PGPR	Stress	References
Triticum aestivum	*Bacillus safensis* *Ochrobactrum pseudogregnonense*	Salt	Chakraborty et al. (2013)
Triticum aestivum	*Pseudomonas putida* Enterobacter cloacae *Serratia ficaria* *P. fluorescens*	Salt	Nadeem et al. (2013)
Oryza sativa	*Alcaligenes faecalis* *Bacillus pumilus* *Ochrobactrum* sp.	Salt	Bal et al. (2013)
Oryza sativa	*P. pseudoalcaligenes* *B. pumilus*	Salt	Jha et al. (2013)
Triticum aestivum	*B. subtilis* *Arthrobacter* sp.	Salt	Upadhyay et al. (2012)
Triticum aestivum	*Azospirillum* sp.	Salt	Zarea et al. (2012)
Triticum aestivum	*Streptomyces* sp.	Salt	Sadeghi et al. (2012)
Persea gratissima	*Pseudomonas* sp. *Bacillus* sp. *Variovorax* sp	Salt	Nadeem et al. (2012)
Zea mays	*Azotobacter chroococcum*	Salt	Rojas-Tapias et al. (2012)
Cicer arietinum	*P. pseudoalcaligenes* *P. putida*	Salt	Patel et al. (2012)
Arachis hypogaea	*Brachybacterium saurashtrense* *Brevibacterium casei* *Haererohalobacter* sp	Salt	Shukla et al. (2012)
Phaseolus vulgaris	*P. extremorientalis* *P. ehlororaphis*	Salt	Egamberdieva (2011)
Triticum aestivum	*Bacillus* *Burkholderia* Enterobacter *Microbacterium* *Paenibacillus*	Salt	Upadhyay et al. (2011)
Lycopersicon esculentum	*P. fluorescens* *P. aeruginosa,* *P. stutzeri*	Salt	Tank and Saraf (2010)
Solanum melongena	*Pseudomonas* sp.	Salt	Fu et al. (2010)
Triticum durum	*Azospirillum* sp.	Salt	Nabti et al. (2010)
Gossypium hirsutum	*P. putida*	Salt	Yao et al. (2010)
Zea maize	*B. megaterium*	Salt	Marulanda et al. (2010)

(continued)

Table. 8.1 (continued)

Crop	PGPR	Stress	References
Raphanus sativus	*Agrobacterium rubi* *Burkholderia gladii* *P. putida* *B. subtilis* *B. megaterium*	Salt	Kaymak et al. (2009)
Hordeum vulgare	*A. brasilense*	Salt	Omar et al. (1994)
L. sativa L. cv. Tafalla	*P. mendocina*	Salt	Kohler et al. (2009, 2010)
Arabidopsis thaliana	*B. subtilis*	Salt	Zhang et al. (2008)
Phaseolus vulgaris	*A. brasilense*	Salt	Dardanelli et al. (2008)
Zea mays	*Bacillus* sp. *Ochrobactrum* sp.	Salt	Principe et al. (2007)
Zea mays	*P. syringae* *P. fluorescens* *E. aerogenes*	Salt	Nadeem et al. (2007)
Arachis hypogaea	*P. fluorescens*	Salt	Saravanakumar and Samiyappan (2007)
L. sativa	*Azospirillum*	Salt	Barassi et al. (2006)
Piper nigrum	*P. fluorescence*	Salt	Paul et al. (2006)
Oryza sativa	*P. pseudoalcaligenes*	Salt	Diby et al. (2005)
Lycopersicon esculentum	*Achromobacter piechaudii*	Salt	Mayak et al. (2004)
Triticum aestivum	*Aeromonas hydrophila* *B. insolitus* *Bacillus sp.*	Salt	Ashraf et al. (2004)
Zea mays	*Azospirillum*	Salt	Hamdia et al. (2004)
C. arietinum *Vicia faba*	*A. brasilense*	Salt	Hamaoui et al. (2001)
Leptochloa fusca	*A. lipoferum* *A. brasilense* *Azoarcus* *Pseudomonas* sp.	Salt	Malik et al. (1997)
Solanum lycopersicum	*Glomus mosseae*	Salt	He et al. (2007)
Glycine max	*Glomus etunicatum*	Salt	Sharifi et al. (2007)
Oryza sativa	*Osmotolerant bacteria*	Drought	Yuwono et al. (2005)
Lycopersicon esculentum *Capsicum annuum*	*Achromobacter piechaudii*	Drought	Mayak et al. (2004)
Triticum aestivum	*Azospirillum*	Drought	Creus et al. (2004, 2005)

(continued)

Table. 8.1 (continued)

Crop	PGPR	Stress	References
Zea mays	*A. brasilense*	Drought	Casanovas et al. (2002)
P. vulgaris	*A. brasilense*	Drought	German et al. (2000)
Lycopersicon esculentum	A. brasilense	Drought	Creus et al. (2005), Molina-Favero et al. (2008)
Zea mays	*Azospirillum lipoferum*	Drought	Cohen et al. (2009)
Triticum aestivum	*Azospirillum* sp.	Drought	Arzanesh et al. (2011)
Arabidopsis thaliana	*Phyllobacterium brassicacearum* strain STM196	Drought	Bresson et al. (2013)
Platycladus orientalis	*Bacillus subtilis*	Drought	Liu et al. (2013)
Glycine max	*P. putida* H-2–3	Drought	Sang-Mo et al. (2014)
Lavandula dentate	*B. thuringiensis*	Drought	Ahmad et al. (2014)
Triticum aestivum	*Rhizobium leguminosarum* (LR-30), *Mesorhizobium ciceri* (CR-30, CR-39) *Rhizobium phaseoli* (MR-2)	Drought	Hussain et al. (2014)
Lycopersicon esculentum *Capsicum annuum*	*Achromobacter piechaudii* ARV8	Drought	Mayak et al. (2004)
Pisum sativum	*Variovorax paradoxus* 5C-2	Drought	Dodd et al. (2005)
Pisum sativum	*Pseudomonas fluorescens* Biotype G (ACC-5)	Drought	Zahir et al. (2008)
Pisum sativum	*V. paradoxus* 5C-2	Drought	Belimov et al. (2009)
Triticum aestivum	ACC deaminase-producing rhizobacteria	Drought	Shakir et al. (2012)
Cicer arietinum	Consortia of *Bacillus* isolate 23-B *Pseudomonas* 6-P *Mesorhizobium ciceris*	Drought	Sharma et al. (2013)
Capsicum annuum	*Bacillus licheniformis* K11	Drought	Hui and Kim (2013)
Triticum aestivum	*Bacillus thuringiensis* AZP2	Drought	Timmusk et al. (2014)
Zea mays	*Pseudomonas putida* GAP-P45	Drought	Sandhya et al. (2010)
Zea mays	*P. fluorescens*	Drought	Ansary et al. (2012)
Lavandula dentate	*B. thuringiensis*	Drought	Ahmad et al. (2014)
Lycopersicon esculentum	*Bacillus polymyxa*	Drought	Shintu and Jayaram (2015)

(continued)

Table. 8.1 (continued)

Crop	PGPR	Stress	References
Oryza sativa	Consortia containing *P. jessenii*, R62, *P. synxantha*, R81 *A. nitroguajacolicus* strainYB3, strain YB5	Drought	Gusain et al. (2015)
Zea mays	*Azospirillum lipoferum*	Drought	Bano et al. (2013)
Phaseolus vulgaris	*Rhizobium etli*	Drought	Suarez et al. (2008)
Zea mays	*A. brasilense*	Drought	Rodriguez et al. (2009)
Arabidopsis thaliana	*Bacillus subtilis* GB03	Drought	Zhang et al. (2010)
Zea mays	*Klebsiella variicola* F2, *Pseudomonas fluorescens* YX2 *Raoultella planticola* YL2	Drought	Gou et al. (2015)
Oryza sativa	*Azoapirillum brasilense* Az39	Drought	Cassan et al. (2009)
Arabidopsis thaliana	*Paenibacillus polymyxa* B2	Drought	Timmusk and Wagner (1999)
Capsicum annuum	*B. licheniformis* K11	Drought	Lim and Kim (2013)
Triticum aestivum	*Bacillus amyloliquefaciens* 511 *Azospirillum brasilense* NO40	Drought	Kasim et al. (2013)
Arabidopsis thaliana	*Pseudomonas chlororaphis* O6	Drought	Cho et al. (2013)
Sugarcane	*Gluconacetobacter diazotrophicus* PAL5	Drought	Vargas et al. (2014)
Arabidopsis thaliana	*Azospirillum brasilense* sp. 245strain	Drought	Cohen et al. (2015)
Cucumis sativa cucumber	*Bacillus cereus* strain AR156 *B. subtilis* strain SM21 *Serratia* sp. strain XY21	Drought	Wang et al. (2012)
Helianthus annuus	*Achromobacter xylosoxidans* (SF2) *Bacillus pumilis* (SF3 and SF4)	Drought	Castillo et al. (2013)
Hyoscyamus niger	*Pseudomonas putida* strain (PP), *Pseudomonas fluorescens* strain (PF)	Drought	Ghorbanpour et al. (2013)
Sorghum bicolor	*Bacillus* spp. Strains KB122, KB129, KB133, KB142	Drought	Grover et al. (2014)
Solanum tuberosum	*Bacillus pumilus* strain DH-11 *Bacillus firmus* strain 40	Drought	Gururani et al. (2013)
Vigna radiata	*Pseudomonas fluorescens* strain Pf1 *Bacillus subtilis* EPB5, EPB22, and EPB 31	Drought	Saravanakumar et al. (2011)
Zea mays	PGPR isolate 1 K, 9 K and KB	Drought	Yasmin et al. (2013)
Zea mays	*Proteus penneri* strain(Pp1) *Pseudomonas aeruginosa* strain (Pa2) *Alcaligenes faecalis* strain (AF3)	Drought	Naseem and Bano (2014)

(continued)

Table. 8.1 (continued)

Crop	PGPR	Stress	References
Zea mays	*Burkholderia phytofirmans* strain PsJN Enterobacter sp. strain FD17	Drought	Naveed et al. (2014)
Zea mays	*Bacillus amyloliquefaciens* strain HYD-B17 *B. licheniformis* strain HYTAPB18 B. *Thuringiensis* strain HYDGRFB19 *Paenibacillus favisporus* strain BKB30 B. *Subtilis* strain RMPB44	Drought	Vardharajula et al. (2011)
Zea mays	*Burkholderia* sp. strainLD-11	Drought	Fan et al. (2015)
Oryza sativa	*Bacillus subtilis* *Bacillus megaterium* *Bacillus* sp.	Heavy metal	
Brassica juncea	*Rhodococcus* sp. *Variovorax paradoxus*	Heavy metal	Belimov et al., (2005)
Pisum sativum	*P. brassicacearum* AM3, *P. marginalis* Dp1	Heavy metal	Safronova et al., (2006)
Trifolium pratense	*Brevibacillus* spp.	Heavy metal	Vivas et al., (2003)
Zea mays	Soil bacteria AN8, AN12	Heavy metal	Hassan et al. (2014)
Cicer aritenum	*Acinetobacter* sp. nbri05	Heavy metal	Srivastava and Singh (2014)
Zea mays	*Acinetobacter* sp. RG30 *Pseudomonas putida* GN04	Heavy metal	Rojas-Tapias et al. (2014)
Zea mays *Helianthus annuus*	*Pseudomonas* sp. TLC 6–6.5-4	Heavy metal	Li and Ramakrishna (2011)
Lolium multiflorum *Glycine max*	*Bradyrhizobium* sp. YL-6	Heavy metal	Guo and Chi (2014)
Brassica napus	*Rahnella* sp. JN6	Heavy metal	He et al. (2013)
Alyssum pintodasilvae	*Arthrobacter nicotinovorans* SA40	Heavy metal	Cabello-Conejo et al. (2014)
Medicago lupulina	*Sinorhizobium meliloti* CCNWSX0020	Heavy metal	Kong et al. (2015)
Triticum aestivum *Zea mays*	*Klebsiella* sp. CIK-502	Heavy metal	Ahmad et al. (2014)
Zea mays	*Ralstonia eutropha* *Chryseobacterium humi*	Heavy metal	Moreira et al. (2014)
Brassica juncea	*Bacillus* sp. MN3–4	Heavy metal	Shin et al. (2012)
Salix caprea	*Burkholeria* sp. RX232	Heavy metal	Kuffner et al. (2010)
Salix caprea	*Microbacterium* sp. EX72	Heavy metal	Kuffner et al. (2010)

(continued)

Table. 8.1 (continued)

Crop	PGPR	Stress	References
Lens culinaris var. Malka	*Rhizobium* sp. RL9	Heavy metal	Wani and Khan (2013)
Solanum nigrum *Zea mays* *Amaranthus hypochondriacus* *Amaranthus mangostanus*	*Rahnella* sp. JN27	Heavy metal	
Sorghum bicolour *Phytolacca acinosa* *Solanum nigrum*	*Bacillus* sp. SLS18	Heavy metal	Luo et al. (2012)
Brassica napus	Cu-resistant isolates belonged to *Firmicutes*, b *Actinobacteria* *Proteobacteria*	Heavy metal	Sun et al. (2010)
Alyssum serpyllifolium *Brassica juncea*	*Pseudomonas* sp. A3R3	Heavy metal	Ma et al. (2011a, b)

plants, and it is a rapid and cheap method for the drought stress management in dryland agriculture.

8.3 Protection Against Salt Stress

Among the various abiotic stresses, salinity was the major factor that decreased the crop yield through affecting the germination, plant phase transition, plant vigor, and yield (Munns and Tester 2008). Saline soils are often considered as barren lands, which are not suitable for plant cultivation. Previous reports indicated that salinity affects ~800 million hectares of land all over the world. Salt stress reduced the crop yield through suppression of photosynthesis, protein synthesis, and metabolism of lipids. The application of PGPR could be useful to resolve this issue. Various beneficial bacteria such as *Azospirillum*, *Azotobacter*, *Rhizobium, Bradyrhizobium*, *Bacillus*, and *Pseudomonas* have been isolated from the unfavorable stressful ecosystems (acid soils, saline, alkaline, desert environments and eroded hill slopes, and they could participate in reclamation of the soil (Selvakumar et al. 2009; Upadhyay et al. 2009). Salinity-resistant PGPR attribute osmotolerance to the plants inhabiting in the saline soil that in turn provide various beneficiary actions such as improved growth (enhanced root and shoot growth), nutrient uptake, chlorophyll content, vigor, and yield. The mobilization of nutrients and regulation of phytopathogens in the rhizosphere soil, as well as the synthesis of 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) and phytohormones, contribute to plant tolerance. Moreover, nutrient circulation in the rhizosphere soil and osmolyte accumulation in plants were also achieved by the PGPR treatment. Higher accumulation of K^+ ion in

the PGPR treatment causes the enhanced K^+/Na^+ ratio, which in turn facilitates the salinity tolerance. Salt stress-mediated oxidative stresses was scavenged by the rhizobacterial antioxidative enzymes. ACC deaminase produced by the PGPR enhanced the plant growth under adverse environmental conditions (Belimov et al. 2001). Furthermore, the production of ACC deaminase by the PGPR plays a role in the regulation of ethylene production. In line with this, a recent study demonstrated that *Distichlis spicata* (halophilic grass)-associated rhizobacteria (*Bacillus* sp. and *Pseudomonas lini*) effectively stimulates the growth of *Arabidopsis* seedlings in saline conditions (Palacio-Rodríguez et al. 2017). The salt tolerance is associated with the phosphate solubilization and production of IAA and siderophore. In addition, ACC deaminase expression and enhanced auxin redistribution in the roots of *Arabidopsis* were also observed. This is emphasized that salt resistant is rendered by the rhizobacterial strains associated with the saline-tolerant crops. It was reported that survival of the seedlings was improved by suppressing the ethylene level in the first few days after sowing and enhanced the root formation (Glick et al. 1998). Wang et al. (2016) demonstrated that ACC deaminase producing rhizobacterial (*Variovorax paradoxus* 5C-2) inoculation in pea plants decreased the Na^+ flow and increased the K^+ uptake and root to shoot K flow under salt stress. PGPR inoculation causes enhanced photosynthetic efficiency and improves the plant growth. Furthermore, multiple stress tolerance was also reported for the microbes inoculated with the plants. For instance, the enhanced resistance to salt and *Fusarium* and *Blumeria* infections was found in the barley plants applied with the *Piriformospora indica* (Waller et al. (2005). Several studies showed the potential application of PGPR in the salt stress tolerance in various plants as represented in Table. 8.1**.** The use of PGPR as microbial inoculants for crop improvement in saline ecosystems is a powerful strategy for saline agriculture management.

8.4 Protection Against Heavy Metal Stress

Apart from the salt and drought stress, plants are continuously exposed to heavy metal stress, which mainly arises from the industrial and other environmental pollution. In addition to having plant growth-promoting traits, certain bacterial strains are also very important in the alleviation of the heavy metal toxicity in plants. In the metal-accumulated soil, PGPR could help in the plant growth and survival (Rajkumar and Freitas 2008). PGPR and mycorrhizal fungi are involved in the reduction of harmful heavy metals which in turn protects the plants from toxicity induced by heavy metals (Denton 2007). Soil microbes secrete acids, phytoantibiotics, proteins, and other chemicals that help in the alleviation of toxic heavy metal-induced stresses in plants (Denton 2007). The facilitation of heavy metal stress tolerance in plants is provided by some PGPR through the increased plant growth by enhanced phytoremediation, mitigation of metal toxicity, altered metal accumulation capability, and enhanced translocation of metals within the plant. PGPR aided the improved plant growth via the decreased metal phytotoxicity and the altered phytoavailability of heavy metals in polluted soil through the detoxification, accumulation,

transformation, and sequestration (Ma et al. 2016). Phytoremediation was strongly influenced by the metal phytotoxicity. The reduction of phytotoxicity by the action of *Bacillus* sp. MN3–4 (lead-resistant bacterium) isolated from the *Alnus firma* (metal hyperaccumulator) through extracellular sequestration and intracellular accumulation, which results in the improved phytoremediation (Shin et al. 2012). Furthermore, the secretion of organic acids and siderophores by PGPR facilitates the increased metal and mineral mobilization and enhanced uptake of heavy metal that leads to increased metal phytoextraction potential of host plants (Chen et al. 2014). For instance, the synthesis of ACC deaminase, IAA production, P solubilization, siderophore production, and plant polymer-hydrolyzing enzymes by the action of *Pseudomonas* sp. A3R3 (Ni-resistant bacteria) isolated from the hyperaccumulator (*A. serpyllifolium*) host plant enhanced the growth of *Brassica juncea* cultivated in the Ni-contaminated soil (Ma et al. 2011a, b). Certain bacterial species include *Pseudomonas* sp. can produce biosurfactants, which are involved in heavy metal mobilization in the contaminated soil (Braud et al. 2006). Sun et al. (2010) reported that improved plant growth and translocation of Cu in *B. napus* with the help of copper-resistant PGPR via the production of ACC deaminase, siderophore synthesis, and arginine decarboxylase activity. The reduction of Cd bioavailability in rhizosphere soils by the action of *B. megaterium* H3 and *Neorhizobium huautlense* T1–17 leads to decreased accumulation of Cd in the polished rice (Li et al. 2017). The multibeneficial action of PGPR included the enhanced plant growth and development and decreased the heavy metal accumulation in plants. Various studies showed the potential application of PGPR in the heavy metal stress tolerance in plants, as represented in Table. 8.1**.** Moreover, the application of microbial communities was more effective in plant growth and development as well as heavy metal degradation as compared to the single microorganism.

8.5 Harmful Aspects of PGPR

PGPR functions in maintaining the soil fertility as well as plant growth and development. Most of the studies emphasized the positive aspects (growth enhancement) of PGPR, whereas some reports indicated the negative action of PGPR (Alstrom and Burns 1989; Saharan and Nehra 2011; Suslow and Schroth 1982). For instance, certain *Pseudomonas* species produces cyanide, which acts as growth promotor as well as suppressor activities. Cyanide acts as a biocontrol agent against certain plant pathogens, and it can also cause adverse effects on plant growth (Martínez-Viveros et al. 2010; Bakker and Schippers 1987). PGPR is also known to produce auxin, which functions as positive and negative fashion depending on their concentration (Eliasson et al. 1989; Vacheron et al. 2013; Young and Mulkey 1997). For example, at low concentration, it enhances plant growth, whereas at a high level, it inhibits root growth (Patten and Glick 2002; Xie et al. 1996). Similar dual roles of PGPR products were reported including the rhizobitoxine produced by *Bradyrhizobium elkanii.* It acts as a suppressor of ethylene synthesis and thus mitigates the harmful effect of stress-stimulated ethylene synthesis on nodulation (Vijayan et al. 2013).

On the other hand, it can induce foliar chlorosis in soybeans (Xiong and Fuhrmann 1996). Although PGPR is very effective for promoting plant growth and development, certain bacterial species may function as growth inhibitory action. However, such a negative role may occur under certain specific conditions and also for some particular traits. Therefore, the selection of a suitable strain is crucial for obtaining maximum benefits regarding improved plant growth and development.

8.6 Concluding Perspectives

Plant growth-promoting rhizobacteria are enriched in the rhizosphere soil (root zones) and serve in the enhancement of plant growth and development. PGPR gives protection to plants against salt, drought, and heavy metal stresses. In general, PGPR was shown to produce growth promoting (phytohormones, siderophores, organic acids, and volatile compounds) and stress-induced metabolites (osmotic solutes, proline, nitric oxides, and induction of antioxidant enzymes) that function in the plant growth promotion and stress mitigation activities. These potential beneficial functions of PGPR indicate that their role in the sustainable agriculture production. Although the genetic engineering and breeding approaches were helpful to generate the abiotic stress-tolerant crops, it required more time and considered as expensive as compared to the PGPR application. The phenotypical, physiological, and molecular mechanism of biotic stress tolerance attributed by the bacterium has been conducted widely (Van Loon et al. 1998). Several studies reported the PGPR-mediated abiotic stress tolerance, whereas details on the mode of action in abiotic stress tolerance were still lacking. Moreover, the detailed molecular mechanisms articulate their role and are still understudied. Therefore, it is warranted that the molecular mechanism behind the stress alleviation and growth promotion is essential to unravel their specific functions.

Acknowledgments This study was supported by a grant (Sanction No.: PDF/2016/002258LS) from the National Post Doctoral Fellowship, Department of Science and Technology, Science and Engineering Research Board, Government of India.

Conflict of interest: The authors declare that they have no conflict of interest.

References

Ahmad I, Akhtar MJ, Zahir ZA, Naveed M, Mitter B, Sessitsch A (2014) Cadmium- tolerant bacteria induce metal stress tolerance in cereals. Environ Sci Pollut Res 21(18):11054–11065

Alstrom S, Burns RG (1989) Cyanide production by rhizobacteria as a possible mechanism of plant growth inhibition. Biol Fertil Soils 7:232–238

Ansary MH, Rahmani HA, Ardakani MR, Paknejad F, Habibi D, Mafakheri S (2012) Effect of *Pseudomonas fluorescens* on proline and phytohormonal statusof maize (*Zea mays* L.) under water deficit stress. Annal. Biol Res 3:1054–1062

Armada E, Roldan A, Azcon R (2014) Differential activity of autochthonous bacteria in controlling drought stress in native Lavandula and Salvia plants species under drought conditions in natural arid soil. Microb Ecol 67:410–420

Arvin P, Vafabakhsh J, Mazaheri D, Noormohamadi G, Azizi M (2012) Study of drought stress and plant growth promoting Rhizobacteria (PGPR) on yield, yield components and seed oil content of different cultivars and species of Brassica oilseed rape. Ann Biol Res 3(9):4444–4451

Arzanesh MH, Alikhani HA, Khavazi K, Rahimian HA, Miransari M (2011) Wheat (*Triticum aestivum* L.) growth enhancement by *Azospirillum* sp. under drought stress. World J Microbiol Biotechnol 27:197–205

Ashraf M, Hasnain S, Berge O, Mahmood T (2004) Inoculating wheat seedlings with exopolysaccharide-producing bacteria restricts sodium uptake and stimulates plant growth under salt stress. Biol Fertil Soils 40(3):157–162

Bakker AW, Schippers B (1987) Microbial cyanide production in the rhizosphere in relation to potato yield reduction and Pseudomonas spp. mediated plant growth-stimulation. Soil Biol Biochem 19:451–457

Bal HB, Nayak L, Das S, Adhya TK (2013) Isolation of ACC deaminase producing PGPR from rice rhizosphere and evaluating their plant growth promoting activity under salt stress. Plant Soil 366(1–2):93–105

Bano Q, Ilyas N, Bano A, Zafar N, Akram A, Ul Hassan F (2013) Effect of *Azospirillum* inoculation on maize (*Zea mays* L.) under drought stress. Pak J Bot 45:13–20

Barassi CA, Ayrault G, Creus CM, Sueldo RJ, Sobrero MT (2006) Seed inoculation with Azospirillum mitigates NaCl effects on lettuce. Sci Hortic-Amst 109(1):8–14

Belimov AA, Safronova VI, Sergeyeva TA, Egorova TN, Matveyeva VA, Tsyganov VE, Borisov AY, Tikhonovich IA, Kluge C, Preisfeld A, Dietz K, Stepanok VV (2001) Characterization of plant growth promoting rhizobacteria isolated from polluted soils and containing 1-aminocycl opropane-1-carboxylate deaminase. Can J Microbiol 47:642–652

Belimov AA, Hontzeas N, Safronova VI, Demchinskaya SV, Piluzza G, Bullitta S, Glick BR (2005) Cadmiumtolerant plant growth-promoting bacteria associated with the roots of Indian mustard (Brassica juncea LCzern.). Soil Biol Biochem 37(2):241–250

Belimov AA, Dodd IC, Hontzeas N, Theobald JC, Safronova VI, Davies WJ (2009) Rhizosphere bacteria containing 1-aminocyclopropane-1-carboxylate deaminase increase yield of plants grown in drying soil via both local and systemic hormone signalling. New Phytol 181:413–423

Braud A, Jezequel K, Vieille E, Tritter A, Labeau T (2006) Changes in extractability of Cr and Pb in a polycotaminated soil after bioaugmentation with microbial prokucers of biosurfactants, organic acids and siderohores. Water Air Soil Pollut 6:3–4

Bresson J, Varoquaux F, Bontpart T, Touraine B, Vile D (2013) The PGPR. strain *Phyllobacterium brassicacearum* STM196 induces a reproductive delayand physiological changes that result in improved drought tolerance in Arabidopsis. New Phytol 200:558–569

Burdman S, Jurkevitch E, Okon Y (2000) Recent advances in the use of plant growth promoting rhizobacteria (PGPR) in agriculture. In: Subba Rao NS, Dommergues YR (eds) Microbial interactions in agriculture and forestry. Science Publishers, Enfield, NH, pp 229–250

Cabello-Conejo MI, Becerra-Castro C, Prieto-Ferna´ndez A, Monterroso C, Saavedra-Ferro A, Mench M (2014) Rhizobacterial inoculants can improve nickel phytoextraction by the hyperaccumulator Alyssum pintodasilvae. Plant Soil 379(1):35–50

Casanovas EM, Barassi CA, Sueldo RJ (2002) *Azospirillum* inoculation mitigates water stress effects in maize seedlings. Cereal Res Commun 30:343–350

Cassan F, Maiale S, Masciarelli O, Vidal A, Luna V, Ruiz O (2009) Cadaverine production by *Azospirillum brasilense* and its possible role in plant growth promotion and osmotic stress mitigation. Eur J Soil Biol 45:12–19

Castillo P, Escalante M, Gallardo M, Alemano S, Abdala G (2013) Effects of bacterial single inoculation and co-inoculation on growth and phytohormone production of sunflower seedlings under water stress. Acta Physiol Plant 35:2299–2309

Chakraborty N, Ghosh R, Ghosh S, Narula K, Tayal R, Datta A, Chakraborty S (2013) Reduction of oxalate levels in tomato fruit and consequentmetabolic remodeling following overexpression of a fungal oxalate decarboxylase1[W]. Plant Physiol 162(1):364–378

Chen L, Luo S, Li X, Wan Y, Chen J, Liu C (2014) Interaction of Cd-hyperaccumulator Solanum nigrum L. and functional endophyte Pseudomonas sp. Lk9 on soil heavy metals uptake. Soil Biol Biochem 68:300–308

Cho SM, Beom R, Yong K, Young C (2013) Transcriptome analysis of induced systemic drought tolerance elicited by *Pseudomonas chlororaphis* O6 in *Arabidopsis thaliana*. Plant Pathol J 29:209–220

Cohen AC, Travaglia CN, Bottini R, Piccoli PN (2009) Participation of abscisicacid and gibberellins produced by endophytic *Azospirillum* in the alleviation ofdrought effects in maize. Botanique 87:455–462

Cohen A, Bottini R, Pontin M, Berli F, Moreno D, Boccanlandro H, Travaglia C, Picocoli P (2015) *Azospirillum brasilense* ameliorates the response of *Arabidopsis thaliana* to drought mainly via enhancement of ABA levels. Physiol Plant 153:79–90

Creus CM, Sueldo RJ, Barassi CA (2004) Water relations and yield in *Azospirillum*-inoculated wheat exposed to drought in the field. Can J Bot 82:273–281

Creus CM, Graziano M, Casanovas EM, Pereyra MA, Simontacchi M, Puntarulo S, Barassi CA, Lamattina L (2005) Nitric oxide is involved in the *Azospirillum brasilense*-induced lateral root formation in tomato. Planta 221:297–303

Dardanelli MS, de Cordoba FJF, Espuny MR, Carvajal MAR, Diaz MES, Serrano AMG (2008) Effect of *Azospirillum brasilense* coinoculated with rhizobium on *Phaseolus vulgaris* flavonoids and Nod factor production under salt stress. Soil Biol Biochem 40(11):2713–2721

Denton B (2007) Advances in phytoremediation of heavy metals using plant growth promoting bacteria and fungi. Basic Biotechnol 3:1–5

Diby P, Bharathkumar S, Sudha N (2005) Osmotolerance in biocontrol strain of Pseudomonas pseudoalcaligenes MSP-538: a study using osmolyte, protein and gene expression profiling. Ann Microbiol 55(4):243–247

Dimkpa C, Weinand T, Asch F (2009) Plant-rhizobacteria interactions alleviate abiotic stress condition. Plant Cell Environ 32:1682–1694

Dimkpa CO, Merten D, Svatoš A, Büchel G, Kothe E (2009a) Metal-induced oxidative stress impacting plant growth in contaminated soil is alleviated by microbial siderophores. Soil Biol Biochem 41:154–162

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? 4th International Crop Science Congress

Egamberdieva D (2011) Survival of *Pseudomonas extremeorientalis* TSAU20 and *P. Chlororaphis* TSAU13 in the rhizosphere of common bean (*Phaseolus vulgaris*) under saline conditions. Plant Soil Environ 57(3):122–127

Egamberdiyeva D (2007) The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. Appl Soil Ecol 36:184–189

Eliasson L, Bertell G, Bolander E (1989) Inhibitory action of auxin on root elongation not mediated by ethylene. Plant Physiol 91:310–314

Fan X, Hu H, Huang G, Huang F, Li Y, Palta J (2015) Soil inoculation with *Burkholderia* sp. LD-11 has positive effect on water-use efficiency in inbred lines of maize. Plant Soil 390:337–349

Farooq M, Wahid A, Kobayashi N, Fujita D, Basra SMA (2009) Plant drought stress: effects, mechanisms and management. Agron Sustain Dev (29):185–212

Fu QL, Liu C, Ding NF, Lin YC, Guo B (2010) Ameliorative effects of inoculation with the plant growth-promoting rhizobacterium *Pseudomonas* sp. DW1 on growth of eggplant (*Solanum melongena* L.) seedlings under salt stress. Agr Water Manag 97(12):1994–2000

German MA, Burdman S, Okon Y, Kigel J (2000) Effects of Azospirillumbrasilense on root morphology of common bean (*Phaseolus vulgaris* L.) underdifferent water regimes. Biol Fertil Soil 32:259–264

Ghorbanpour M, Hatami M, Khavazi K (2013) Role of plant promoting rhizobacteria on antioxidant enzyme activities and tropane alkaloid production of *Hyoscyamus niger* under water deficit stress. Turk J Biol 37:350–360

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

Gou W, Zheng P, Chen F, Zhang L, Cui Z, Cao M, Zhang L, Hu J (2015) Accumulation of choline andglycinebetaine and drought stress tolerance induced in maize (*Zea mays*) by three plant growth promoting rhizobacteria (pgpr) strains. Pak J Bot 47:581–586

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

Grover M, Madhubala R, Ali SZ, Yadav SK, Venkateswarlu B (2014) Influence of Bacillus spp. strains on seedling growth and physiological parameters of sorghum under moisture stress conditions. J Basic Microbiol 54:951–961

Guo J, Chi J (2014) Effect of Cd-tolerant plant growth-promoting rhizobium on plant growth and Cd uptake by *Lolium multiflorum* Lam. and *Glycine max* (L.) Merr. In Cd-contaminated soil. Plant Soil 375(1):205–214

Gururani MA, Upadhyaya CP, Baskar V, Venkatesh J, Nookaraju A, Park SW (2013) Plant growth-promoting rhizobacteria enhance abiotic stress tolerance in *Solanum tuberosum* through inducing changes in the expression of ROS- scavenging enzymes and improved photosynthetic performance. J Plant Growth Regul 32:245–258

Gusain YS, Singh US, Sharma AK (2015) Bacterial mediated amelioration of drought stress in drought tolerant and susceptible cultivars of rice (*Oryza sativa* L.). Afr J Biotechnol 14:764–773

Hamaoui B, Abbadi JM, Burdman S, Rashid A, Sarig S, Okon Y (2001) Effects of inoculation with *Azospirillum brasilense* on chickpeas (*Cicer arietinum*) and faba beans (*Vicia faba*) under different growth conditions. Agronomie 21(6–7):553–560

Hamdia MBE, 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(2):165–174

Hassan W, Bano R, Bashir F, David J (2014) Comparative effectiveness of ACCdeaminase and/or nitrogen-fixing rhizobacteria in promotion of maize (*Zea mays* L.) growth under lead pollution. Environ Sci Pollut Res 21(18):10983–10996

He Z, He C, Zhang Z, Zou Z, Wang H (2007) Changes of antioxidative enzymes and cell membrane osmosis in tomato colonized by arbuscular mycorrhizae under NaCl stress. Colloids Surf B: Biointerfaces 59:128–133

He H, Ye Z, Yang D, Yan J, Xiao L, Zhong T (2013) Characterization of endophytic *Rahnella* sp. JN6 from *Polygonum pubescens* and its potential in promoting growth and Cd, Pb, Zn uptake by *Brassica napus*. Chemosphere 90(6):1960–1965

Hui LJ, Kim SD (2013) Induction of drought stress resistance by multi functional PGPR *Bacillus licheniformis* K11 in pepper. Plant Pathol J 29:201–208

Hussain MB, Zahir ZA, Asghar HN, Asghar M (2014) Exo polysaccharides producing rhizobia ameliorate drought stress in wheat. Int J Agric Biol 16:3–13

Jha M, Chourasia S, Sinha S (2013) Microbial consortium for sustainable rice production. Agroecol Sustain Food Syst 37(3):340–362

Kasim WA, Osman ME, Omar MN, Abd El-Daim IA, Bejai S, Meijer J (2013) Control of drought stress in wheat using plant growth promoting bacteria. J Plant Growth Regul 32:122–130

Kaymak HC, Guvenc I, Yarali F, Donmez MF (2009) The effects of biopriming with PGPR on germination of radish (*Raphanus sativus* L.) seeds under saline conditions. Turk J Agric For 33(2):173–179

Kohler J, Hernandez JA, Caravaca F, Roldan 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(2–3):245–252

Kohler J, Caravaca F, Roldan A (2010) An AM fungus and a PGPR intensify the adverse effects of salinity on the stability of rhizosphere soil aggregates of *Lactuca sativa*. Soil Biol Biochem 42(3):429–434

Kong ZY, Mohamad OA, Deng ZS, Liu XD, Glick BR, Wei GH (2015) Rhizobial symbiosis effect on the growth, metal uptake, and antioxidant responses of Medicago lupulina under copper stress. Environ Sci Pollut Res 22:12479–12489

Kuffner M, Maria SD, Puschenreiter M, Fallmann K, Wieshammer G, Gorfer M (2010) Culturable bacteria from Zn- and cd-accumulating *Salix caprea* with differential effects on plant growth and heavy metal availability. J Appl Microbiol 108(4):1471–1484

Li K, Ramakrishna W (2011) Effect of multiple metal resistant bacteria from contaminated lake sediments on metal accumulation and plant growth. J Hazard Mater 189(1–2):531–539

Li Y, Pang HD, He LY, Wang Q, Sheng XF (2017) Cd immobilization and reduced tissue cd accumulation of rice (Oryza sativa wuyun-23) in the presence of heavy metal-resistant bacteria. Ecotoxicol Environ Saf 138:56–63

Lim JH, Kim SD (2013) Induction of drought stress resistance by multi-functional PGPR *Bacillus licheniformis* K11 in pepper. Plant Pathol J 29:201–208

Liu F, Xing S, Ma H, Du Z, Ma B (2013) Cytokin in producing, plant growth promoting rhizobacteria that confer resistance to drought stress in *Platycladus orientalis* container seedlings. Appl Microbiol Biotechnol 97:9155–9164

Luo S, Xu T, Liang C, Chen J, Chan R, Xiao X (2012) Endophyte-assisted promotion of biomass production and metal-uptake of energy crop sweet sorghum by plant-growth- promoting endophyte *Bacillus* sp. SLS18. Appl Microbiol Biotechnol 93(4):1745–1753

Ma Y, Prasad MNV, Rajkumar M, Freitas H (2011a) Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. Biotechnol Adv 29:248–258

Ma Y, Rajkumar M, Luo Y, Freitas H (2011b) Inoculation of endophytic bacteria on host and non-host plants effects on plant growth and Ni uptake. J Hazard Mater 195:230–237

Ma Y, Rajkumar M, Zhang C, Freitas H (2016) Beneficial role of bacterial endophytes in heavy metal phytoremediation. J Environ Manag 174:14–25

Malik KAB, Rakhshanda S, Mehnaz G, Rasul MS, Mirza S (1997) Association of nitrogen-fixing plant-growth-promoting rhizobacteria (PGPR) with kallar grass and rice. Plant Soil 194(1–2):37–44

Marasco R, Rolli E, Vigani G, Borin S, Sorlini C, Ouzari H (2013) Are drought-resistance promoting bacteria cross-compatible with different plant models? Plant Signal Behav 8:e26741. https://doi.org/10.4161/psb.26741

Martínez-Viveros O, Jorquera MA, Crowley DE, Gajardo G, Mora ML (2010) Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. J Soil Sci Plant Nutr 10:293–319

Marulanda A, Azcon R, Chaumont F, Ruiz-Lozano JM, Aroca R (2010) Regulation of plasma membrane aquaporins by inoculation with a bacillus megaterium strain in maize (*Zea mays* L.) plants under unstressed and salt-stressed conditions. Planta 232(2):533–543

Mayak S, Tirosh T, Glick BR (2004) Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. Plant Sci 166:525–530. https://doi.org/10.1016/j.plantsci.2003.10.025

Molina-Favero C, Creus CM, Simontacchi M, Puntarulo S, Lamattina L (2008) Aerobic nitric oxide production by *Azospirillum brasilense* Sp245 and itsinfluence on root architecture in tomato. Mol Plant Microb Interact 2:1001–1009

Moreira H, Marques AP, Franco AR, Rangel AO, Castro PM (2014) Phytomanagement of cd-contaminated soils using maize (*Zea mays* L.) assisted by plant growth-promoting rhizobacteria. Environ Sci Pollut Res 21(16):9742–9753

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59:651–681

Nabti E, Sahnoune M, Ghoul M, Fischer D, Hofmann A, Rothballer M (2010) Restoration of growth of durum wheat (*Triticum durum* var. waha) under saline conditions due to inoculation with the rhizosphere bacterium *Azospirillum brasilense* NH and extracts of the marine alga *Ulva lactuca*. J Plant Growth Regul 29(1):6–22

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(10):1141–1149

Nadeem SM, Zahir ZA, Naveed M, Asghar HN, Arshad M (2010) Rhizobacteria capable of producing ACC-deaminase may mitigate salt stress in wheat. Soil Sci Soc Am J 74(2):533–542

Nadeem SM, Shaharoona B, Arshad M, Crowley DE (2012) Population density and functional diversity of plant growth promoting rhizobacteria associated with avocado trees in saline soils. Appl Soil Ecol 62:147–154

Nadeem SM, Zahir ZA, NaveedM NS (2013) Mitigation of salinity induced negative impact on the growth and yield of wheat by plant growth-promoting rhizobacteria in naturally saline conditions. Ann Microbiol 63(1):225–232

Naseem H, Bano A (2014) Role of plant growth-promoting rhizobacteria and their exopolysaccharide in drought tolerance in maize. J Plant Interact 9:689–701

Naveed M, Mitter B, Reichenauer TG, Wieczorek K, Sessitsch A (2014) Increased drought stress resilience of maize through endophytic colonization by *Burkholderia phytofirmans* PsJN and Enterobacter sp. FD 17. Environ Exp Bot 97:30–39

Omar SA, Abdel-Sater MA, Khallil AM, Abdalla MH et al (1994) Growth and enzyme activities of fungi and bacteria in soil salinized with sodium chloride. Folia Microbiol 39(1):23–28

Palacio-Rodríguez R, Coria-Arellano JL, López-Bucio J, Sánchez-Salas J, Muro-Pérez G, Castañeda-Gaytán G, Sáenz-Mata J (2017) Halophilic rhizobacteria from Distichlis spicata promote growth and improve salt tolerance in heterologous plant hosts. Symbiosis 73:179. https://doi.org/10.1007/s13199-017-0481-8

Patel D, Jha CK, Tank N, Saraf M (2012) Growth enhancement of chickpea in saline soils using plant growth-promoting rhizobacteria. J Plant Growth Regul 31(1):53–62

Patten CL, Glick BR (2002) Role of Pseudomonas putida indole acetic acid in 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 Microb Biot 22(4):369–374

Podile AR, Kishore GK (2006) Plant growth-promoting rhizobacteria. In: Gnanamanickam SS (ed) Plant-associated bacteria. Springer, Dordrecht, pp 195–230

Principe A, Alvarez F, Castro MG, Zachi L, Fischer SE, Mori GB, Jofre E (2007) Biocontrol and PGPR features in native strains isolated from saline soils of Argentina. Curr Microbiol 55(4):314–322

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

Rodriguez RJ, Henson J, Van Volkenburgh E, Hoy M, Wright L, Beckwith F (2008) Stress tolerance in plants via habitat-adapted symbiosis. ISME J 2:404–416. https://doi.org/10.1038/ismej.2007.106

Rodriguez SJ, Suarez R, Caballero MJ, Itturiaga G (2009) Trehalose accumulation in *Azospirillum brasilense* improves drought tolerance andbiomass in maize plants. FEMS Microbiol Lett 296:52–59

Rojas-Tapias D, Moreno-Galvan A, Pardo-Diaz S, Obando M, Rivera D, Bonilla R (2012) Effect of inoculation with plant growth-promoting bacteria (PGPB) on amelioration of saline stress in maize (*Zea mays*). Appl Soil Ecol 61:264–272

Rojas-Tapias DF, Bonilla R, Dussán J (2014) Effect of inoculation and co-inoculation of *Acinetobacter* sp. RG30 and *Pseudomonas putida* GN04 on growth, fitness, and copper accumulation of maize (*Zea mays*). Water Air Soil Pollut 225(12):1–13

Sadeghi A, Karimi E, Dahaji PA, Javid MG, Dalvand Y, Askari H (2012) Plant growth promoting activity of an auxin and siderophore producing isolate of Streptomyces under saline soil conditions. World J Microb Biot 28(4):1503–1509

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

Saharan BS, Nehra V (2011) Plant growth promoting rhizobacteria: a critical review. Life Sci Med Res 21(1):30

Sandhya V, Ali SKZ, Grover M, Reddy G, Venkateswaralu B (2010) Effect ofp lant growth promoting *Pseudomonas* spp. on compatible solutes antioxidant status and plant growth of maize under drought stress. Plant Growth Regul 62:21–30

Sang-Mo K, Radhakrishnan R, Khan AL, Min-Ji K, Jae-Man P, Bo-Ra K, Dong-Hyun S, In-Jung L (2014) Gibberellin secreting rhizobacterium, *Pseudomonas putida* H-2-3 modulates the hormonal and stress physiology of soybean to improve the plant growth under saline and drought conditions. Plant Physiol Biochem 84:115–124

Saravanakumar D, Samiyappan R (2007) ACC deaminase from Pseudomonas fluorescens mediated saline resistance in groundnut (*Arachis hypogea*) plants. J Appl Microbiol 102(5):1283–1292

Saravanakumar D, Kavino M, Raguchander T, Subbian P, Samiyappan R (2011) Plant growth promoting bacteria enhance water stress resistance in green gram plants. Acta Physiol Plant 33:203–209

Selvakumar G, Joshi P, Mishra PK, Bisht JK, Gupta HS (2009) Mountain aspect influences the genetic clustering of psychrotolerant phosphate solubilizing pseudomonads in the Uttarakhand Himalayas. Curr Microbiol 59(4):432–438. https://doi.org/10.1007/s00284-009-9456-1

Shafi M, Zhang G, Bakht J, Khan MA, Islam UE, Khan MD (2010) Effect of cadmium and salinity stresses on root morphology of wheat. Pakistan J Bot 42(4):2747–2754

Shahzadi N, Basharat A, Shahida H (2012) Growth promotion of *Vigna mungo* L. by Pseudomonas spp. exhibiting auxin production and ACC-deaminase activity. Ann Microbiol 62:411–417

Shakir MA, Asghari B, Arshad M (2012) Rhizosphere bacteria containing ACC deaminase conferred drought tolerance in wheat grown under semi-aridclimate. Soil Environ 31:108–112

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

Sharma P, Khanna V, Kumar PI (2013) Efficacy ofaminocyclopropane-1-carboxylic acid (ACC)-deaminase-producing rhizobacteria in ameliorating water stress in chickpea under axenic conditions. Afr J Microbiol Res 7:5749–5757

Shin M-N, Shim J, You Y, Myung H, Bang K-S, Cho M et al (2012) Characterization of lead resistant endophytic Bacillus sp. MN3-4 and its potential for promoting lead accumulation in metal hyperaccumulator Alnus firma. J Hazard Mater 199-200:314–320

Shintu PV, Jayaram KM (2015) Phosphate solubilising bacteria (*Bacillus polymyxa*) an effective approach to mitigate drought in tomato (*Lycopersicon esculentum* mill). Trop Plant Res 2:17–22

Shukla PS, Agarwal PK, Jha B (2012) Improved salinity tolerance of Arachis hypogaea (L.) by the interaction of halotolerant plantgrowth- promoting rhizobacteria. J Plant Growth Regul 31(2):195–206

Srivastava S, Singh N (2014) Mitigation approach of arsenic toxicity in chickpea grown in arsenic amended soil with arsenic tolerant plant growth promoting *Acinetobacter* sp. Ecol Eng 70(70):146–153

Suarez R, Wong A, Ramirez M, Barraza A, OrozcoMdel C, Cevallos MA (2008) Improvement of drought tolerance and grain yield in common bean by over expressing trehalose-6-phosphate synthase in rhizobia. Mol Plant Microb Interact 21:958–966

Sun LN, Zhang YF, He LY, Chen ZJ, Wang QY, Meng Q (2010) Genetic diversity and characterization of heavy metal-resistant-endophytic bacteria from two copper-tolerant plant species on copper mine wasteland. Bioresour Technol 101(2):501–509

Suslow TV, Schroth MN (1982) Role of deleterious rhizobacteria asminor pathogens in reducing crop growth. Phytopathology 72:111–115

Sziderics AH, Rasche F, Trognitz F, Sessitsch A, Wilhelm E (2007) Bacterial endophytes contribute to abiotic stress adaptation in pepper plants (*Capsicum annuum* L.). Can J Microbiol 53:1195–1202

Tank N, Saraf M (2010) Salinity-resistant plant growth promoting rhizobacteria ameliorates sodium chloride stress on tomato plants. J Plant Interact 5(1):51–58

Tardieu F, Tuberosa R (2010) Dissection and modelling of abiotic stress tolerance in plants. Curr Opin Plant Biol 13:206–212

Timmusk S, Wagner EG (1999) The plant-growth-promoting rhizobacterium *Paenibacillus polymyxa* induces changes in *Arabidopsis thaliana* gene expression: a possible connection between biotic and abiotic stress responses. Mol Plant Microb Interact 12:951

Timmusk S, Islam A, Abd El D, Lucian C, Tanilas T, Ka nnaste A (2014) Drought-tolerance of wheat improved by rhizosphere bacteria from harsh environments: enhanced biomass production and reduced emissions of stress volatiles. PLoSOne 9:1–13

Upadhyay SK, Singh DP, Saikia R (2009) Genetic diversity of plant growth promoting rhizobacteria isolated from rhizospheric soil of wheat under saline condition. Curr Microbiol 59(5):489–496. https://doi.org/10.1007/s00284-009-9464-1

Upadhyay SK, Singh JS, Singh DP (2011) Exopolysaccharide-producing plant growth-promoting rhizobacteria under salinity condition. Pedosphere 21(2):214–222

Upadhyay SK, Singh JS, Saxena AK, Singh DP (2012) Impact of PGPR inoculation on growth and antioxidant status of wheat under saline conditions. Plant Biol 14(4):605–611

Vacheron J, Desbrosses G, Bouffaud M, Touraine B, Moenne-Loccoz Y, Muller D (2013) Plant growth-promoting rhizobacteria and root system functioning. Front Plant Sci 4:1–19

Van Loon LC, Bakker PAHM, Pieterse CMJ (1998) Systemic resistance induced by rhizosphere bacteria. Annu Rev Phytopathol 36:453–483

Vardharajula S, Ali SZ, Grover M, Reddy G, Bandi V (2011) Drought-tolerant plant growth promoting Bacillus spp.: effect on growth osmolytes, and antioxidant status of maize under drought stress. J Plant Interact 6:1–14

Vargas L, Santa Brigida AB, Mota Filho JP, de Carvalho TG, Rojas CA (2014) Drought tolerance conferred to sugarcane by association with glucon acetobacter diazotrophicus: a transcriptomic view of hormone pathways. PLoS One 9(12):e114744. https://doi.org/10.1371/journal.pone.0114744

Vijayan R, Palaniappan P, Tongmin SA, Elavarasi P, Manoharan N (2013) Rhizobitoxine enhances nodulation by inhibiting ethylene synthesis of Bradyrhizobium elkanii from Lespedeza species: validation by homology modelingand molecular docking study. World J Pharm Pharm Sci 2:4079–4094

Vivas A, Marulanda A, Ruiz-Lozano JM, Barea JM, Azcón R (2003) Influence of Bacillus spp on physiological activities of two arbuscular mycorrhizal fungi and plant responses to PEG-induced drought stress. Mycorrhiza 13:249–256

Waller F, Achatz B, Baltruschat H, Fodor J, Becker K, Fischer M et al (2005) The endophytic fungus Piriformospora indica reprograms barley to salt-stress tolerance, disease resistance, and higher yield. Proc Natl Acad Sci U S A 102:13386–13391. https://doi.org/10.1073/pnas.0504423102

Wang C, Yang W, Wang C, Gu C, Niu D, Liu H, Wang Y, Guo J (2012) Induction of drought tolerance in cucumber plants by a consortium of three plant growth- promoting rhizobacterium strains. PLoS One 7:e52565

Wang A, Dodd IC, Belimov AA, Jiang F (2016) Rhizosphere bacteria containing 1-aminocyclopropane-1- carboxylate deaminase increase growth and photosynthesis of pea plants under salt stress by limiting Na^+ accumulation. Funct Plant Biol 43:161–172. https://doi.org/10.1071/FP15200

Wani PA, Khan MS (2013) Nickel detoxification and plant growth promotion by multi metal resistant plant growth promoting *Rhizobium* species RL9. Bull Environ Contam Toxicol 91(1):117–124

Xie H, Pasternak JJ, Glick BR (1996) Isolation and characterization of mutants of the plant growth-promoting rhizobacterium Pseudomonas putida GR12-2 that overproduce indole acetic acid. Curr Microbiol 32:67–71

Xiong K, Fuhrmann JJ (1996) Comparison of rhizobitoxine-induced inhibition of betacystathionase from different bradyrhizobia and soybean genotypes. Plant Soil 186:53–61

Yang J, Kloepper J, Ryu C (2009) Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci 14:1–4

Yao LX, Wu ZS, Zheng YY, Kaleem I, Li C (2010) Growth promotion and protection against salt stress by *Pseudomonas putida* Rs-198 on cotton. Eur J Soil Biol 46(1):49–54

Yasmin H, Bano A, Samiullah A (2013) Screening of PGPR isolates from semi-arid region and their implication to alleviate drought stress. Pak J Bot 45:51–58

Young KS, Mulkey TJ (1997) Effect of ethylene antagonists on auxin-induced inhibition of intact primary root elongation in maize (*Zea mays*). J Plant Biol 40:256–260

Yuwono T, Handayani D, Soedarsono J (2005) The role of osmotolerant rhizobacteria in rice growth under different drought conditions. Aust J Agric Res 56:715–721

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

Zarea MJ, Hajinia S, Karimi N, Goltapeh EM, Rejali F, Varma A (2012) Effect of *Piriformospora indica* and *Azospirillum* strains from saline or non-saline soil on mitigation of the effects of NaCl. Soil Biol Biochem 45:139–146

Zhang H, Kim MS, Sun Y, Dowd SE, Shi H, Pare PW (2008) Soil bacteria confer plant salt tolerance by tissue-specific regulation of the sodium transporter HKT1. Mol Plant-Microbe Interact 21(6):737–744

Zhang H, Murzello C, Sun Y, Kim X, Mi-S R, Jeter RM, Zak JC, Scot Dowd E, Pare PW (2010) Choline and osmotic-stress tolerance induced in *Arabidopsis* by the soil microbe *Bacillus subtilis* (GB03). Mol Plant Microb Interact 23:1097–1011

Check for updates

9 Plant Growth-Promoting Rhizobacteria (PGPR) and Their Action Mechanisms in Availability of Nutrients to Plants

Hassan Etesami and Sina M. Adl

Abstract

One of the main obstacles to plant growth is the lack of the availability of nutrient elements in many agricultural environments in the world, especially the tropics where soils can be extremely low in nutrients. Using different mechanisms of action, plant growth-promoting rhizobacteria (PGPR) participate in geochemical nutrition cycles and determine their access to plants and the microbial community of the soil. Use of these bacteria as bio-inoculants will increase the availability of nutrient elements in soil, help to minimize the chemical fertilizer application, reduce environmental pollution, and promote sustainable agriculture. Considering comprehensive reviews previously published on plant growth enhancement mechanisms, this review focuses on what is known about the action mechanisms underlying the increase of the availability of nutrient elements as an effect of microbial colonization especially PGPR. In this chapter, some of the most important mechanisms and processes regarding the effects of PGPR on the availability and hence uptake of nutrient elements by plant are reviewed. The awareness of such mechanisms can be important for the selection and hence production of microbial inoculums, which are appropriate for biological fertilization as substituting or decreasing the need of using chemical fertilizers in crops. In this review, special consideration is given to the role of PGPR in the availability of nitrogen (N), phosphorus (P), potassium (K), and sulfur (S) as macronutrients and iron (Fe) and manganese (Mn) as micronutrients.

H. Etesami (✉)
Department of Soil Science, Faculty of Agricultural Engineering & Technology, University College of Agriculture & Natural Resources, University of Tehran, Tehran, Iran
e-mail: hassanetesami@ut.ac.ir

S. Adl
Department of Soil Sciences, University of Saskatchewan, Saskatoon, Canada

© Springer Nature Singapore Pte Ltd. 2020
M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_9

Keywords
PGPR · Nutrient elements · Availability of nutrients · Action mechanisms

9.1 Introduction

Many of the plant-related microorganisms are known for their ability to promote plant growth (Compant et al. 2010). Plants produce a wide range of organic compounds between 6% and 21% of the carbon fixed including sugars (such as glucose, xylose, fructose, maltose, sucrose, and ribose), organic acids (such as citric, malic, lactic, succinic, oxalic, and pyruvic acids), amino acids, fatty acids, nucleotides, putrescine, and vitamins, which can be used as nutrients or signals by microbial populations. These signal molecules can also be used to link plants and microbes (Lugtenberg 2015). Plant-associated microorganisms, on the other hand, regulate the growth and morphogenesis of plant or activate plant immunity by releasing small molecules or volatile compounds and phytohormones (Ortíz-Castro et al. 2009). Symbionts, pathogens, epiphytes, or endophytes are four ways in which microorganisms are associated with plants (Iniguez et al. 2005). The microorganisms can colonize different parts of the plant, which are grouped into three groups based on their colonization area: rhizosphere (in the vicinity of root) microorganisms, rhizoplane (on the surface of root) microorganisms, and endophytic microorganisms. Endophytes are plant-associated microorganisms that are isolated from the tissues that reside without damage to the host (Andrews and Harris 2000), while those isolated from rhizoplane and phylloplane surfaces are called epiphytes (Azevedo et al. 2000; Petrini et al. 1989; Sturz et al. 2000). There are three basic types of ecological based microbial interactions: the neutral, negative, and positive interaction that is commonly found between microorganisms and plants (Whipps 2001). Most microorganisms are commensals in which the microorganisms interact safely with host plants that have no significant effects on the overall host's growth and physiology (Beattie 2007). In negative interactions, phytopathogenic microorganisms produce phytotoxic substances such as hydrogen cyanide (HCN) or ethylene, which negatively affects plant growth and physiology (Khalid et al. 2004). In contrast to these deleterious microorganisms, some of these microorganisms can promote plant growth and development either directly or indirectly (Glick 2012a, 2014, 2015a, b). Soil bacteria that are useful for plant growth by colonizing the plant root are commonly referred to as plant growth-promoting rhizobacteria (PGPR) (Hayat et al. 2010). Majority of credible group of PGPRs belong to genera *Frankia*, *Acinetobacter*, *Arthrobacter*, *Azotobacter*, *Azospirillum Streptomyces* spp., *Bacillus*, *Enterobacter*, *Burkholderia*, *Bradyrhizobium*, *Rhizobium*, *Serratia*, *Thiobacillus*, and *Pseudomonas* (Dimkpa et al. 2009; Gray and Smith 2005; Vessey 2003). It has been reported that PGPRs are beneficial to plants in various ways (Hayat et al. 2010; Lugtenberg and Kamilova 2009; Paul 2012). Although the precise mechanisms of stimulating plant growth remain largely speculative, a possible explanation includes (i) improving soil structure and bioremediating the polluted

soils by sequestering toxic heavy metal species and degrading xenobiotic compounds; (ii) improving abiotic stress resistance; (iii) biological nitrogen fixation (BNF); (iv) producing numerous plant growth regulators, like abscisic acid (ABA), gibberellic acid (GA), cytokinins (CK), and auxin, *i.e.*, indole-3-acetic acid (IAA); (v) solubilization and mineralization of nutrients, particularly mineral phosphate; (vi) protecting plants from phytopathogens by controlling or inhibiting them like antibiotic production, production of siderophores, induction of systemic resistance, chelation of available Fe in the rhizosphere, synthesis of extracellular enzymes to hydrolyze the fungal cell wall, and competition for niches within the rhizosphere; (vii) producing siderophores; and (viii) reducing the level of ethylene in the root of developing plants by production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Braud et al. 2009; Hayat et al. 2010).

In all, plants require 17 essential elements, 14 of which are taken up in inorganic forms by the roots. The absence or paucity of any one of these essential elements will commonly lead to plant death or inability to complete its life cycle. In the presence of nutrient deficiencies, even at asymptomatic levels, performance of crop, yield, and quality of crop are often at risk (Jewell et al. 2010). Since the nutrients in soils are generally bound to inorganic and organic soil constituents, or alternatively present as insoluble precipitates, plenty of nutrients are not available to plants for root absorption. PGPR play an essential role in the environment by contributing to the release of key nutrients from primary minerals that are required not only for their own nutrition but also for that of plants (Uroz et al. 2009). Use of these bacteria as bio-inoculants will increase the availability of nutrients in soil, help to minimize the chemical fertilizer application, reduce environmental pollution, and promote sustainable agriculture. PGPR have been proved to be vital for circulation of plant nutrients in many ways. Researchers are studying these microbes for the past 30 years to understand the action mechanisms employed by PGPR to support plant growth. Awareness of the mechanisms operated by these bacteria in promoting plant growth is a prerequisite for the development of new management strategies for sustainable agriculture. In the following, some of the most important mechanisms and processes regarding the effects of PGPR on the availability and hence uptake of nutrient elements are reviewed.

9.2 Action Mechanisms of PGPR of Providing Nutrients for Plants

PGPR enhance plant growth and health by the beneficial mechanisms which are direct or indirect. Any mechanism that protects the plant against infections (biological stress) or helps the plant grow healthy under abiotic stress is considered as indirect mechanics, whereas any mechanism that directly increases plant growth through the provision of nutrients or the production of growth regulators is considered as a direct mechanism (Goswami et al. 2016). This section focuses on plant growth promotion by PGPR directly. Generally, modes of action of PGPR of providing nutrients for plants are as below.

9.2.1 Increasing Nutrient Supply of Plants

In the absence of a nutrient in the soil, PGPR can provide the nutrient for plants, such as nitrogen (N) by fixing atmospheric nitrogen (N_2) (Fig. 9.1a). In the rhizosphere, there are microorganisms able to fix N_2 forming specialized structures (*e.g., Rhizobium* and related genera) or simply establishing associative relationships (*e.g., Azospirillum* and *Acetobacter*). Furthermore, some bacteria (*e.g.,* ammonifiers and nitrifiers) convert organic N compounds into inorganic forms (*i.e.,* NH_4^+ and NO_3^-) that are available for root uptake.

9.2.2 Increasing Nutrient Availability to Plants

A large proportion of nutrients are unavailable for the root uptake by plants because the nutrients in soils are generally bound to organic and inorganic soil constituents, or alternatively present as insoluble precipitates. Therefore, in these conditions, there are these nutrients in soil but their solubility is low and PGPR enhance the availability of these nutrients to plants by different mechanisms such as enhancing the solubility of phosphorus (P) and iron (Fe) (Fig. 9.1b). On the other hand, the increase of PGPR-derived ion concentration would help the uptake of nutrients by roots because one of the mechanisms of ion transport to plant roots is diffusion movement, which is caused by differences in concentration.

9.2.3 Enhancing Plant's Greater Access to Soil Nutrients

In these conditions, there is nutrient in soil and its solubility is also high but plants do not have any or more access to it. Therefore, PGPR enhance the access of plants to the nutrient and more uptake of it by increasing the root growth of plant by different mechanisms. The most important mechanisms involved in root elongation by bacteria are the production of IAA and ACC deaminase (Fig. 9.1c). Since one of the mechanisms of ion transport to plant roots is root interception (growth of roots throughout the soil mass), which is a physical contact resulted by root growth, it may be concluded that PGPR by IAA production and subsequently increased root length can enhance plant's greater access to soil nutrients. Therefore, a good root system is a prerequisite for nutrient acquisition. It is a commonplace that the contact between the nutrient and the root of the plant may be necessary before it may be taken up. However, both availability and efficiency largely depend on the contact between nutrients and the root. In general, it has well been known that many PGPR may reduce the growth rate of the primary root (Dobbelaere et al. 1999), increase the number and/or length of lateral roots (Chamam et al. 2013; Combes-Meynet et al. 2011), and stimulate root hair elongation in vitro (Contesto et al. 2008; Dobbelaere et al. 1999). Consequently, the uptake of minerals and water, and thus the growth of the whole plant, can be increased. Some of these effects, including increased root and shoot biomass, are also documented for PGPR-inoculated plants

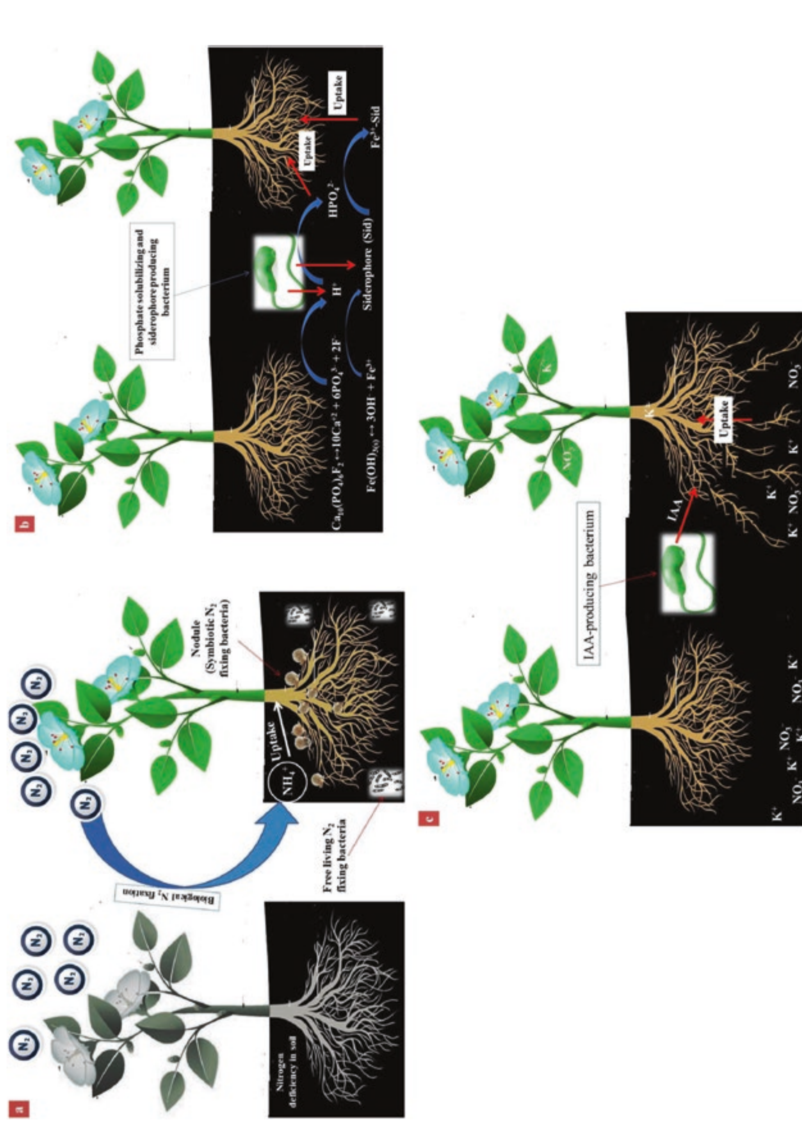


Fig. 9.1 Modes of action of PGPR of providing nutrients for plants. **(a)** Bacteria can provide the nutrients in soil which is lacking, **(b)** bacteria can increase insoluble nutrients availability to plants, and **(c)** bacteria can enhance plant greater access to soil nutrients

growing in soil (El Zemrany et al. 2006; Veresoglou and Menexes 2010; Walker et al. 2012).

9.3 Essential Plant Nutrients

Only 17 elements have been found to be absolutely essential for plant growth and metabolism that plants require to complete in their life cycle, based upon the criteria for essentiality of an element. These elements are further divided into two broad categories on the basis of their quantitative requirements: (i) macronutrients including carbon (C), hydrogen (H), oxygen (O_2), nitrogen (N), phosphorus (P), potassium (K), sulfur (S), calcium (Ca), and magnesium (Mg) and (ii) micronutrients or trace elements including manganese (Mn), iron (Fe), copper (Cu), molybdenum (Mo), zinc (Zn), nickel (Ni), chlorine (Cl), and boron (B). Among the essential elements mentioned above, O_2, H, and C are mainly obtained from CO_2 and H_2O, while the others are absorbed from the soil as mineral nutrition. Crop nutrition is affected by several factors. These factors can be internal or genetic factors (plant factors) and external factors (soil factors). Both types play significant roles in the nutrition processes that we can observe in crops. Availability of the nutrients is the resultant of a complex of soil factors. Among soil factors, soil pH is one of the most important factors affecting nutrient availability in the soil, which may either increase or decrease nutrient availability (Fig. 9.2). As shown in Fig. 9.2, maximum availability for the majority of nutrients is at pH = 6.5 (soils with pH levels higher or lower than 6.0 and 7.0), *i.e.,* under slightly acidic conditions. Among nutrient elements, N, K, and S solubility are less affected by pH, but still are to some extent. P, however, is affected. For example, at acidic pH, phosphate ions react with aluminum (Al) and Fe and they become less soluble compounds and these ions also react rapidly with Ca and Mg to form the same less soluble compounds at alkaline pH greater than pH 7.5. Availability of microelements increases with acidity, with the exception of molybdenum. The effect of soil pH on chloride availability is also neutral. In addition to pH, the availability of S, Fe, and Mn is also affected by redox reactions. In this review, special consideration is given to the role of PGPR in the availability of N, P, K, and S as macronutrients and Fe and Mn as micronutrients.

9.3.1 Nitrogen (N)

N is an essential element in plant development and a limiting nutrient for both natural and agricultural ecosystems. Although there are about 78% N_2 in the atmosphere, this form of N is not available to plants. Since there is a triple bond between the two N atoms, making the molecule almost inert, N2 cannot be directly assimilated by living cells. However, certain bacteria genera acquired an enzyme complex that uses N2 and converts it into organic N-containing molecules in the cytoplasm. Ammonium (NH_4^+) and nitrate (NO_3^-) are the predominant inorganic forms of N in soils. Plants absorb the available N in the soil through their roots in the form of NH_4^+ and NO_3^-.

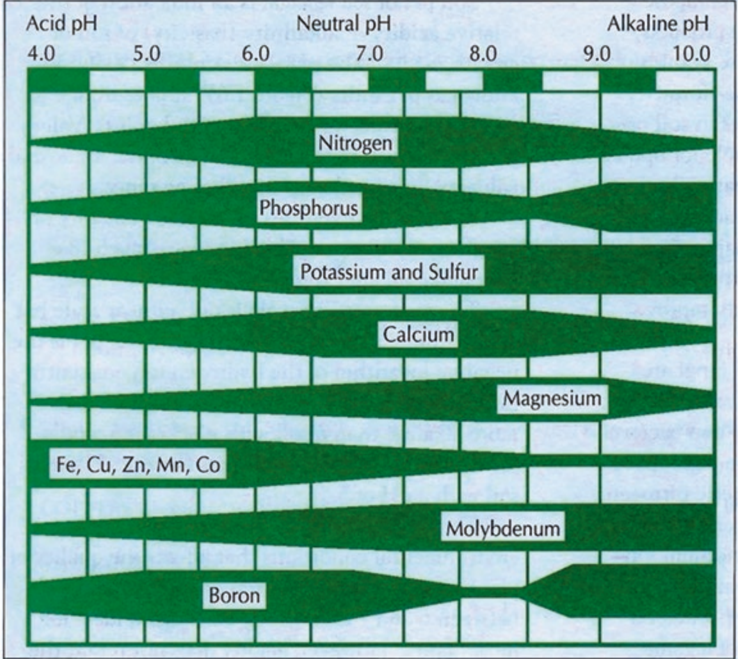


Fig. 9.2 Availability ranges of nutrient elements depending on soil pH

9.3.1.1 N_2-Fixing Bacteria (NFB)

Biological nitrogen fixation (BNF) is the process by which N_2 is reduced to ammonia by a specialized group of microorganisms called diazotrophs. All the nitrogen-fixing organisms are prokaryotes (archaebacteria and eubacteria). Diazotrophic bacteria possessing the trait of N_2 fixation are classified into three subgroups: symbiotic, free living, and associative. Root/legume-associated symbiotic bacteria possess the specificity and infect the roots to produce nodule. Several types of symbiotic biological N_2-fixing associations are known. The most prominent among them is the legume-bacteria (strains of *Rhizobium*) relationship. The amount of N_2 fixed by legumes into usable N can be substantial (176×10^{12} g year^{-1}). The legume host plant provides the bacteria with their necessary carbohydrates and possibly all the other nutrients they require in the exchange for the fixed N_2 by the bacteria. In this biological process, nodule-forming rhizobia inhabit the roots of leguminous plants and through a symbiotic relationship convert atmospheric N_2 to a form the plant can use (Fig. 9.1a). The total BNF is estimated to be twice as much as the total nitrogen fixation by nonbiological processes (80×10^{12} g year^{-1}). Associative nitrogen-fixing bacteria are a wide variety of the diazotrophs that form associative and/or endophytic relationships with a wide variety of plant roots including those of cereals and colonize the root surface of nonleguminous plants but do not inhabit specialized growth structures on their host plants (Franche et al. 2009). This relationship is

described as a nonspecific and loose symbiosis. In other words, associative nitrogen fixation is commonly defined as nitrogen fixation by a free-living diazotroph under the direct influence of a host (Dalton and Kramer 2006). These bacteria do not possess specificity to plants such as *Azospirillum, Burkholderia, Enterobacter, Gluconoacetobacter, Herbaspirillum, Azoarcus* spp., *and Klebsiella* (Dalton and Kramer 2006). Due to a very close relationship established between associative NFB and plants, the fixed N_2 (some excess N) by these bacteria can also be taken up by the plant and the microbes can utilize plant-derived carbon compounds to fuel the nitrogen fixation reaction. Furthermore, plants may provide suitable conditions for protecting the nitrogenase complex from exposure to oxygen. Generally, these bacteria can make only a small contribution to the nitrogen nutrition of the plant because nitrogen fixation is an energy-expensive process, and large amounts of organic nutrients are not continuously available to microbes in the rhizosphere. Bacterial genera such as *Klebsiella*, *Azotobacter*, *Azoarcus*, *Bacillus*, *Enterobacter*, *Xanthobacter*, *Beijerinckia*, *Achromobacter* spp., *Arthrobacter* spp., *Clostridium* spp., *Corynebacterium* spp., *Herbaspirillum* spp., *Pseudomonas* spp., *Rhodopseudomonas*, *Rhodospirillum*, *Azomonas*, and *Derxia* (Saharan 2011; Saxena and Tilak 1998) are examples of the NFB that live independently of other organisms (any plant species). These bacteria are also named as free-living nitrogen-fixing bacteria. Almost all of the nitrogen fixed by free-living NFB is used by these bacteria.

9.3.1.2 Action Mechanisms of Bacteria in Providing N for Plant

Biological N_2 Fixation (BNF)

Many associated bacteria can fix N_2 so that they could provide N to the plant. N_2-fixing PGPR can increase plant N uptake by different processes. The N cycle is biologically influenced. PGPR have a central role in almost all aspects of N availability. In terms of availability of N to plants, some bacteria (diazotrophs) can convert N_2 into ammonia by the process termed biological nitrogen fixation (BNF) and using a complex enzyme system known as nitrogenase. Mechanism of BNF has been well known and documented (Franche et al. 2009; Santi et al. 2013). In nitrogen fixation process, 16 moles of ATP and a supply of electrons and protons (hydrogen ions) are needed to produce two ammonia molecules from a mole of N_2 gas (the equation below). Nitrogenase enzyme catalyzes the nitrogen fixation reaction:

$$\mathrm{N_2} + 8\mathrm{H^+} + 8\mathrm{e^-} + 16\mathrm{Mg}\quad ATP \rightarrow 2NH_3 + \mathrm{H_2} + 16\mathrm{Mg}\quad ADP + 16\mathrm{P_i}$$

In addition to rhizobial bacteria associated with legume plants, numerous nitrogen-fixing species have also been identified that are able to colonize the root surface and, in some cases, the root interior of a variety of pasture grasses and cereal crops (non-leguminous plants) (Franche et al. 2009).

Mineralization of Organic Nitrogenous Compounds

Another mechanism of bacteria in making N available to plant is mineralization of organic forms of N in soil (Fig. 9.3). Many bacteria degrade organic matter and release fixed N for reuse by other organisms (nonlegume plant). Each part of a legume crop that remains after harvest (*e.g.,* roots, leaves, and nodules) can supply N to the soil system during the decomposition of plant materials. In addition, these plants do not use the entire N they receive from the atmosphere and return extra N to the soil. During the decomposition of plant matter, dead bacteria, and root exudates including N, organic N is once again converted to inorganic ammonium and released into the soil. The process that converts organic N to ammonium is called mineralization (conversion of organic N to inorganic forms) and plays a significant role in the management of N. The first step of mineralization is called aminization, in which microorganisms including bacteria break down complex proteins to simpler amino acids, amides, and amines. Ammonification is the second step of

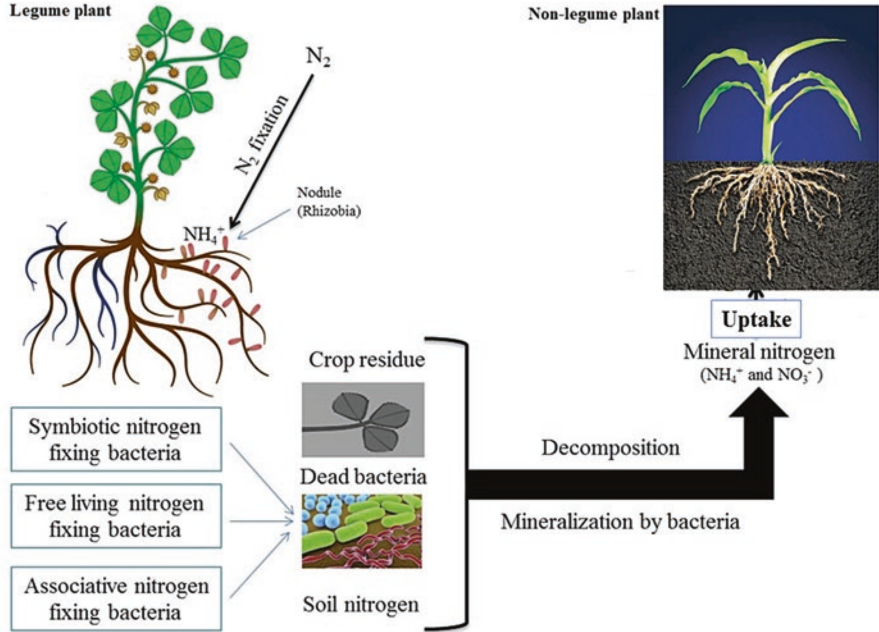


Fig. 9.3 Mechanisms increasing the availability of N in the rhizosphere to leguminous and nonleguminous plants. In legumes and a few other plants, the bacteria live in small growths on the roots called nodules. Within these nodules, nitrogen fixation is done by the bacteria, and the NH_3 produced is absorbed by the plant. Almost all of the nitrogen fixed goes directly into the leguminous plant. Little leaks into the soil for a neighboring nonlegume plant. However, other plants (nonleguminous plants) benefit from nitrogen fixing bacteria when the bacteria die and release nitrogen to the environment, or when the bacteria (associative and free living) live in close association with the plant, or by the release of ammonium or simple organic nitrogen compounds through the decomposition of organic matter obtained from vegetation (roots, leaves, fruits) of leguminous plants

mineralization in which amino (NH_2) groups are converted to ammonium. Again, microorganisms, including bacteria, do this. The two steps of nitrification (conversion of ammonium to nitrate) are also performed by microbial activity. *Nitrosomonas* (obligate autotrophic bacteria) convert ammonium to nitrite. *Nitrobacter* species perform the second step of nitrification, which converts nitrite to nitrate. This step quickly converts ammonium into nitrite, and thus nitrite concentration in soils is usually low.

Immobilization of Soluble Inorganic N

Immobilization, or the temporary tying up of inorganic N by soil microorganisms decomposing plant residues, is not strictly a loss process. A large proportion of the total fixed N will be locked up in the biomass or in the dead remains of organisms. Immobilized N will be unavailable to plants for a time, but will eventually become available as residue decomposition proceeds and populations of microorganisms decline. Therefore, it may be concluded that PGPR by immobilization of inorganic N (NO_3^-) can make more N available for plants because immobilization decreases the loss of soluble NO_3^-, which is highly mobile and is easily lost from the soil system by leaching and denitrification (conversion of NO_3^- to N gases).

Increased Root System of Plant

Nutrient presence in soil and its solubility may be high, but still plants do not have any access to it due to limitations in root growth or activities. Since essential nutrients are absorbed from the soil by the root, a good root growth is a prerequisite for increasing plant growth (Mills et al. 1996). Root hairs, along with the rest of the root surface, are the major sites of water and nutrient uptake. In an important analysis and review of the literature, Kuzyakov and Xu (2013) argued that microorganisms were more effective than roots at obtaining nutrients from the soil. Thus microorganisms win in the competition for nitrogen against roots. However, over the duration of the growing season, as root biomass increases it will outcompete microorganisms. Key to this dynamic is the high turnover rate of microbes consumed by the soil food web, in contrast to the continuously increasing root surface for membrane transport and overall biomass. PGPR increase root system of plants by production of IAA and ACC deaminase. Rhizobacterium-mediated root proliferation has been well proved (Diby et al. 2005). Plants treated with PGPR have better root with a subsequent increase of nutrient and water uptake. Promotion of root growth results in a larger root surface and can, therefore, have positive effects on water acquisition and nutrient uptake (Diby et al. 2005b; Paul and Sarma 2006) that is expected to move nutrient (*e.g.,* N) from soil to root (mechanism of mass flow in ion transport to plant roots). Phytohormone IAA, whose biosynthesis requires L-tryptophan (L-Trp) as a precursor, is primarily involved in stimulating the proliferation of lateral roots in plants; thereby root surface area is increased and they absorb more water and soil minerals (Egamberdieva and Kucharova 2009; Lugtenberg and Kamilova 2009). Under both biotic (*e.g.,* phytopathogen attacks) and abiotic (*e.g.,* heavy metals, flooding, and salinity) stresses, plant produces ethylene up to the level that is inhibitory to root growth (Arshad et al. 2007; Chen et al.

2013; Khalid et al. 2006; Nadeem et al. 2009). An enzyme ACC deaminase produced by many soil microflora including PGPR (He et al. 2010; Kumar et al. 2009) degrades ACC (an immediate precursor for ethylene in plants) and decreases the ethylene biosynthesis in plant tissues (Saleem et al. 2007; Shaharoona et al. 2007; Zahir et al. 2009). Many PGPR produce IAA and enzyme ACC deaminase that undoubtedly affect root growth, leading to the formation of root systems with increased exploratory capacity. Plant growth-promoting non-rhizobial bacteria can help the fixation of N by enhancing the capacity of rhizobial bacteria to colonize plant roots and increasing the number of nodules (Masciarelli et al. 2014). In addition, IAA-producing PGPRs by increasing root exudates can have a positive role in N_2 fixation. It has been reported that the phenolics and aldonic acids that are directly secreted by the roots of N_2-fixing legumes act as the main signal for the bacteria that form root nodules where N_2 is reduced to ammonia (Dakora and Phillips 2002). Overall, IAA and ACC deaminase-producing PGPR increase root surface area and length (Potters et al. 2009, 2007; Ryan et al. 2008; Vessey 2003) and thus increase the access of plants to nutrients and water absorption.

IAA Production

A member of the group of phytohormones, IAA is usually considered to be the most important native auxin. Almost most rhizospheric bacteria (usually more than 80% of bacteria) have the ability to produce this hormone (Khalid et al. 2004). At present, IAA-producing PGPR are the most well-studied phytohormone producers (Spaepen et al. 2007; Tsavkelova et al. 2006). The majority of root-related bacteria, which have a positive effect on plant growth, produce IAA (Hayat et al. 2010). An increase in the number of lateral roots and root hairs causes addition of root surface available for nutrients and water uptake (Fig. 9.1c). Higher water and nutrient uptake by inoculated roots causes an improved water status of plant, which in turn could be the main factor enhancing plant growth (Dalla Santa et al. 2004; Egamberdieva 2009; Egamberdieva and Kucharova 2009; Mostajeran et al. 2002). Inoculation of various plant species with such bacteria leads to increased root growth and/or enhanced formation of lateral roots and root hairs (Dimkpa et al. 2009) that can result in enhanced uptake of nutrients such as N. In addition to the production of IAA, GA and other growth regulators produced by PGPR can support increased root length, root surface area, and number of root tips, leading to enhanced uptake of nutrients (Egamberdieva and Kucharova 2009). By increasing nutrient availability via mechanisms such as producing plant growth-promoting (PGP) products, the symbiotic, free-living, and associative NFB and other PGPR can also enhance plant growth directly. The production of IAA appears to be widespread in associative NFB and has since been confirmed in a number of other genera including *Azospirillum*, *Herbaspirillum*, and *Pseudomonas* (Pedraza et al. 2004). Although the growth-promoting effects of *Azospirillum* have been well documented, the exact mechanism of growth promotion goes beyond nitrogen fixation to include nitrate reduction, phytohormone production, production of undefined signal molecules that can interfere with plant metabolism, and enhancement of mineral uptake by plants in response to root elongation (Okon and Itzigsohn 1995). Morphological plant root changes have been observed repeatedly upon *Azospirillum* inoculation and have

been attributed to the production of PGP substances, CK and GA, with auxin production being quantitatively the most important (Spaepen et al. 2008). Specific evidence for the interference of IAA produced by *Azospirillum* in root development was obtained in many cases. In a study (El-Khawas and Adachi 1999), the inoculation of IAA-producing *A. brasilense* to the roots of rice resulted in an increase in root length, root surface, root dry matter, and development of lateral roots and root hair in comparison with uninoculated roots. Similarly, IAA-producing *A. brasilense* Cd induced many roots and increased root length of soybean plants (Molla et al. 2001). More direct evidence for the importance of IAA was provided when several IAA-attenuated mutants were compared with their parental wild types for their effect on plant growth. A mutant of *A. brasilense* with low production of phytohormones, but high N_2-fixing activity, did not enhance root growth over uninoculated controls (Kundu et al. 1997).

Bacterial IAA, by loosening plant cell walls (Chaintreuil et al. 2000; Chi et al. 2005; James et al. 2002; Sevilla et al. 2001), can also promote an increase in root exudation (carbon exudation) that provides additional nutrients to support the growth of rhizosphere bacteria. Due to IAA bacterial derived root exudation, the increased microbial population enhances microbial respiration and subsequently reduces oxygen. Reduced oxygen supply in the root zone has been shown to enhance nitrogenase activity in rhizosphere organisms (Döbereiner et al. 1972). In addition, the correlation of nitrogenase activity and photosynthate flux indicates that carbon exudates are a major regulatory factor in diazotrophic activity in the rhizosphere (Dalton and Kramer 2006). Bacterial IAA is also involved in many processes of nodule formation by rhizobia in legume plants. Founder cell specification, nodule initiation and differentiation (IAA accumulation), nodule numbers, vascular bundle formation, and cell division and differentiation are some of the processes of nodule formation mediated by bacterial IAA. These three latter events are more necessary for nodule formation (Glick 2012b; Theunis 2005). In addition, IAA-producing bacteria, by increasing the root system, provide more active sites for more bacteria colonization. As an example, Parmar and Dadarwal (1999) reported that increased root growth provides more active sites and provides access to nodulation for rhizobia in chickpea plant. In another study, the presence of PGPR in the vicinity of the root can improve the ability of rhizobia to compete with indigenous populations to nodulation. Therefore, it is suggested to pay more attention in selecting microbial inoculants with high phytohormone production to potentially increase the uptake of N. In addition to hormone production, associative fixing bacteria may also benefit hosts plants in a variety of ways including improved nutrient cycling or uptake (especially through production of siderophores for iron uptake) (Dobbelaere et al. 2003). Bacterial IAA production also stimulates the activity of the enzyme ACC deaminase involved in the degradation of the ethylene precursor ACC (Glick 2005). In general, IAA and ACC deaminase work in concert to stimulate root elongation (Etesami et al. 2015a, 2014).

ACC Deaminase Activity

PGPR contain the enzyme ACC deaminase; it can act to modulate the level of ethylene in plants (Glick 2014; Singh et al. 2011). This enzyme is responsible for the

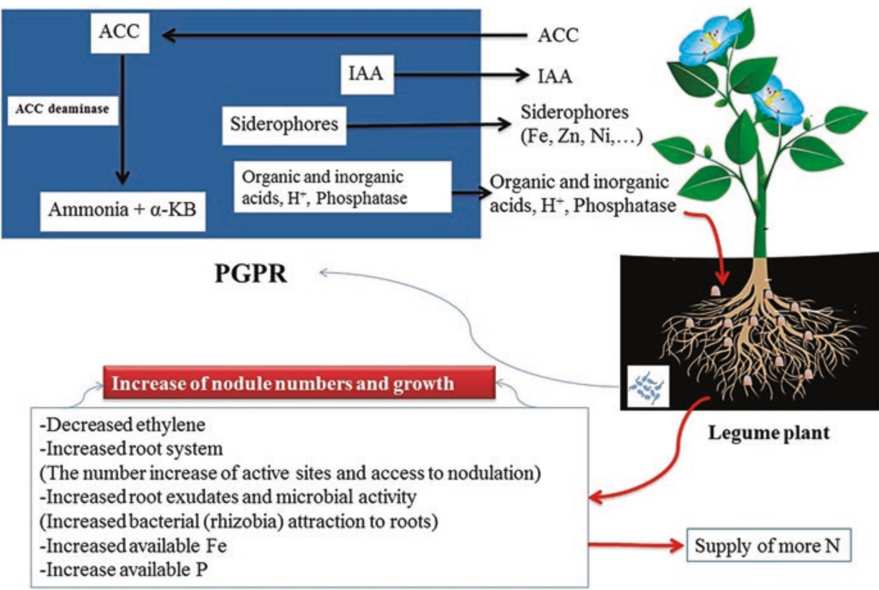


Fig. 9.4 Mechanisms by which PGPR may affect nodule number and nitrogen fixation in a legume plant

cleavage of the plant ethylene precursor, ACC, into ammonia and α-ketobutyrate (Glick et al. 2007). Plants that are inoculated with bacteria that produce enzyme ACC deaminase can adjust their ethylene levels and thus help the wider root system (Arshad et al. 2007; Safronova et al. 2006; Stearns et al. 2005). The ACC deaminase trait has been extensively studied in PGPR (Glick 2005) such as the genera *Achromobacter*, *Acidovorax*, *Alcaligenes*, *Enterobacter*, *Klebsiella*, *Methylobacterium*, *Pseudomonas*, *Rhizobium*, and *Variovorax* (Esquivel-Cote et al. 2010). In general, ACC deaminase-containing PGPRs may act as a sink for ACC. It has been well known that under stressful conditions, nodulation, nitrogenase activity, N_2 fixation, and total N content in legume plants are reduced. One of the main reasons for this decrease may be due to the production of stress-induced ethylene. Ethylene inhibits the elongation of infection threads and, consequently, the formation of nodules in most legumes (Sugawara et al. 2006). Extra ethylene production can also inhibit root prolongation, growth of lateral roots, and root hair growth (Belimov et al. 2009; Mayak et al. 2004; Saleem et al. 2007), which subsequently result in decrease in the nodule number of root. Fe deficiency also decreases nodule mass and particularly leghemoglobin content, number of bacteroids, and nitrogenase activity (Garcia et al. 2015; Tang et al. 1990). The deficiency of P supply and availability also remains a severe limitation of N_2 fixation and symbiotic interactions (Pereira and Bliss 1989). It has been well known that PGPR can alleviate the effect of these stresses on legume plant and increase N_2 fixation by different ways (Fig. 9.4).

9.3.2 Phosphorus (P)

After N, the essential mineral element that most frequently limits the growth of plants is phosphorus (P), which is taken up only in monobasic ($H_2PO_4^-$) or dibasic (HPO_4^{2-}) soluble forms. P is found mainly in inorganic fractions, which are either adsorbed into the soil's inorganic surfaces or found as sparingly available precipitates, and in organic forms that are either adsorbed, incorporated within biomass, or associated with soil organic matter (Richardson and Simpson 2011). Even in soils with abundant P ranging from 400 to 1200 mg kg^{-1} of soil, usually only about 1% of the soil P is actually in a readily available, soluble form, and over 90% is generally bound tightly to soil particles and inorganic minerals such as apatite, hydroxyapatite, and oxyapatite or appear as one of several organic forms including inositol phosphate (soil phytate), phosphomonoesters, and phosphotriesters (Khan et al. 2007b), which require mineralization before they become plant available (Jewell et al. 2010). P is an integral part of various biochemical substances such as nucleic acids, phospholipids, nucleotides, and phosphoproteins. Calcium concentration, soil pH, proportion of organic matter, type and proportion of clay, soil moisture, soil texture, root density, and exudates are among the parameters that have been able to influence the availability of soil P to the plant (Barber 1995). Parameters including high soil pH, high soil $CaCO_3$, low soil organic matter, and drought decrease P availability to plants in the calcareous soils of Iran, with arid and semiarid climates. As previously mentioned, the soil pH for optimum P availability is 6.5. P reactions in soil are pH dependent. At high or neutral pH, phosphate is converted to less soluble compounds such as dicalcium phosphate dihydrate or octacalcium phosphate. In some cases it may eventually convert to hydroxyapatite. P may react with Al and Fe to form low-solubility Fe- and Al-phosphates such as strengite and varescite under acidic conditions. The limitation in bioavailability of P from the soil along with the fact that this element is essential for plant growth suggests that the inability to obtain sufficient amount of P restricts plant growth (Khan et al. 2007b). Plants are well adapted to the uptake of P from low-concentration soil solution under low-P conditions (Jungk 2001). Plants have been demonstrated to alter the rhizosphere with specific exudates, commonly organic acids or enzymes, to improve the availability of nutrients such as phosphate (Hong et al. 2008; Park et al. 2007; Xiao et al. 2007). Furthermore, by inhibiting primary root growth, promoting lateral root growth, and enhancing root hair development and cluster root formation, which all promote P acquisition by plants, plants adjust their root architecture to low-P conditions (Jain et al. 2007; Ma et al. 2003; Niu et al. 2013; Osmont et al. 2007). Lateral roots have been known to play an important role in the absorption of P via different ways such as solubilizing insoluble P (Lynch 2007) and increasing the absorptive surface of the root system (Pérez-Torres et al. 2008) and soil exploration (Zhu et al. 2005).

9.3.2.1 Phosphate-Solubilizing Bacteria (PSB)

It has been known that strategies mentioned above are often not efficient enough to meet the needs of the plants growing especially in calcareous and alkaline soils. Therefore, using phosphate-solubilizing bacteria (PSB) for providing accessible forms of P for plants is necessary when it is scant or unavailable in soils. The conversion of insoluble phosphate compounds (both organic and inorganic) in a form accessible to the plant is an important trait of PSB. Solubilization of insoluble P by microorganisms was reported by Pikovskaya (1948). In soil, PSB constitute 1–50% of the total respective population. PSB have been isolated, using serial plate dilution method or by enrichment culture technique, from almost all areas, including from rhizosphere and non-rhizosphere soils, rhizoplane, phyllosphere, and rock P deposit area soil and even from stressed soils (Zaidi et al. 2009).

The ability to solubilize insoluble inorganic phosphate compounds such as hydroxyapatite, tricalcium phosphate, rock phosphate, and dicalcium phosphate has been reported in PGPR strains belonging to various genera (Khan et al. 2009b; Ramaekers et al. 2010). A significant number of microbial species show the capacity of solubilizing P; these include actinobacteria, bacteria, fungi, and even algae. The solubilization of insoluble phosphates has been reported in most known bacterial genera (*e.g.*, *Streptomyces* sp., *Agrobacterium* sp., *Azospirillum brasilense*, *Bacillus* sp., *B. circulans*, *B. cereus*, *B. fusiformis*, *B. pumilus*, *B. megaterium*, *B. mycoides*, *B. polymyxa*, *B. coagulans*, *B. subtilis*, *Rhodococcus*, *Klebsiella*, *Vibrio proteolyticus*, *Alcaligenes* sp., *Aerobacter aerogenes*, *Achromobacter* sp., *Enterobacter, Thiobacillus ferrooxidans*, *T. thiooxidans*, *Xanthomonas* sp., *Actinomadura oligospora*, *Brevibacterium* sp., *Citrobacter* sp., *Arthrobacter*, *Serratia*, *Chryseobacterium*, *Gordonia*, *Phyllobacterium*, *Xanthobacter agilis*, *Delftia* sp., *Azotobacter*, *Xanthomonas*, *Pantoea*, *Pseudomonas* sp., *P putida*, *P. striata*, *P. fluorescens*, *P. calcis*, *Flavobacterium* sp., *Nitrosomonas* sp., *Erwinia* sp., *Micrococcus* sp., and *Nitrobacter* sp.) (Sharma et al. 2013). By mobilizing inorganic and organic P, symbiotic nitrogenous rhizobia like *Rhizobium leguminosarum* bv. *Trifolii* and *Rhizobium* species nodulating *Crotalaria* species also improved plant P nutrition (Abril et al. 2007; Sridevi et al. 2007; Zaidi et al. 2009). Of the bacterial genera mentioned above, *Pseudomonas and Bacillus* were reported as the most important bacterial genera that were able to effectively solubilize insoluble phosphates.

9.3.2.2 Action Mechanisms of P Solubilization by PSB

Solubilization and mineralization of P in rhizosphere are the most common modes of action implicated in PGPR that increase the nutrient availability to the host plant (Glick 2012a; Rashid et al. 2004b). PGPR play an important role in all three major components of the soil P cycle (*i.e.,* dissolution–precipitation, sorption–desorption, and mineralization–immobilization). An example is the PSB, which dissolve various sparingly soluble P sources such as $Ca_3(PO_4)_2$ (Rodriguez et al. 2004) and $Zn_3(PO_4)_2$ (Saravanan et al. 2007) by lowering pH of the rhizosphere soil and making P available for plant uptake. By solubilizing and mineralizing reactions, and immobilizing P into microbial biomass and/or forming sparingly available forms of inorganic and organic soil P, PSB and their interactions in soil play a critical role in

mediating the distribution of P between the available pool in soil solution and the total soil P. Overall, phosphate-solubilizing PGPRs can either convert these insoluble phosphates into available forms through acidification, chelation, exchange reactions, release of complexing or mineral dissolving compounds (*e.g.,* organic acid anions, protons, hydroxyl ions, CO_2), secretion of siderophores, IAA production, ACC deaminase activity, and release of organic acids (Chung et al. 2005; Glick 2012a) or mineralize organic phosphates by secreting a variety of different extracellular phosphatases, catalyzing the hydrolysis of phosphoric esters (Gyaneshwar et al. 2002; Van Der Heijden et al. 2008). Each organism can act in one or more than one way to bring about the solubilization of insoluble P. Though it is difficult to pinpoint a single mechanism, production of organic acids and consequent pH reduction appear to be of great importance. In the following section, different mechanisms involved in the solubilization and mineralization of insoluble P by PSB are discussed.

Production of Organic Acids

One of the most known mechanisms of P solubilization by PSB is associated with the production of organic and inorganic acids and proton excretion. H^+ excretion is originated from NH_4^+ assimilation by plant and PSB (Parks et al. 1990). For some microorganisms, NH_4^+-driven proton release seems to be the sole mechanism to promote P solubilization. An HPLC analysis of the culture solution of *Pseudomonas* sp., in contrast to the expectation, did not detect any organic acid while solubilization occurred (Illmer and Schinner 1995). According to these authors, probable cause for the dissolution of phosphate without acid production is the release of protons originated from assimilation of NH_4^+.

Organic acids (*e.g.,* acid, oxalic acid, citric acid, lactic acid, tartaric acid, and aspartic) are the product of the microbial metabolism, mostly by oxidative respiration or by fermentation of organic carbon sources (*e.g.,* glucose) (Trolove et al. 2003) or by oxidation of the soil organic matter or being added as manure. Furthermore, PGPR (*e.g.,* IAA producers) can enhance the amount of root exudates. Root exudates include a huge diversity of organic nutrients (*i.e.,* organic acids, phytosiderophores, sugars, vitamins, amino acids, nucleosides, mucilage) and signals that attract microbial populations, especially PSB able to metabolize plant-exuded compounds and proliferate in this microbial habitat (Badri and Vivanco 2009; Drogue et al. 2013; Khan et al. 2007a; Pothier et al. 2007; Sharma et al. 2013).

Organic acids produced by PSB and plant decrease the rhizosphere pH favoring the solubility of precipitated P forms. Organic anions produced by PSB can also compete with phosphates for fixation sites or even replace phosphate (anion exchange of phosphate) sorbed on the surfaces of soil clays (kaolinite, goethite, montmorillonite, and amorphous Al oxides). They also can enhance the chelation of the cations (Al^{3+}, Fe^{3+}, and Ca^{2+}) bound to P or the formation of soluble complexes with metal ions associated with insoluble P avoiding thus the precipitation of phosphate (Osorio Vega 2007; Rashid et al. 2004a; Whitelaw 1999) and thus P is released. The monovalent anion phosphate $H_2PO_4^-$ is a major soluble form of inorganic phosphate, which usually occurs at lower pH. However as the pH of the soil environment

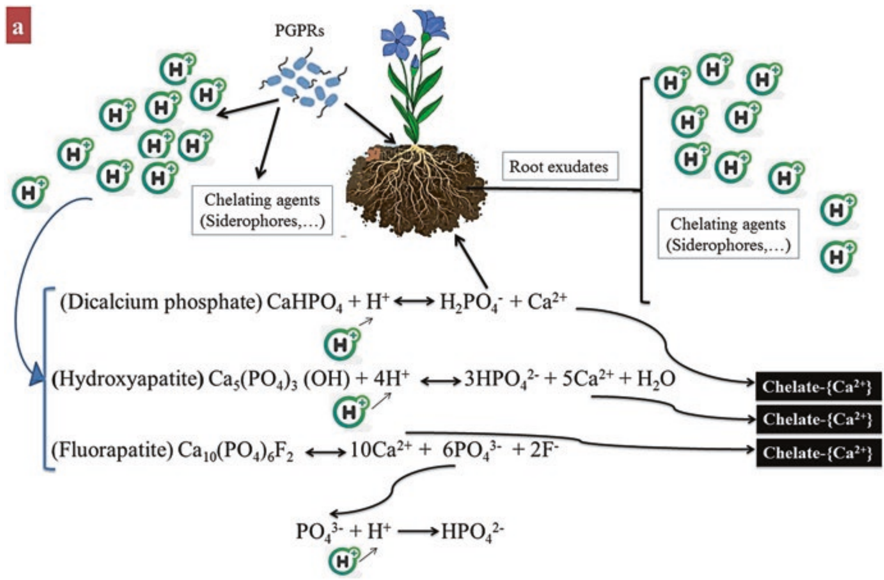
increases the divalent and trivalent forms of Pi (HPO_4^{-2} and HPO_4^{-3}, respectively) occur. Thus, the synthesis and discharge of organic acid by the phosphate-solubilizing PGPR strains into the surrounding environment acidify the cells and their surrounding environment that ultimately leads to the release of P ions from the P mineral by H^+ substitution for the cation bound to phosphate (Goldstein 1994). When phosphate-solubilizing PGPRs are inoculated to neutral or alkaline soils, the acid production decreases the rhizosphere pH, favoring thus the solubility of calcium phosphates and apatites (Fig. 9.5a). If the activity of H^+ increases in the reactants of the reactions of the solubility of dicalcium phosphate and hydroxyapatite, these reactions proceed. In addition, the sequestering of Ca^{2+} by organic anions or other chelating agents such as siderophores favors the reactions. In acid soils, the minerals variscite and strengite control the solubility of phosphate. The presence of organic acids assists the formation of complexes with Al^{3+} and Fe^{3+} ions, which in turn facilitates the dissolution of these minerals. If Fe^{3+} and Al^{3+} are sequestered via chelation with organic anions, the reactants of the reactions of the solubility of strengite and variscite proceed to the right (Fig. 9.5b).

Production of Inorganic Acids

PSB have also been shown to solubilize insoluble phosphates by producing inorganic acids (*e.g.,* HCl) (Kim et al. 1997). Bacteria of the genera *Nitrosomonas* and *Thiobacillus* species (microbial sulfur oxidation) and other bacteria can also dissolve phosphate compounds by producing nitric and sulfuric acids, and carbonic acid formed as a result of the decomposition of organic residues, respectively (Azam and Memon 1996), decreasing soil pH. Therefore, elemental sulfur can be inoculated with *Thiobacillus* to enhance the P solubility of apatite, and hence plant biomass (Stamford 2003). However, the effectiveness of inorganic acids is lower than that of organic acids in solubilizing insoluble phosphates (Kim et al. 1997). The other mechanism is the production of H_2S, which reacts with ferric phosphate to yield ferrous sulfate with concomitant release of phosphate (Swaby and Sperber 1958). Overall, acidification does not seem to be the only mechanism of solubilizing insoluble phosphates by phosphate-solubilizing PGPRs because the ability to reduce PH in some cases is not related to the ability to solubilize P minerals (SubbaRao 1982).

Production of IAA and ACC Deaminase

PGPR can enhance the capacity of plants to acquire P from soil through increased root growth either by hormonal stimulation of root growth, branching, or root hair development (*e.g.,* production enzymes that alter plant ethylene precursors, such as ACC deaminase or production of IAA) or by an extension of existing root systems (Hayat et al. 2010; Richardson et al. 2009). ACC deaminase can affect plant root growth by degrading the precursor for the production of the stress hormone, ethylene. Increased level of ethylene production in plant can decrease root growth. As a consequence, the enzymes can also indirectly influence P effect on root growth as well as its uptake by plant, because ethylene can adjust root architectural response to P availability in the soil. Under stresses such as P deficiency, the increased



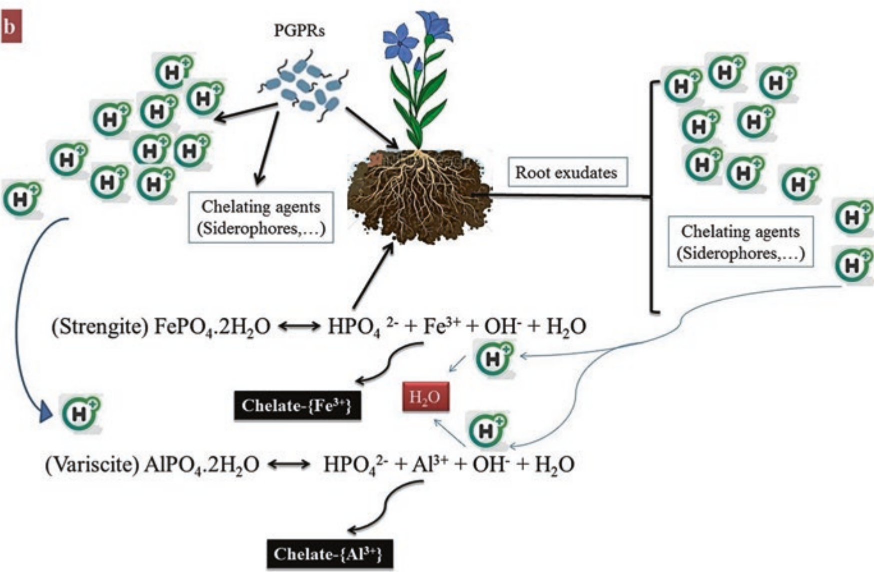


Fig. 9.5 (**a**) Role of PGPR in enhancing the capacity of plants to acquire P from soil through alteration of sorption equilibria that may result in increased net transfer of orthophosphate ions into soil solution. Organic anions and protons are particularly effective in solubilizing precipitated forms of P (e.g., Ca phosphates under alkaline conditions and (**b**) Fe and Al under acidic conditions), chelating metal ions that are commonly associated with complexed forms of soil P (as is for the role of siderophores in mediating Fe availability)

production of stress can adversely affect plant response to P and decrease the number of root hairs (Borch et al. 1999).

Bacterial IAA can increase the root exudates and root system. Organic acids (*e.g.,* gluconic and citric acid) found in the root exudates in turn result in acidification of the rhizosphere (Amir and Pineau 2003; Dakora and Phillips 2002; Jones et al. 2003). In addition, production of CO_2 by respiration processes (due to release of carbohydrates, amino acids, lipids, and vitamins by roots and subsequently stimulation of microorganisms in the soil), pump of H^+ in nutrient uptake by plant and microbes, organic matter decomposition, and N_2 fixation by the symbiosis *Rhizobium* legume (Marschner and Rimmington 1988) are some of the responsible mechanisms for acidification of rhizosphere than the bulk soil. By the complexation of essential ions, the organic acids play an important role in the increase of mobility of the elements for plant uptake. Acid pH is common for the rhizosphere environment due to proton extrusion through the root cell membrane (Spaepen et al. 2007). The acidification can also contribute to plant growth by mobilizing nutrients such as P and micronutrient. Increase in the acidity of the surrounding soil can occur by releasing proton and organic acids from the seeds and roots and absorbing nutrient ions by the plant (Hartmann et al. 2008). Altered root morphology of inoculated plants may enhance P uptake. In addition, the prevalence of root hair and lengths is also associated with an increase in the absorption of relatively immobile elements such as P. A large number of phosphate-solubilizing PGPR (Ahemad 2012; Ahemad and Khan 2010; He et al. 2010; Misra et al. 2012; Oves et al. 2013) in soils have been reported to secrete IAA that is absorbed by plant roots to increase the endogenous pool of plant IAA (Glick et al. 2007). Datta et al. (1982) reported that a P-solubilizing and IAA-producing strain of *B. firmus* increased the grain yield and P uptake of rice in a P-deficient soil amended with rock phosphate.

In general, stimulation of root growth or greater elongation of root hairs by specific microorganisms may enhance plant P nutrition indirectly by allowing greater exploration of soil, rather than by direct increase in the availability of soil P. It is presumed that the supply and availability of P to the root surface are influenced by the root and microbial processes. According to the materials listed above, it may be suggested that IAA-producing PGPR (due to having a role in enhancing root exudates and root surface area) can also solubilize insoluble phosphates similar to phosphate-solubilizing bacteria (Fig. 9.6) (Dobbelaere et al. 1999; Lambrecht et al. 2000; Steenhoudt and Vanderleyden 2000).

Production of Siderophores

Siderophores are complexing agents that have a high affinity for Fe(III) and are produced by almost all microorganisms in response to Fe deficiency. Siderophores, in the case of iron deficiency, act as a solubilizing agent for Fe from organic compounds or minerals. Some of the produced siderophores (~500 known siderophores) are exclusively used by microbial species and strains that produce them and some of them are used by a wide variety of plants and microorganisms (Crowley 2006). The ability to produce siderophores by phosphate-solubilizing PGPR is well established (Caballero-Mellado et al. 2007; Hamdali et al. 2008; Vassilev et al. 2006). The

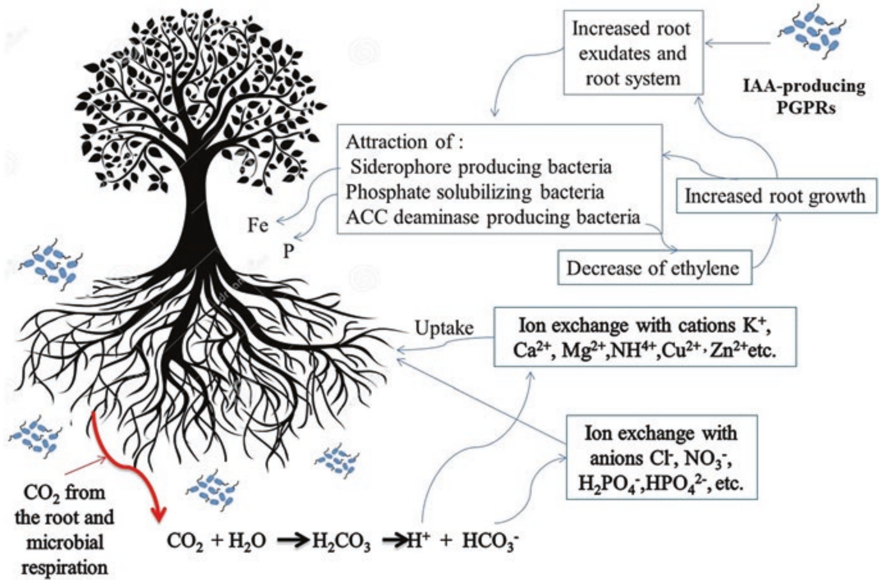


Fig. 9.6 The schematic representation of role of IAA-producing PGPR in the availability of nutrient elements (e.g., P) to plant by affecting plant (root) growth and hence plant root exudates

siderophores can increase the availability of P for plants either by chelating cations (*e.g.,* Ca^{2+}, Fe^{+3}, and Al^{3+}) forming precipitations with P or by exchange of ligands. Considering the dominance of mineral dissolution over ligand exchange by organic acid anions as a P-solubilizing mechanism (Parker et al. 2005), the potential role of siderophores in enhancing the availability of P should be clear (Sharma et al. 2013).

Production of Exopolysaccharides (EPS)

The role of exopolysaccharides (EPS) in the microbial mediated solubilization of P has also been confirmed (Yi et al. 2008). Microbial EPS, produced by some bacteria and fungi, are polymers composed mainly of carbohydrates and secreted outside the cell wall of microbes. However, these organic compounds may be homo- or heteropolysaccharides and may also contain a number of different organic and inorganic substituents. In general, the composition and structures of EPS are very diverse (Sutherland 2001). It has been known that EPS-producing PGPR (*e.g., Enterobacter* sp., *Arthrobacter* sp., and *Azotobacter* sp.) have the ability to solubilize tricalcium phosphate (TCP) (Yi et al. 2008). However, more studies are needed to understand the relationship between phosphate solubilization and EPS production (Sharma et al. 2013).

Mineralization of Organic P

In addition to mechanisms involved in releasing P from inorganic compounds, the release of phosphatase enzymes that mineralize organic P compounds has also been

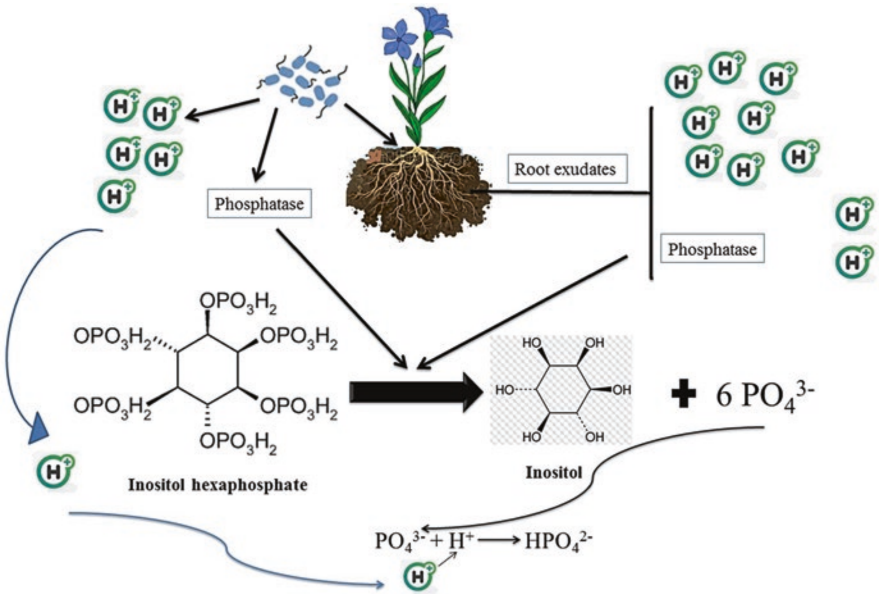


Fig. 9.7 The role of PGPR in the release of phosphatase enzymes mineralizing organic P compounds and releasing inorganic P (HPO_4^{2-})

suggested as another mechanism involved (Fig. 9.7). Organic P solubilization is also called mineralization of organic P. Mineralization of soil organic P plays an imperative role in P cycling of a farming system. Organic P may constitute 4–90% of the total soil P (Khan et al. 2009b). Organic P of organic compounds can be released in soil by enzymes of phosphatase. Phosphate-solubilizing PGPR similarly produce a range of phosphatases and when cultured in laboratory media have the capacity to utilize P from various forms of organic P that occur in soil. These enzymes, depending on their pH, are divided into acid and alkaline phosphatase, both of which can be produced by phosphate-solubilizing PGPR depending on the external conditions (Jorquera et al. 2008; Kim et al. 1998). So it is clear that alkaline phosphatases usually dominate in neutral and alkaline soils, while acid phosphatases are abundant in acidic soils (Renella et al. 2006). Although the roots of plants can produce acid phosphatase, they rarely produce large quantities of alkaline phosphatase, suggesting that this is a potential niche for phosphate-solubilizing PGPR (Criquet et al. 2004). It is also difficult to differentiate between root- and phosphate-solubilizing PGPR-produced phosphatases (Richardson et al. 2009); however, some evidence suggests that microbial phosphatases have a higher affinity for phosphorus compounds than plant phosphates and are also effective in releasing orthophosphate from soil organic P (Tarafdar et al. 2001). NSAPs (nonspecific acid phosphatases) dephosphorylate phospho-ester or phosphoanhydride bonds of organic matter. Among the variety of phosphatase enzyme classes released by phosphate-solubilizing PGPR, phosphomonoesterases (often just called phosphatases) are the

most abundant and best studied (Nannipieri et al. 2011). Inositol phosphate is a dominant form of organic phosphorus found in many soils (Turner 2006). Phytases specifically cause release of P from phytate degradation. Phytate in its original form is the main source of the inositol and the main form of P stored in plant seeds and pollen, and the main component of P is organic matter (Richardson et al. 1994). Bünemann (2008) reported that up to 60% of the total organic P may typically be hydrolyzed by phosphatases with highest amounts being released by phytases (monoester phosphatases active against phytate). Phosphonatases and C–P lyases also can cleave the C–P bond of organophosphonates (Rodríguez et al. 2006).

Immobilization of Inorganic P

Phosphate-solubilizing PGPR decompose organic amendments added to soil (*e.g.,* manures and plant residues) and mineralize organic P along with that of soil organic matter. However, in the long run, all of the microbial phosphorus is potentially available to plants, and it has been suggested that the immobilization of phosphorus in biomass is an important mechanism for regulating the supply of P in soil solution (Seeling and Zasoski 1993) and for maintaining it in labile forms that are protected (in a temporal sense) from reactions with soil (Olander and Vitousek 2004). In general, PGPR in the presence of labile C serve as a sink for P, by rapidly immobilizing it even in low-P soils; therefore phosphate-solubilizing PGPR become a source of P to plants upon its release from their cells. Release of P immobilized by phosphate-solubilizing PGPR primarily occurs when cells die due to changes in environmental conditions, starvation, or predation. Environmental changes, such as drying–rewetting or freezing–thawing, can result in so-called flush events, a sudden increase in available P in the solution due to an unusually high proportion of microbial cell lysis (Butterly et al. 2009). According to the theory of sink, phosphate-solubilizing PGPR remove and assimilate phosphorus from the liquid and thus activate the indirect dissolution of calcium phosphate compounds by sequentially removing P from the liquid medium. For instance, the P content in the biomass of *Pseudomonas* sp. and *P. aurantiogriseum* was similar to that observed in non-phosphate-solubilizing PGPR (Illmer et al. 1995) which can be explained by the fact that the P content in biomass of organisms is consistently correlated with the decomposition of P containing organic substrates (Dighton and Boddy 1989).

9.3.2.3 Promotion of Plant Growth by PSB

Besides making soluble P accessible for uptake by plants, there have been a number of reports on plant growth promotion by these microorganisms (Sharma et al. 2013). There are studies showing that phosphate-solubilizing microorganisms under controlled conditions and, in some cases, in field conditions have increased plant P nutrition and subsequently plant growth (Gyaneshwar et al. 2002; Harvey et al. 2009; Jakobsen et al. 2005; Khan et al. 2009a, 2007a; Whitelaw 1999; Zaidi et al. 2009). Following inoculation of *Ricinus communis* and *Helianthus annuus* with P-solubilizing *Psychrobacter* sp. SRS8 (Ma et al. 2010), wheat with P-solubilizing *Pseudomonas* sp. (Babana and Antoun 2006), and peanut with P-solubilizing *Pantoea* J49 (Taurian et al. 2010), an increase in growth and P uptake of these plants over

Table 9.1 Plant growth-promoting substances released by PSB

Bacterial isolates	PGP traits	References
Pseudomonas aeruginosa strain OSG41	Production of IAA and siderophores	Oves et al. (2013)
Pseudomonas sp.	Production of IAA	Singh et al. (2013)
Acinetobacter haemolyticus RP19	Production of IAA	Misra et al. (2012)
Pseudomonas putida	Production of IAA and siderophores	Ahemad and Khan (2011b, 2012c, d)
Pseudomonas fluorescens strain Psd	Production of IAA and siderophores	Upadhyay and Srivastava (2010)
Bacillus thuringiensis	Production of IAA	Sandip et al. (2011)
Pseudomonas aeruginosa	Production of IAA and siderophores	Ahemad and Khan (2010b, 2011a, d, 2012a)
Pseudomonas sp. TLC 6-6.5-4	Production of IAA and siderophore	Li and Ramakrishna (2011)
Bacillus sp.	Production of IAA	Karuppiah and Rajaram (2011)
Klebsiella sp.	Production of IAA and siderophores	Ahemad and Khan (2011c, e, 2012b)
Enterobacter asburiae	Production of IAA and siderophores	Ahemad and Khan (2010a)
Bacillus species PSB10	Production of IAA and siderophores	Wani and Khan (2010)
Arthrobacter sp. MT16, *Microbacterium* sp. JYC17, *Pseudomonas chlororaphis* SZY6, *Azotobacter vinelandii* GZC24, and *Microbacterium lactium* YJ7	Production of ACC deaminase, IAA, and siderophore	He et al. (2010)
Pseudomonas sp.	Production of IAA and siderophore	Tank and Saraf (2009)
Enterobacter aerogenes NBRI K24 and *Rahnella aquatilis* NBRI K3	Production of ACC deaminase, IAA, and siderophore	Kumar et al. (2009)
Enterobacter sp.	Production of ACC deaminase, IAA, and siderophore	Kumar et al. (2008)
Burkholderia	Production of ACC deaminase, IAA, and siderophore	Jiang et al. (2008)
Pseudomonas aeruginosa	Production of ACC deaminase, IAA, and siderophore	Ganesan (2008)

uninoculated plants was observed. In addition to solubilizing P, phosphate-solubilizing PGPR also promote plant growth through N_2 fixation (He et al. 2010), lowering ethylene levels (Jiang et al. 2008; Kumar et al. 2009), siderophore production (Ahemad and Khan 2012a, b), and phytohormone secretion (Misra et al. 2012; Oves et al. 2013) (Table 9.1).

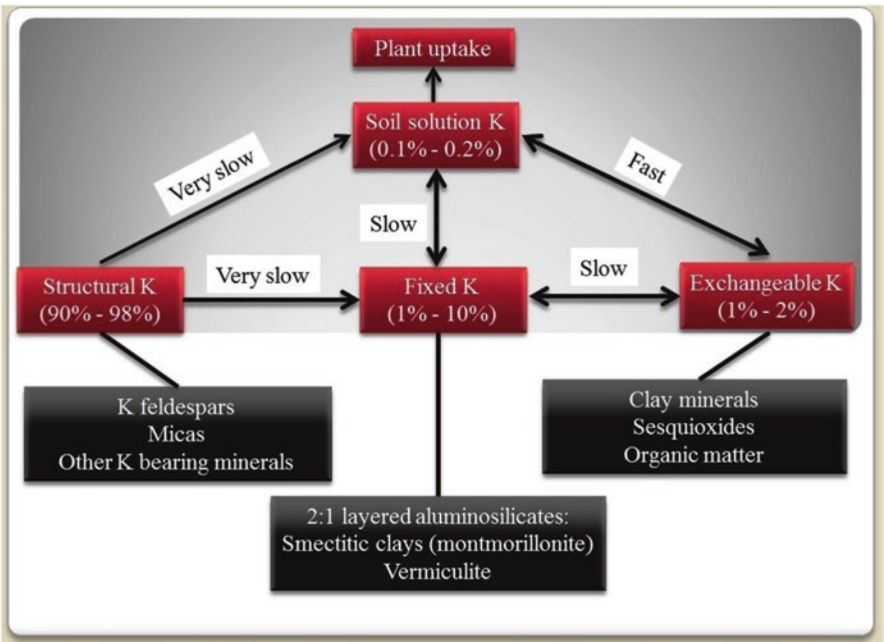


Fig. 9.8 Forms of potassium (K) in the soil and their plant availability

9.3.3 Potassium (K)

Potassium (K), one of the most important macronutrients, plays an important role in plant growth that is required in adequate quantities for all crops to achieve their maximum yield. K together with N and P forms the NPK chemical fertilizer used in both intensive and extensive agriculture. Non-exchangeable K, exchangeable K, mineral non-exchangeable K, and K in soil solution (water-soluble K) are four forms of K in the soil (Fig. 9.8). Although K deposits are generally large in soil, most soil K is not directly available for plant capture (Zörb et al. 2014). Mineral form makes up more than 90–98% of soil K (Sparks 1987), which is tightly bound, and most of it is unavailable for plant uptake. The potassium present in the soil solution is absorbed by the plants. Owing to soil erosion, introduction of high-yielding crop varieties and hybrids during green revolution, low application of K fertilizer, imbalanced fertilizer application, intensive cropping, runoff, leaching, and presence of insoluble K sources, the K availability to plants is decreasing (Xiafang and Weiyi 2002; Zörb et al. 2014). As a consequence, K deficiency is becoming one of the major constraints in crop production, and therefore many crops do respond to K fertilization in soils. In this situation, the role of PGPR is gaining importance in modern agriculture for sustainable crop production, which can enhance K availability in soil by their activities. The use of K-solubilizing microorganisms is one of the effective technologies to fulfill the K requirement of crops.

9.3.3.1 KSB (Potassium-Solubilizing Bacteria)

Soil bacteria, fungi, and actinobacteria are important in the cycling of mineral elements. Among these microbes, bacteria are the important players in this system. The bacteria involved in the solubilization of K from K-bearing minerals are called KSB (potassium-solubilizing bacteria). Potassium-solubilizing PGPRs have the ability to convert insoluble/mineral K into available K in soil making them available to the plants (Diep and Hieu 2013; Gundala et al. 2013; Keshavarz Zarjani et al. 2013; Zeng et al. 2012). KSB play an important role in the natural K cycle (Meena et al. 2014; Parmar and Sindhu 2013; Sindhu et al. 2014b). A wide range of bacteria including *Pseudomonas*, *Burkholderia*, *Acidithiobacillus ferrooxidans*, *Enterobacter hormaechei, Paenibacillus glucanolyticus, Arthrobacter* spp.*, Paenibacillus mucilaginosus, P. glucanolyticus, Bacillus mucilaginosus*, *B. edaphicus*, and *B. circulans* have been reported to release K from K-bearing minerals (Basak and Biswas 2009; Keshavarz Zarjani et al. 2013; Li et al. 2006; Lian et al. 2002; Prajapati et al. 2013; Sangeeth et al. 2012; Sheng 2005; Sheng and He 2006; Uroz et al. 2007; Zhang et al. 2013).

9.3.3.2 Action Mechanisms of KSB in the Availability of K

The K-bearing minerals are a major source of insoluble K in soils (Mengel and Kirkby 2001). The minerals are biotite, feldspar, mica, vermiculite, muscovite, orthoclase, illite/smectite, etc. These minerals supply slowly available K to plants. Clay minerals are selective for K ions and release K slowly from the lattice wedge sites (Mengel and Kirkby 2001). It has been known that KSB by converting mineral K into available K have a significant role in providing K to plants. At this time, there is little information on the mechanisms used by KSB to solubilize K. The K solubilization is a complex phenomenon affected by many factors, *e.g.,* amount of mineral, microorganisms involved, nutritional status of soil, soil mineral type, size of mineral, and environmental factors (Sindhu et al. 2016). Like the basic mechanism of PGPR for solubilizing P, potassium-solubilizing PGPR also solubilize K through the production of organic acids. Extracellular polysaccharides, production of capsular polysaccharides, hydroxyl anion, siderophores, organic ligands, extracellular enzymes, and formation of biofilms on the rhizospheric mineral surfaces are also involved in dissolution of minerals to release K (Balogh-Brunstad et al. 2008; Barker et al. 1998; Basak and Biswas 2012; Das and Pradhan 2016; Keshavarz Zarjani et al. 2013; Liermann et al. 2000; Liu et al. 2006; Meena et al. 2015; Parmar and Sindhu 2013; Sheng and He 2006; Singh et al. 2015; Uroz et al. 2009; Vandevivere et al. 1994). In general, some of the direct mechanisms used by KSB (Fig. 9.9) include (i) acidolysis, (ii) chelation, (iii) oxidation, and (iv) production of carbon dioxide (CO_2), explained separately in the following sections.

Acidolysis

Acidolysis is defined as decomposition resulting from the interaction of a compound and an acid. The major mechanism involved in mineral weathering is acidification. Although K appears to be less affected directly by soil pH, lowering the pH is one of the mechanisms for KSB to solubilize K. Minerals are known to be

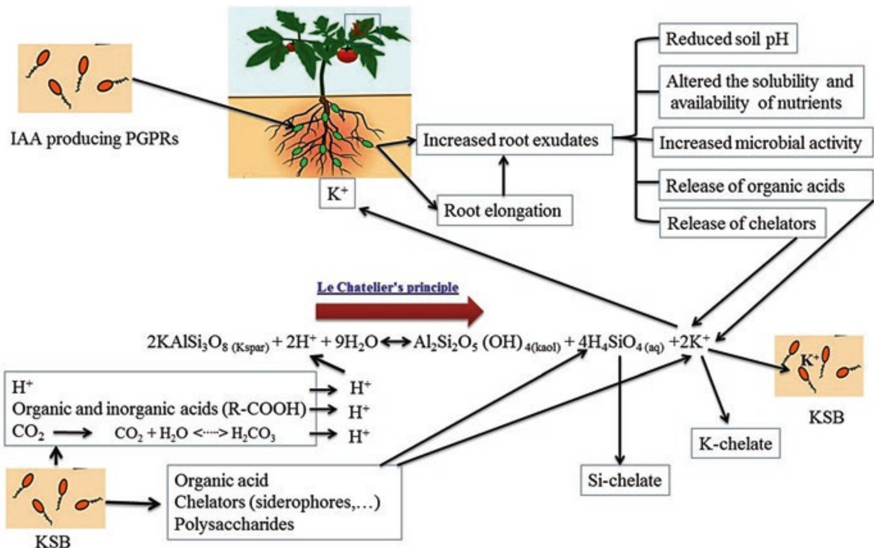


Fig. 9.9 The role of IAA producing and K solubilizing PGPR in the availability of K to plant by different mechanisms

susceptible to various biological by-products of bacterial metabolism, including protons, organic acids, and more complex molecules (Uroz et al. 2009). K-bearing mineral weathering in the rhizosphere is a proton attack as a result of microbial production of organic and inorganic acids, followed by removal of the products of weathering (cations dissolved from the mineral) by the production of complex-forming agents (organic acids, extracellular polymeric substances, siderophores, etc.) and/or by cellular cation uptake (Shelobolina et al. 2012), which induces the releasing of K. According to Le Chatelier's principle, after removal of the products of weathering by the production of complex-forming agents or by cellular cation uptake, the equilibrium shifts to the left to produce more K (Fig. 9.9).

Acidity in soils can be generated from several sources. K-solubilizing PGPR can also have a significant role in acid production. CO_2 is released from decomposition of soil organic matter (SOM) by soil microorganisms and respiration from plant roots and soil fauna. After being hydrated, CO_2 is converted into carbonic acid ($CO_2 + H_2O \leftrightarrow H_2CO_3$) (Fig. 9.9). In addition, decomposition of organic materials and sulfide oxidation by microorganisms result in the production of organic acids and sulfuric acid, respectively. Nitric and nitrous acids are also produced by nitrifying bacteria. The hydrogen ion released by these acids can react with aluminosilicate minerals (feldspars, micas, clays, etc.). For example, hydrogen ion can convert K feldspar (a primary mineral) into kaolinite, a secondary mineral (Fig. 9.9). As a result of this reaction and the breakdown of K-feldspar, H^+ is used up and K^+ is released to solution, a kind of ion-exchange reaction.

In general, such acidolysis by organic acids produced by KSB can either directly dissolve the mineral K as a result of slow releases of exchangeable K or readily available exchangeable K or chelate by both Al and Si ions associated with K mineral (Römheld and Kirkby 2010). For example, KSB had the ability to weather phlogopite through acidic dissolution and aluminum chelation of the crystal network (Abou-el-Seoud and Abdel-Megeed 2012; Meena et al. 2014). Increasing evidence also exists for a mechanism of direct silicate precipitation by bacteria via metal sorption at the cell membrane (Konhauser and Ferris 1996; Urrutia and Beveridge 1994).

As mentioned above, bacterial IAA increases root system and promotes an increasing amount of root exudation. The IAA-derived root system increase enhances the bacterial effect on mineral mobilization due to increased surface area for reactivity and helps improve nutrient uptake and mobilization of minerals (Gahoonia et al. 1997). Some of the roles of bacterial mediated root exudation in weathering K-bearing minerals include the following: (i) root exudation of high concentrations of organic acid anions can lower rhizosphere pH (Dakora and Phillips 2002); (ii) root exudates help by indirectly providing the substrates for the production of weathering metabolites by bacteria (Gahoonia et al. 1997); and (iii) root exudates include complex-forming agents (organic acids, extracellular polymeric substances, siderophores, etc.) (Shelobolina et al. 2012).

It has been reported that solubilization of K-bearing minerals by KSB is due to the production of organic acids like citric acid, tartaric acids, 2-ketogluconic acid, oxalic acid, gluconic acid, malic acid, propionic, fumaric, glycolic, and succinic acid (Keshavarz Zarjani et al. 2013; Prajapati and Modi 2012; Prajapati et al. 2012; Sheng and He 2006; Wu et al. 2005), which convert insoluble K (*i.e.,* mica, muscovite, biotite, feldspar) to soluble form of K (soil solution form) with the net result of increasing the availability of the nutrients to the plants. Gluconic, oxalic acids, α-ketogluconic, and succinic citric are the most efficient acids released by K-solubilizing PGPR strains that are effectively involved in the solubilization of insoluble K. In addition, the various types of organic acids produced by KSB differed with different organisms (Table 9.2) (Maurya et al. 2014; Verma et al. 2014; Zhang and Kong 2014).

Chelation Process

Chelation is a type of bonding of ions and molecules to metal ions. It involves the formation or presence of two or more separate coordinate bonds between a polydentate (multiple bonded) ligand and a single central atom. Usually these ligands are organic compounds. Chelating molecules might increase the dissolution rates of cations by forming strong bonds with them or with mineral surfaces (Uroz et al. 2009). Complex-forming agents (*e.g.,* organic acids, extracellular polymeric substances, siderophores) produced by K-solubilizing PGPR or in root exudates form a complex with cations dissolved from K-bearing minerals, removing the products of weathering. According to Le Chatelier's principle, with the uptake of K by plant or K-solubilizing PGPRs and/or removal of K by forming complex, the equilibrium is disturbed and K will be drawn upon from the non-exchangeable and soil mineral

Table 9.2 Mechanisms used by KSB for K solubilization

KSB	Action mechanism	References
Enterobacter hormaechei	Organic acids	Prajapati et al. (2013)
Paenibacillus mucilaginosus	Tartaric, citric, and oxalic acids	Liu et al. (2012)
Bacillus mucilaginosus	Acidification	Abou-el-Seoud and Abdel-Megeed (2012)
Burkholderia glathei	Siderophores and organic ligands	Calvaruso et al. (2007)
Burkholderia	Acidification and complexation	Uroz et al. (2007)
Bacillus circulans	Lipo-chitooligosaccharides production	Lian et al. (2002)
Bacillus mucilaginosus	IAA production	Han and Lee (2005)
Bacillus mucilaginosus *Bacillus edaphicus*	Production of citric, tartaric, and oxalic acid	Sheng (2005)
Bacillus spp.	Gluconic acid	Gundala et al. (2013)
Bacillus mucilaginosus	Polysaccharides	Liu et al. (2006)
Bacillus edaphicus	Production of organic acids like oxalic acid and tartaric acids and production of capsular polysaccharides (CPS)	Sheng and He (2006)
Rhizobium tropici	Production of tartaric acids and extracellular polysaccharides	Wang et al. (2015)
Pseudomonas aeruginosa	Acetic, citric, and oxalic acids	Badr et al. (2006)
Bacillus, Clostridium, and *Thiobacillus*	production of mucilaginous capsules containing of exopolysaccharides	Groudev (1987)
Cladosporium cladosporioides and *Penicillium* sp.	Production of protons, organic acids, siderophores, and organic ligands as chelating agents	Kumar et al. (2015), Meena et al. (2014, 2015)

fraction (Fig. 9.9). K-solubilizing PGPR secrete organic acids which act as chelating agents and quickly dissolve rock and silicon ions, ultimately releasing the K ions into the soil (Bennett et al. 1998).

Štyriakova et al. (2003) showed that K solubilization occurred by buildup of complex between organic acids and metal ions such as Fe^{2+}, Al^{3+}, and Ca^{2+}. Organic acids can either directly enhance dissolution by either a proton- or a ligand-mediated mechanism or indirectly enhance dissolution by the formation of complexes in solution with reaction products (Ullman and Welch 2002). Liu et al. (2006) demonstrated that polysaccharides strongly adsorbed the organic acids and attached to the surface of the mineral, resulting in an area of high concentration of organic acids near the mineral. It was suggested that the extracellular polysaccharides adsorbed SiO_2 and this affected the equilibrium between the mineral and fluid phases and led to the reaction toward SiO_2 and K^+ solubilization. Adhering to mineral surfaces and extracting nutrients from mineral particles by electron transfer, breaking the oxygen links, and chelating ions present in solution through their carboxyl and hydroxyl groups are some of the action mechanisms of organic acids and chelating molecules on mineral weathering (Welch et al. 2002). The latter indirectly accelerates the dissolution rate of the mineral by creating an imbalance between cation and anion

concentrations in the solution. Bacterial organic acids have been found to facilitate the weathering of minerals through the formation of metal organic complexes with silicon ions to bring the K into solution, in addition to directly dissolving K from rocks (Bennett et al. 1998). According to previous discoveries, acidification does not seem to be the only solubilization mechanism, so that the ability to reduce pH in some cases is not consistent with the ability to solubilize K minerals by K-solubilizing PGPR (Liu et al. 2006; Sheng and Huang 2001; Subhashini and Kumar 2014; Zhang and Kong 2014). In general, acidolysis and complexolysis processes can be simultaneously used by K-solubilizing PGPR to impact mineral stability. *Agrobacterium* and *Bacillus* strains were described for their ability to weather phlogopite via aluminum chelation and acidic dissolution of the crystal network (Leyval and Berthelin 1989). Some of the selected examples about mechanisms used by KSB to solubilize K have been shown in Table 9.2.

Oxidation

Microbial Fe(II) oxidation as an additional mechanism of microbial weathering of K-bearing minerals having Fe (II) (e.g., biotite) in the rhizosphere has been reported, contributing to soil formation and providing K and Fe for plant nutrition (Shelobolina et al. 2012). Microbial oxidation of structural Fe (II) led to biotite changes similar to those found in nature, including a decrease in the unit cell b dimension toward dioctahedral levels and Fe and K release. Structural Fe (II) oxidation can entangle either direct enzymatic oxidation, followed by solid-state mineral transformation, or indirect oxidation as a result of forming aqueous Fe, followed by electron transfer from Fe (II) in the mineral to Fe (III) in solution. These cells indirectly attack biotite through oxidation of the sorbed Fe (II) in indirect oxidation that was generated because of electron exchange between structural Fe (II) and surface Fe (III) in the biotite (Shelobolina et al. 2012).

Production of CO_2

The weathering of K-bearing minerals may be the result of carbonic acid formation from the respiratory CO_2 release of the microorganisms and its subsequent reaction with water (Barker et al. 1998). Bacterial IAA can attract more rhizosphere bacteria in the rhizosphere by increasing more amount of root exudation, resulting in more production of CO_2. In addition, CO_2 can directly release K from K-bearing minerals (Rosenqvist et al. 2014). For example, CO_2 can convert K feldspar into muscovite and release K (Fig. 9.10). In general, K-solubilizing PGPR can dissolve K-bearing minerals by production of organic acids, IAA, siderophore, and polysaccharides. Previous studies showed K-solubilizing PGPR with other PGP traits. For example, Zhao et al. (2008) isolated bacterial strains with multiple beneficial activities such as IAA production, K solubilization, and siderophore production. Prajapati et al. (2013) reported that inoculation with IAA-producing PGPR strain *Enterobacter hormaechei* enhanced the root and shoot length of okra and mobilized K efficiently in plant when feldspar was added to the soil. The plants with more fibrous, branching roots increase nutrient (K)-absorbing surface. Nadeem et al. (2009) reported that ACC deaminase-containing rhizobacteria improved the uptake of K in maize

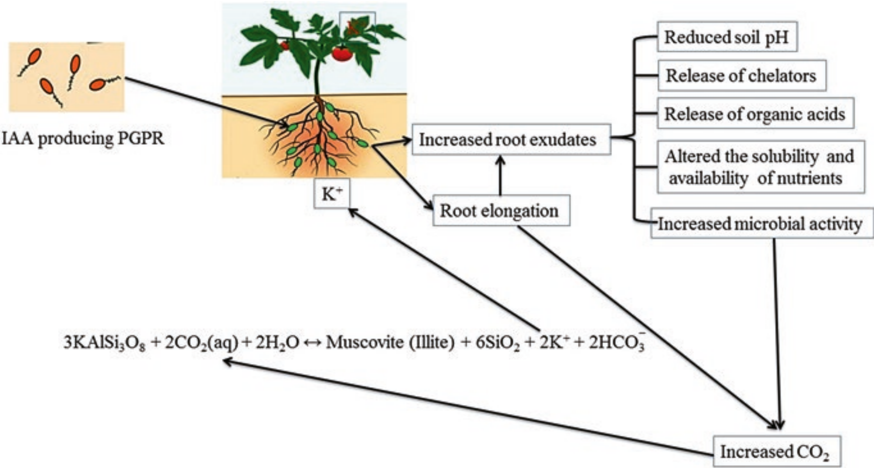


Fig. 9.10 The direct role of CO_2 in releasing K from K-bearing minerals

under salinity stress. They observed that these strains were strong colonizers of plant roots. The increased colonization by the K-solubilizing PGPR made the plants capable to explore more soil that might have improved the uptake of K indirectly. Previous studies showed that the solubilization and release of K through organic acids by K-solubilizing PGPR resulted in plant growth promotion leading to enhanced yield and production, which have been well reviewed (Ahmad et al. 2016; Bahadur et al. 2014; Das and Pradhan 2016; Meena et al. 2016; Sharma et al. 2016; Sindhu et al. 2014a, 2016; Velázquez et al. 2016; Zahedi 2016). The above discussion shows that bacterial strains have a number of potential mechanisms to solubilize K from insoluble sources and the contribution of these mechanisms in weathering of K-bearing minerals is variable. Among these mechanisms, the production of organic acids is one of the major mechanisms used by K-solubilizing PGPR. It has also been observed that K solubilization by the bacteria is affected by a large number of soil and environmental factors (Uroz et al. 2009).

9.3.3.3 KSB and Increased Availability of K and Other Nutrients

As discussed above, the production of organic acids is one of the major mechanisms used by KSB to solubilize K-bearing minerals and release K. The availability of some plant nutrients is also greatly affected by soil pH. Therefore, these bacteria can also provide the availability of nutrients such as P, Fe, Zn, Cu, and Mn. According to Prajapati et al. (2013), K-solubilizing PGPR can not only activate the insoluble K mineral but also change that into available P, Zn, and Fe. Sheng (2005) and Sheng et al. (2008) showed that inoculation of cotton and rape plants with the K-solubilizing PGPR strain *Paenibacillus edaphicus* NBT enhanced the N and P content in both plants and soil. Increases ranging from 26% to 30% were found in both plants when illite was added to soil as a source of insoluble K. The plant dry weight and the uptake of both K and N by tobacco seedlings enhanced significantly

with respect to uninoculated controls mainly when the strain inoculated was *Klebsiella variicola* XF11 (Zhang and Kong 2014). Lin et al. (2002) indicated significant increases in K and P uptake in tomato plants inoculated with silicate-dissolving bacterium (*B. mucilaginosus*) compared with the non-inoculated plants. K-solubilizing PGPR also resulted in increased biomass and enhanced contents of P and K in sorghum plants than uninoculated control (Zheng and Tu 2005). Inoculation of K-solubilizing PGPR combined with K- and P-bearing minerals caused increase in dry matter yield of sorghum plants along with P and K uptake and also improved fertility in three different soils, i.e., clay, sandy, and calcareous soils (Badr et al. 2006). The organic acids and siderophores generated by PGPR could play a crucial role in the solubilization of elements such as K, Si, and Fe from the liquid medium containing acid-leached soil, muscovite, and biotite (Liu et al. 2006). In general, the K-solubilizing PGPR contribute to exudation of soluble compounds, decomposition of soil organic matter, and mobilization and mineralization of other nutrients (Abhilash et al. 2013; Archana et al. 2013; Diep and Hieu 2013; Rajawat et al. 2012; Zeng et al. 2012).

9.3.4 Sulfur (S)

Sulfur (S), an essential macronutrient required for growth, is increasingly becoming a limitation to crop yield and quality as a result of a reduction in atmospheric S levels due to the increasing use of low-S fuels and enhanced emission controls and crop varieties removing S from soil more rapidly (Fowler et al. 2005; Irwin et al. 2002). Since crop plants have become increasingly dependent on the soil to supply the S, these changes have had an important effect on agriculture. S is needed for the synthesis of proteins and a number of essential vitamins and cofactors and also is a constituent of the essential amino acids cysteine, cystine, and methionine. In agricultural soils, most of the soil S (>95%) is present in an organic form (Gahan and Schmalenberger 2014) as a heterogeneous mixture of forms, partly included in microbial biomass and partly in the soil organic matter. In addition, S present in soil is approximately 95% organically bound largely in one of the two major forms: sulfate esters and carbon-bonded S (sulfonates or amino acid sulfur) (Kertesz and Mirleau 2004). Sulfonates and sulfate esters are not directly available to plants which depend upon microorganisms in rhizosphere and soil for mobilizing these organic forms (Kertesz et al. 2007). S occurs in a variety of oxidation states with three oxidation states of −2 (sulfide and reduced organic sulfur), 0 (elemental sulfur), and +6 (sulfate) being the most significant in nature. Plants obtain S in the form of sulfate (SO_4^{2-}), which is the dominant plant-available source of S and constitutes less than 5% of the total soil S (Autry and Fitzgerald 1990), while to a lesser extent atmospheric reduced S may be utilized (Leustek et al. 2000). Chemical or biological agents help transformation of S from one state to another. A biogeochemical cycle which characterizes these transformations includes many oxidation-reduction reactions.

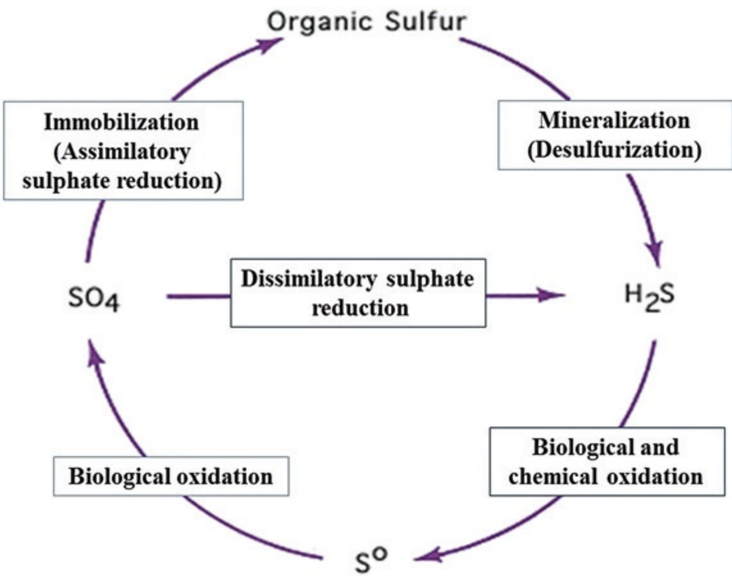


Fig. 9.11 The major processes of transformation involved in the cycling of S in soil

9.3.4.1 Action Mechanisms of Sulfur (S) Availability by PGPR

Similar to some other nutrients, S is also subjected to biological alterations in soil by the soil bacteria. The major processes of transformation involved in the cycling of S in soil are (i) mineralization of organic S to the inorganic form (H_2S), (ii) immobilization or assimilation of S into organic compounds by plants or microorganisms, (iii) oxidation of S and inorganic S compounds, and (iv) reduction of S and incompletely oxidized inorganic compounds of S (Fig. 9.11). Due to having indirect and direct functions in these processes, microorganisms especially bacteria can increase the availability of S to plants, which are explained as follows.

Mineralization of Sulfur (S)

Plant S nutrition depends primarily on the uptake of inorganic sulfate. Due to interconversion of sulfate ester-S and carbon-bonded S to inorganic sulfate by soil microorganisms, it has been shown that the sulfate ester and sulfonate pools of soil S are also plant bioavailable (Kertesz and Mirleau 2004). Aerobic and anaerobic heterotrophic bacteria (*Pseudomonas* and *Clostridium*) release S from sulfate-esters using sulfatases ($R\text{-}O\text{-}SO_3^- + H_2O \rightarrow ROH + H^+ + SO_4^{2-}$); however, release of S from sulfonates is catalyzed by a bacterial multicomponent monooxygenase system (Gahan and Schmalenberger 2014). Splitting of the C-O bond of aliphatic sulfate esters and the O-S bond of aromatic sulfate esters is performed by the enzymes of alkylsulfatase and arylsulfatase, respectively (Kertesz 2000). Some bacteria such as *Pseudomonas, Klebsiella, Salmonella, Enterobacter, Serratia,* and *Comamonas* are able to mobilize sulfate esters (Hummerjohann et al. 2000). Sulfide can be produced

by anaerobic bacteria as a result of the breakdown of proteins to amino acids and further degradation of amino acids to sulfide (R-SH → R + H_2S) (desulfurization).

Immobilization of Sulfur (S)

Sulfur (S) immobilization is microbial conversion of inorganic S compounds to organic S compounds, first to sulfate esters and subsequently to carbon-bound sulfur (Kertesz and Mirleau 2004). S is used for the biosynthesis of amino acids and proteins by bacteria (assimilatory sulfate reduction) in this process. Bacteria reduce only enough sulfates to meet their nutritional requirements for S. During the study period, soil microorganisms were capable of binding all the available sulfate into microbial biomass. In general, PGPR in the presence of labile C serve as a sink for S, by rapidly immobilizing it; therefore PGPR become a source of S to plants upon its release from their cells. Release of S immobilized by PGPR primarily occurs when cells die.

Oxidation of Sulfur (S)

Sulfur (S) oxidation is the process by which a variety of microorganisms convert hydrogen sulfide (H_2S) into elemental sulfur (S_0) by partial oxidation, or sulfate (SO_4^{2-}), which can be used by the plants, while the acidity produced by oxidation helps to solubilize plant nutrients and improve alkali soils (Wainwright 1984). Instead of H_2S, also other sulfur compounds like thiosulfate ($S_2O_3^{2-}$) and tetrathionate ($S_4O_6^{2-}$) can be converted to sulfate ($S_0 \rightarrow H_2S \rightarrow S_2O_3^{2-} \rightarrow S_4O_6^{2-} \rightarrow S_3O_6^{2-} \rightarrow SO_3^{2-} \rightarrow SO_4^{2}$). Thiobacilli (*e.g.,* bacteria of the genus *Thiobacillus*) play an important role in S oxidation in soil. *Beggiatoa, Sulfolobus*, *Thermothrix*, *Thiobacillus*, and *Thiothrix*, known as colorless sulfur-oxidizing bacteria (Janssen et al. 1998), are the most important chemolithotrophic sulfur-oxidizing bacteria (SOB). S oxidation is the most important step of S cycle, which improves soil fertility and decreases pH soil and subsequently increases the availability of micronutrients and P. Photoautotrophic or chemolithotrophic sulfide-oxidizing bacteria use sulfide as an electron donor and convert it to S or sulfate (Robertson and Kuenen 2006). The CO_2 and oxygen (aerobic species) or nitrate and nitrite (anaerobic species) are used as the terminal electron acceptors in photoautotrophic and chemolithotrophic sulfide-oxidizing bacteria, respectively. Light energy and oxidizing reactions supply directly energy needed for metabolism of photoautotrophic SOB and chemolithotrophic SOB, respectively. Among photoautotrophic sulfide-oxidizing bacteria, the green SOB like *Chlorobium*, *Prosthecochloris*, *Chloroherpeton*, *Pelodictyon,* and *Ancalochloris* and purple SOB such as *Chromatium*, *Allochromatium*, *Thioalkalicoccus*, *Thiococcus*, *Thiorhodococcus*, *Thiocystys*, and *Thiospirillum* (Tang et al. 2009) are the most frequent. In addition to being oxidized biologically, oxidation of the reduced S compound is also carried out chemically by ferric iron as the oxidizing agent, and iron-oxidizing bacteria are utilized to regenerate the ferric iron (Fe^{3+}) for further use (Pagella and De Faveri 2000).

Reduction of Sulfur (S)

In addition to reductive reactions, bacteria have also a major role in reductive reactions of the biological S cycle (Kertesz and Mirleau 2004). Sulfate, in turn, can be reduced back to sulfide by sulfate-reducing bacteria such as *Desulfovibrio* and *Desulfatomaculum* (Tang et al. 2009). Sulfate in the absence of oxygen functions as a terminal electron acceptor in metabolic pathways for anaerobic respiration and is converted to sulfide (dissimilatory sulfate reduction). Although dissimilatory sulfate reduction results in decrease of plant-available sulfate, H_2S produced by the reduction of sulfate is further oxidized by some of the green and purple phototrophic bacteria to release elemental sulfur (S_0). The latter can also be oxidized to sulfate by SOB once again.

9.4 Action Mechanisms of PGPR in Availability of Micronutrients

9.4.1 Production of Organic and Inorganic Acids

Since most of the nutrients (micronutrients especially) tend to be less available when soil pH is above 7.5 (Fig. 9.2), it seems that decreasing soil pH (a slightly acidic pH, *e.g.,* 6.5–6.8) is one of the action mechanisms of PGPR in the availability of these nutrients to plants. Due to strongly being adhered to soil particles, these metals are not easily available for uptake by plants in most of the metalliferous soils (Gamalero and Glick 2012). In this context, PGPR producing acid such as PSB, sulfur-oxidizing bacteria (SOB), and nitrifying bacteria are very promising agents. By secreting low-molecular-weight organic acids, the PGPR listed above can solubilize the insoluble and biologically unavailable metals and subsequently facilitate the bioavailability of these nutrients for plant uptake (Fig. 9.12) (Becerra-Castro et al. 2011, 2011; Li and Ramakrishna 2011; He et al. 2013).

9.4.2 Production of Chelating Agents

In addition to soil pH, chelation process can increase nutrient availability to plants by different ways (Fig. 9.13). Hence, the natural chelating agents produced by PGPR may be considered as the other action mechanism of PGPR in the availability of nutrients to plants. Hydroxamate siderophores, organic acids, and amino acids are some of the most important substances possessing this nature, which are naturally produced by soil microorganisms. These substances are essential in natural ecosystems to solubilize and transport these nutrients to plant roots. For example, iron occurs mainly as Fe^{3+} and forms insoluble hydroxides and oxyhydroxides, and thus is not easily available to both plants and microorganisms (Ahemad and Kibret 2014). Under iron-limiting conditions to acquire Fe, PGPR secrete low-molecular-weight siderophores, which are iron chelators with exceptionally strong affinity for ferric iron (Fe^{3+}) (Schalk et al. 2011). Despite their preferential affinity for Fe^{3+}, they

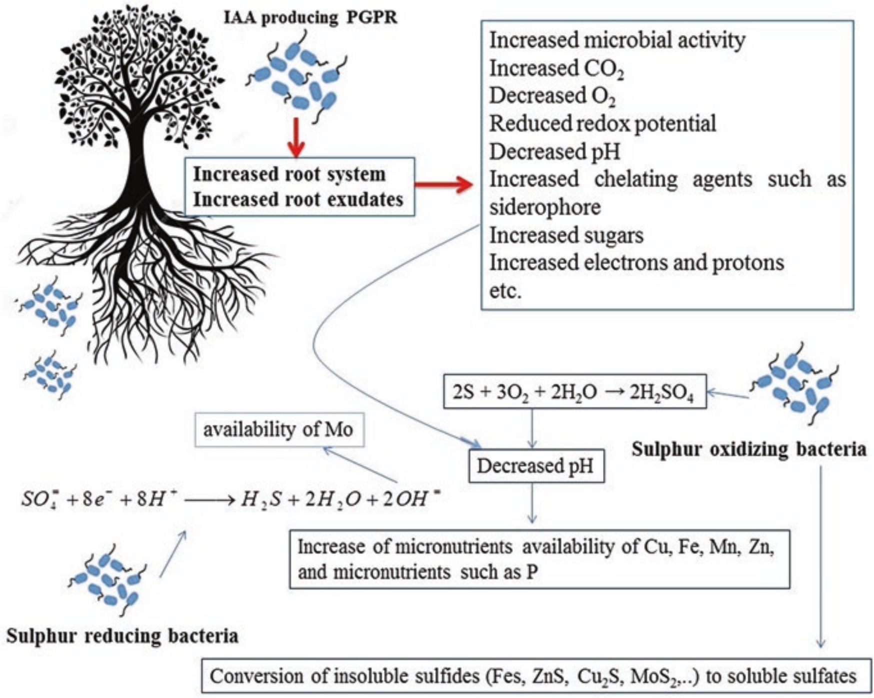


Fig. 9.12 The role of PGPR in increasing the availability of micronutrients to plant by different mechanisms

can also chelate several other metals such as Mg^{2+}, Mn^{2+}, chromium (Cr^{3+}), gallium (Ga^{3+}), cadmium (Cd), Zn^{2+}, Cu^{2+}, Ni, arsenic (As) and lead (Pb), and radionuclides, including plutonium (Pu^{4+}) with variable affinities (Nair et al. 2007; Rajkumar et al. 2010; Schalk et al. 2011). Glycolic, oxalic, malonic, tartaric, lactic, citric, α-ketogluconic, piscidic, succinic, malic, valeric, and formic are some of the known organic acids with chelating properties similar to siderophores (Panhwar et al. 2013). In addition to producing chelating agents, PGPR such as K-solubilizing PGPR can increase metal bioavailability in metal-stressed soils by producing biosurfactant, which aids in metal release from soil particles (Gamalero and Glick 2012; Singh and Cameotra 2013).

9.4.3 Production of IAA

As mentioned above, phytohormone (IAA)-producing PGPR can enhance indirectly the availability of micronutrients by improving root development and growth and root exudates. The exudates released by roots of plant also attract the wide range of PGPR with other plant growth-promoting traits such as siderophore production, phosphate solubilizing, and ACC deaminase production. These bacteria

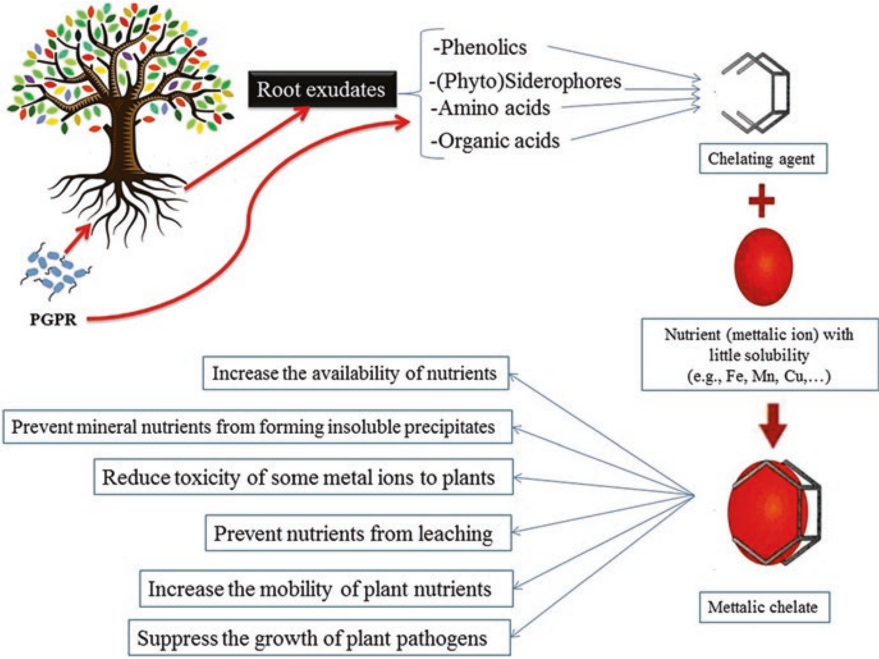


Fig. 9.13 The role of chelating agents produced by PGPR in increasing nutrient availability to plants by different ways

can also enhance the availability of micronutrients by siderophore as a chelating agent, production of acid, and decrease of ethylene and subsequent increase of root system (Fig. 9.6). Root exudates can also act as binding material/cementing agents of soil and, thus, improve soil structure and regulate and maintain the microbial population near the root surface. Microbial activity near the root surface plays an important role in the development and rooting pattern of the plant. In addition, attracted PGPR themselves also produce many exudates, which are very helpful in plant nutrition and growth. The presence of various composites of amino acids, organic acids, sugars, vitamins, purines, adenine, guanine, cytidine, uridine, enzymes (*e.g.,* phosphatase), and some gaseous molecules (*e.g.,* CO_2) in root exudates (Dakora and Phillips 2002) enhances the availability of micronutrients. A fraction of these exudates are further metabolized by PGPR in the vicinity as C and N sources, and some bacterium-oriented molecules are subsequently retaken up by plants for growth and development (Sheng and He 2006). Another nutritional effect that organic acids have in root exudates is acidification of the rhizosphere, which enhances the availability of micronutrients. In general, PGPR, especially IAA-producing bacteria, can increase the availability of micronutrients in the soil directly by the production of different compounds (such as carboxylates, phenolic compounds, etc.) or indirectly through affecting plant growth and hence the production of root exudates (Fig. 9.14) (Badri and Vivanco 2009). To understand the action

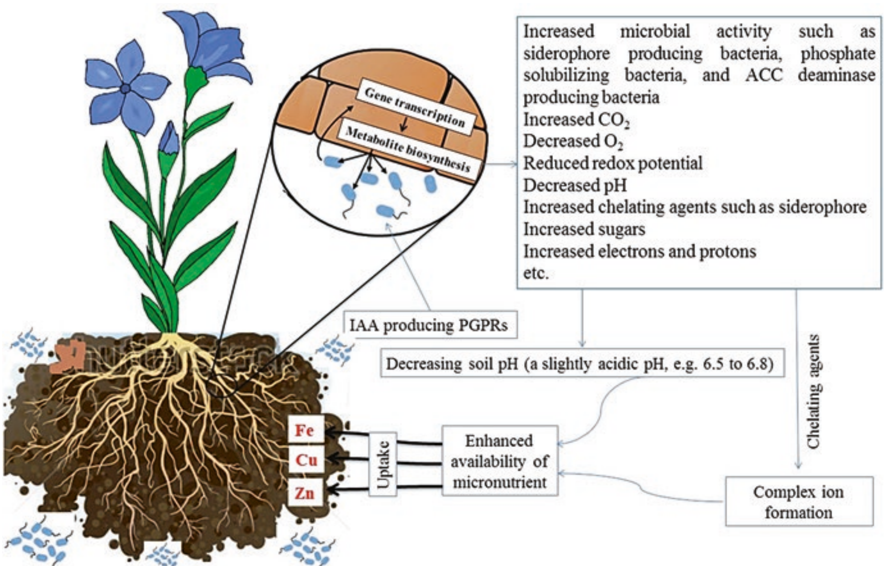


Fig. 9.14 Impact of PGPR on micronutrients acquisition and root functioning. PGPRs can modulate root development and growth through the production of phytohormones such as IAA, secondary metabolites. PGPR also influence plant nutrition via ACC deaminase, solubilization of phosphorus, or siderophore production, and modify root physiology by changing gene transcription and metabolite biosynthesis in plant cells

mechanism of PGPR in the availability of micronutrients to plant, two nutrient elements Fe and Mn are explained in more detail as follows.

9.5 Iron (Fe)

As an essential nutrient for plants, iron (Fe) is crucial for a variety of cellular functions and essential physiological processes, including respiration and photosynthesis and a necessary cofactor for many enzymatic reactions (Zuo and Zhang 2010). Under aerobic conditions, Fe exists predominantly as Fe^{3+} and reacts to form highly insoluble hydroxides and oxyhydroxides that are basically unavailable to plants and microorganisms (Desai and Archana 2011; Zuo and Zhang 2010). High soil pH reduces while acidic soil conditions increase Fe availability. As pH increases by one unit, activity of Fe^{3+} decreases by 1000-fold. In most soils, Fe is present in large quantities, but mainly in forms that are not available to plants (Schmidt 2003; Wintergerst et al. 2007). It has been reported that most plants need the concentrations of soluble Fe at 10^{-4} to 10^{-8} M (optimal soils usually slightly acidic) for their optimal growth. However, 10^{-9} M or lower concentrations of soluble Fe (calcareous or alkaline soils with low bioavailable Fe) are insufficient for plant growth and plants may develop a Fe deficiency-associated leaf chlorosis as a disease symptom.

There are many factors that affect the availability of Fe in the soil. The availability of Fe strongly depends on soil redox potential (redox change) and pH. When decreasing redox potential and pH, availability of Fe increases. PGPR can increase the availability of Fe by decreasing soil redox potential and pH.

9.5.1 Fe Acquisition Strategies by Plants

Despite the abundance of Fe in soils, its availability for plants and microbes is low. Plants and microorganisms have evolved active strategies of Fe uptake. Mobilization of Fe is the prerequisite for uptake of Fe into the roots, which is achieved by two different strategies in the plant kingdom. These strategies are named as strategy I and strategy II. In strategy I, all plant species (monocotyledonous and dicotyledonous plants), except grasses, acidify the rhizosphere and produce organic products. In addition, Fe^{3+} is reduced into Fe^{2+} by a Fe chelate reductase enzyme, converting Fe (III) to Fe (II) (Hartmann et al. 2008). Subsequently, Fe^{2+} can be taken up by a membrane-bound Fe (II) transporter. However, strategy II grasses handle Fe deficiency by the synthesis and secretion of siderophores and uptake of them by the activation of the Fe^{3+} siderophore transporter in the plasmalemma of root cells (Altomare and Tringovska 2011; Charlson and Shoemaker 2006; Curie et al. 2001; Guerinot 2010; Wintergerst et al. 2007).

9.5.2 Action Mechanisms of PGPR in Fe Availability

It has been known that strategies I and II are often not efficient enough to meet the needs of the plants growing especially in calcareous and alkaline soils. Studies show that many IAA- and siderophore-producing PGPR strains could improve iron nutrition (Etesami et al. 2015a; Jin et al. 2006; Ramos-Solano et al. 2010).

9.5.2.1 Production of Siderophores

Most plant-associated bacteria can produce iron chelators called siderophores in response to low iron levels in the rhizosphere. Siderophores are low-molecular-weight organic compounds, which have high affinity to bind some elements such as Fe^{3+} as well as other metal ions and increase their availability (Boukhalfa and Crumbliss 2002). Several examples of increased Fe uptake in plants with concurrent stimulation of plant growth as a result of PGPR inoculations have been reported (Barzanti et al. 2007; Burd et al. 2000; Carrillo-Castañeda et al. 2002; Lemanceau et al. 2009). Many studies have demonstrated that microbial siderophores can be used as Fe source for plants with strategies I and II (Fernández and Winkelmann 2005; Jin et al. 2006; Johnson et al. 2002; Rasouli-Sadaghiani et al. 2014; Robin et al. 2008; Siebner-Freibach et al. 2003; Vansuyt et al. 2007).

According to results of previous studies, it is most probable that the bacterial siderophore is not absorbed by the plant, and iron is obtained through a reduction-based mechanism (Johnson et al. 2002). Ferric siderophores are transported into

cells via specific Fe-siderophore membrane receptors, allowing siderophore release for subsequent reuse (Lemanceau et al. 2009) (Fig. 9.15). It has been found that the ability of siderophores in supplying Fe^{+3} to the root surfaces and in the intracellular spaces of root cells is the most important function of these chelating compounds in the gramineous plant nutrition. Accordingly, the higher concentrations of Fe^{+3} ions, which are available to the root phytosiderophores, enhance their subsequent absorption by plants (Sharma et al. 2003). It has been shown that ligand exchange is another theory on the supply of Fe by siderophores (Latour et al. 2009; Yehuda et al. 1996). This theory has been suggested for iron, showing that Fe supplied by siderophores interacts with phytosiderophores in a ligand exchange reaction and is finally absorbed by the phytosiderophores. This theory confirms the indirect role of siderophore in Fe uptake (Shweta et al. 2008). Among different known microbial siderophores, plants are capable of using hydroxamates, ferrichrome, rhodotorulic acid, desferrioxamine B, agrobactin, as well as catecholate-hydroxamate (Fernández and Winkelmann 2005). Generally, compared to most microorganisms, most plants can grow at very low concentrations of Fe (O'Sullivan and O'Gara 1992); therefore many plants can take up iron in the presence of siderophore-producing microorganisms. In addition to high affinity for Fe (III) ions, siderophores can also form complexes with other bivalent ions (and including phosphates and other micronutrients) that can be assimilated by the plant (Ramos-Solano et al. 2010).

9.5.2.2 Production of IAA

As previously mentioned, the production of root and microbial exudates is an important parameter determining the availability of nutrients in the rhizosphere. Organic acids present in root and microbial exudates result in acidification of the rhizosphere (Dakora and Phillips 2002), which can, in turn, contribute to plant growth by mobilizing nutrients such as Fe. Because of the function of IAA in secreting root exudates (rhizodeposits) and increasing rooting system and since these exudates are involved in acidifying rhizosphere and in providing a reducing condition required for converting Fe^{3+} to Fe^{2+}, it may be suggested that IAA-producing PGPR can also solubilize insoluble Fe sources and induce plant growth and iron uptake in a similar manner to strategy I in plants (Fig. 9.15). For example, protons and electrons are secreted within carbon compounds as undissociated acids or compounds with reducing capabilities. Some of the compounds in root exudates are able to form Fe complexes that improve availability. High-molecular-weight components (i.e., proteins, mucilage) and low-molecular-weight compounds (*i.e.,* secondary metabolites, organic acids, carbohydrates, amino acids, phenolics) are typically the dominant soluble-reduced carbon compounds in rhizodeposits (Badri and Vivanco 2009; Wen et al. 2007). The consumption of O_2, due to respiration by the root (increase of root system due to bacterial IAA) and associated microflora (increase of microflora activity due to production of more root exudates), can also result in steep redox gradients in the rhizosphere (Etesami et al. 2015a, b; Hartmann et al. 2008). Likewise, chelating agents present in root and microbial exudates such as organic acids are capable of chelating Fe^{3+} and making it available to plant roots in a similar manner to strategy II in plants (Fig. 9.15).

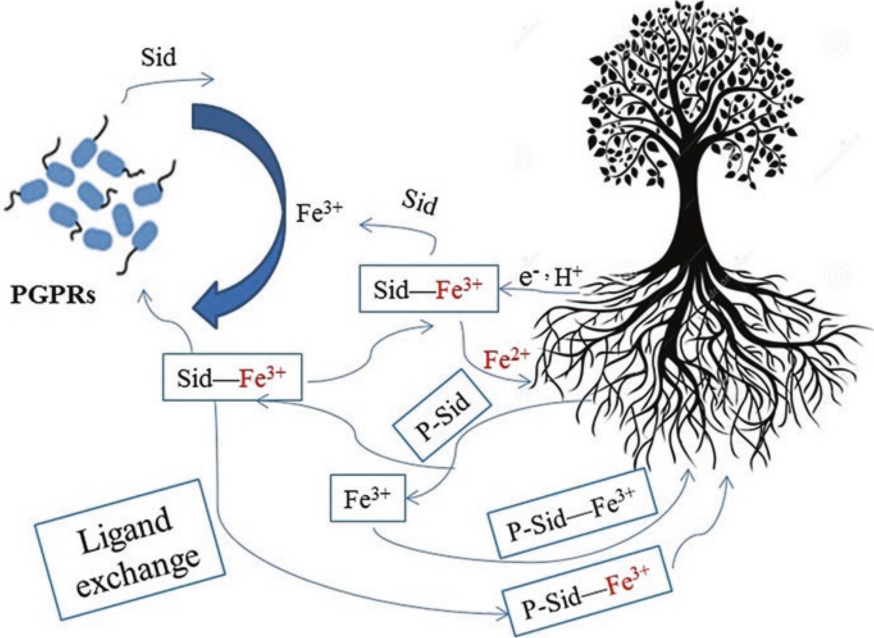


Fig. 9.15 Schematic representations of role of siderophore (Sid)-producing PGPR in enhancing iron availability for plant. In non-grass species (Strategy I), acidification of the rhizosphere occurs in part through the activity of a plasma membrane H^+-ATPase. This H^+ excretion contributes to the solubilization of Fe^{+3}, which is reduced to Fe^{+2} by the FRO_2 ferric chelate reductase, transferring electrons (e^-) from NADPH to Fe^{+3} (Lemanceau et al. 2009). In grasses, Strategy II involves the synthesis of phytosiderophores (P-Sid). P-Sid is secreted from the roots by an uncharacterized mechanism into the rhizosphere where it chelates Fe^{+3}. The Fe^{+3}–P-Sid complex is then transported into the epidermal cells of the roots. PGPRs do not take up Fe^{+3}-Sid complexes, but rather obtain iron through a reduction-based mechanism involving Fe-Sid membrane receptors, acquiring Fe^{2+} while releasing Sid for subsequent reuse. Sid increases the Fe^{+3} pools in the rhizosphere, increasing Fe^{+3} available to the root P-Sid. P-Sid that has a higher affinity for Fe^{3+} than Sid may acquire it via ligand exchange

9.6 Manganese (Mn)

As a micronutrient, manganese (Mn) is essential for many plant functions (as a component of enzymes) and is also involved in photosynthesis and root growth. Mn as free Mn^{2+} in the soil is readily available to plants, and as oxides is of low solubility. The proportion of Mn in various forms in the soil is dependent both on chemical reactions and on microbial activity. As previously mentioned (Fig. 9.2), high soil pH greatly reduces the solubility of soil Mn, and therefore its availability to roots. Thus, Mn deficiency is most likely to occur in soils that are alkaline or have been limed. Mn is a nutrient element and its availability in the rhizosphere is affected by two major factors, namely redox condition and pH. In oxidized soils, Mn is present in its oxidized form, Mn^{4+}, in the low-soluble mineral pyrolusite. It has been known that

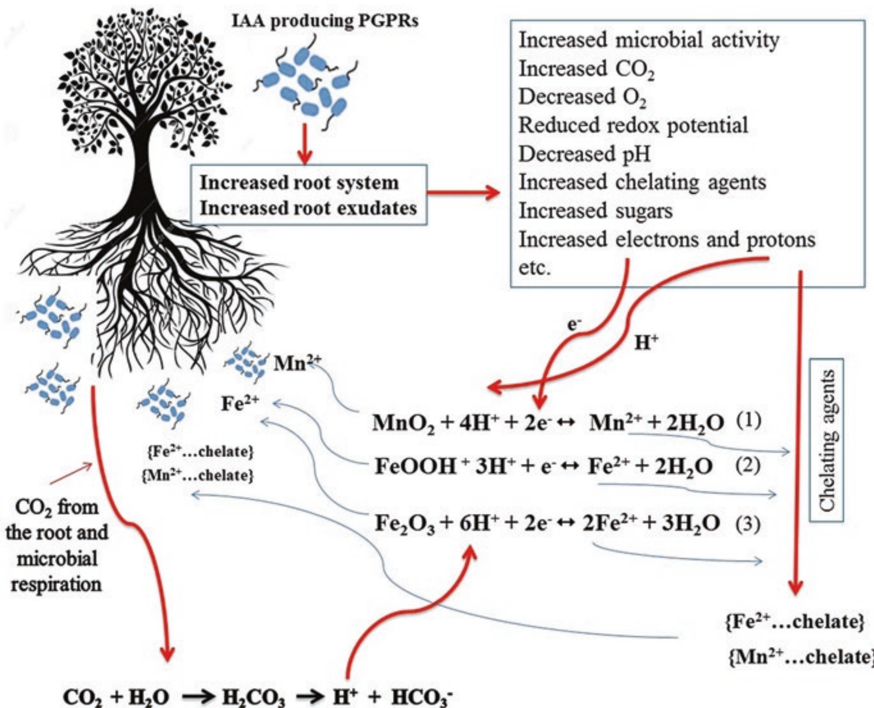


Fig. 9.16 The schematic representation of role of PGPR in the availability of Mn and Fe to plant by affecting plant (root) growth and hence plant root exudates. The electrons and protons required to the reduction of Mn and Fe in reactions (1), (2), and (3) are supplied by the decomposition of carbonaceous compounds and the proton excretion system of root cells, respectively. The roots and PGPR by producing chelating agents (phenolic compounds, organic acids) can form soluble complex with Mn, Fe, and other elements avoiding the reprecipitation of them

some PGPR can increase the availability of this element to plants. For example, PGPR such as *Bacillus*, *Pseudomonas*, and *Geobacter* could reduce oxidized Mn^{4+} to Mn^{2+}, which is the chemical form that is metabolically useful for plants (Osorio Vega 2007). These bacteria can affect Mn availability in the soil mostly by affecting plant growth and hence plant root exudates (Dutta and Podile 2010; Miransari 2011). Increased root exudates originated from bacterial activities in turn supply electrons (by the decomposition of organic molecules present in root exudates) and protons (by the proton excretion system of root cells) required for the reduction of Mn in the following reaction:

$$MnO_2 + 4H^+ + 2e^- \leftrightarrow Mn^{2+} + 2H_2O$$

Consequently, the activity of Mn reducers is highly favored in the rhizosphere (Osorio Vega 2007). By producing electron and H^+, applications of organic matter (OM) can also favor the reduction of Mn (Hue et al. 2001). Therefore, in alkaline soils where Mn usually is insoluble the rhizosphere effect and application of OM can be beneficial. In addition, roots and PGPR can produce chelating agents (phenolic compounds, organic acids) that form soluble complex with Mn, Fe, and other elements avoiding the reprecipitation of them (Fig. 9.16).

9.7 Concluding Remarks and Future Perspectives

It is evident that PGPR have a high potential to be used in the management of nutrient-deficient soils. Using PGPR to increase the availability of nutrition elements in soil is an attractive proposition for developing a more sustainable agriculture. These PGPR have an important role in the cycling of nutrient elements in soil-plant systems and it is anticipated that better understanding of their contribution to mobilizing soil nutrients and plant nutrient nutrition will provide an opportunity for developing more nutrient-efficient and sustainable agricultural systems and improved knowledge of ecosystem function. Developing the proper formulation and delivery systems to ensure survival and effective establishment of target PGPR within the rhizosphere is a main requirement for prosperous deployment of bacterial inoculants (Richardson and Simpson 2011). Increased knowledge concerning the beneficial interactions of PGPR with plants and a proper screening will be of special importance for sustainable agriculture that depends on biological processes and resources, rather than on the use of agrochemicals for maintaining soil fertility and plant health. Previous studies clearly demonstrate the presence of one or more than one type of PGP character in majority of the bacterial strains. A bacterial strain possessing multiple PGP traits is expected to indicate better response than those having single PGP characteristic. However, it would be desirable to examine whether all the traits of PGP are expressed concurrently or at different phases of growth of the bacteria. In other words, since many PGPR possess several of PGP traits simultaneously, different mechanisms at various times during the life cycle of the plant can be used. However, the exact modes by which PGPR promote plant growth at a specific step in life cycle are not fully understood (Bhattacharyya and Jha 2012), which need further studies in the future. In addition, certain issues, such as what should be an ideal and universal delivery system, how to improve the efficacy of biofertilizers, how to stabilize PGPR in soil systems, and how nutritional and root exudation aspects could be controlled in order to get maximum benefits from PGPR application, have not been well known until this moment, which are needed to be addressed by scientists in the future. Biotechnological and molecular approaches may possibly develop more understanding about PGPRs' mode of actions that will result in more successful plant–microbe interaction and prosperous application of the beneficial bacteria (Khalid et al. 2009).

Acknowledgments We wish to thank University of Tehran and University of Saskatchewan for providing the necessary facilities for this study.

References

Abhilash P, Dubey RK, Tripathi V, Srivastava P, Verma JP, Singh H (2013) Remediation and management of POPs-contaminated soils in a warming climate: challenges and perspectives. Environ Sci Pollut Res 20:5879–5885

Abou-el-Seoud II, Abdel-Megeed A (2012) Impact of rock materials and biofertilizations on P and K availability for maize (Zea maize) under calcareous soil conditions. Saudi J Biol Sci 19:55–63

Abril A, Zurdo-Pineiro J, Peix A, Rivas R, Velazquez E (2007) Solubilization of phosphate by a strain of Rhizobium leguminosarum bv. trifolii isolated from Phaseolus vulgaris in El Chaco Arido soil (Argentina). In: First international meeting on microbial phosphate solubilization. Springer, Dordrecht, pp 135–138

Ahemad M (2012) Implications of bacterial resistance against heavy metals in bioremediation: a review. J Inst Integr Omics Appl Biotechnol 3(3):39–46

Ahemad M, Khan M (2010) Phosphate solubilizing Enterobacter asburiae strain PS2. Afr J Microbiol Res 5:849–857

Ahemad M, Khan M (2010a) Plant growth promoting activities of phosphate-solubilizing Enterobacter asburiae as influenced by fungicides. Eur Asian J Biosci 4:88–95

Ahemad M, Khan MS (2010b) Phosphate-solubilizing and plant-growth-promoting Pseudomonas aeruginosa PS1 improves greengram performance in quizalafop-p-ethyl and clodinafop amended soil. Arch Environ Contam Toxicol 58:361–372

Ahemad M, Khan M (2011a) Toxicological assessment of selective pesticides towards plant growth promoting activities of phosphate solubilizing Pseudomonas aeruginosa. Acta Microbiol Immunol Hung 58:169–187

Ahemad M, Khan MS (2011b) Assessment of plant growth promoting activities of rhizobacterium Pseudomonas putida under insecticide-stress. Microbiol J 1:54–64

Ahemad M, Khan MS (2011c) Effects of insecticides on plant-growth-promoting activities of phosphate solubilizing rhizobacterium Klebsiella sp. strain PS19. Pestic Biochem Physiol 100:51–56

Ahemad M, Khan MS (2011d) Pseudomonas aeruginosa strain PS1 enhances growth parameters of greengram [Vigna radiata (L.) Wilczek] in insecticide-stressed soils. J Pest Sci 84:123–131

Ahemad M, Khan MS (2011e) Toxicological effects of selective herbicides on plant growth promoting activities of phosphate solubilizing Klebsiella sp. strain PS19. Curr Microbiol 62:532–538

Ahemad M, Khan MS (2012a) Alleviation of fungicide-induced phytotoxicity in greengram [Vigna radiata (L.) Wilczek] using fungicide-tolerant and plant growth promoting Pseudomonas strain. Saudi J Biol Sci 19:451–459

Ahemad M, Khan MS (2012b) Biotoxic impact of fungicides on plant growth promoting activities of phosphate-solubilizing Klebsiella sp. isolated from mustard (Brassica campestris) rhizosphere. J Pest Sci 85:29–36

Ahemad M, Khan MS (2012c) Effect of fungicides on plant growth promoting activities of phosphate solubilizing Pseudomonasputida isolated from mustard (Brassica compestris) rhizosphere. Chemosphere 86:945–950

Ahemad M, Khan MS (2012d) Evaluation of plant-growth-promoting activities of rhizobacterium Pseudomonas putida under herbicide stress. Ann Microbiol 62:1531–1540

Ahemad M, Kibret M (2014) Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. J King Saud Univ-Sci 26:1–20

Ahmad M, Nadeem SM, Naveed M, Zahir ZA (2016) Potassium-solubilizing bacteria and their application in agriculture. In: Potassium solubilizing microorganisms for sustainable agriculture. Springer, New Delhi, pp 293–313

Altomare C, Tringovska I (2011) Beneficial soil microorganisms, an ecological alternative for soil fertility management. In: Lichtfouse E (ed) Genetics, biofuels and local farming systems. Springer Netherlands, Dordrecht, pp 161–214. https://doi.org/10.1007/978-94-007-1521-9_6

Amir H, Pineau R (2003) Release of Ni and Co by microbial activity in New Caledonian ultramafic soils. Can J Microbiol 49:288–293

Andrews JH, Harris RF (2000) The ecology and biogeography of microorganisms on plant surfaces. Ann Revi Phytopathol 38:145–180

Archana D, Nandish M, Savalagi V, Alagawadi A (2013) Characterization of potassium solubilizing bacteria (KSB) from rhizosphere soil BIOINFOLET-A. Quart J Life Sci 10:248–257

Arshad M, Saleem M, Hussain S (2007) Perspectives of bacterial ACC deaminase in phytoremediation. Trends Biotechnol 25:356–362. https://doi.org/10.1016/j.tibtech.2007.05.005

Autry A, Fitzgerald J (1990) Sulfonate S: a major form of forest soil organic sulfur. Biol Fertil Soils 10:50–56

Azam F, Memon G (1996) Soil organisms. In: Bashir E, Bantel R (eds) Soil science. National Book Foundation, Islamabad, pp 200–232

Azevedo JL, Maccheroni W Jr, Pereira JO, de Araújo WL (2000) Endophytic microorganisms: a review on insect control and recent advances on tropical plants. Electron J Biotechnol 3:15–16

Babana A, Antoun H (2006) Effect of Tilemsi phosphate rock-solubilizing microorganisms on phosphorus uptake and yield of field-grown wheat (Triticum aestivum L.) in Mali. Plant and Soil 287:51–58

Badr MA, Shafei AM, Sharaf El-Deen SH (2006) The dissolution of K and P-bearing minerals by silicate dissolving bacteria and their effect on sorghum growth. Res J Agric Biol Sci 2:5–11

Badri DV, Vivanco JM (2009) Regulation and function of root exudates. Plant Cell Environ 32:666–681

Bahadur I, Meena VS, Kumar S (2014) Importance and application of potassic biofertilizer in Indian agriculture. Res J Chem Sci ISSN 2231:606X

Balogh-Brunstad Z, Keller C, Gill R, Bormann B, Li C (2008) The effect of bacteria and fungi on chemical weathering and chemical denudation fluxes in pine growth experiments. Biogeochemistry 88:153–167

Barber SA (1995) Soil nutrient bioavailability: a mechanistic approach. John Wiley & Sons, New York

Barker WW, Welch SA, Chu S, Banfield JF (1998) Experimental observations of the effects of bacteria on aluminosilicate weathering. Am Mineral 83:1551–1563

Barzanti R, Ozino F, Bazzicalupo M, Gabbrielli R, Galardi F, Gonnelli C, Mengoni A (2007) Isolation and characterization of endophytic bacteria from the nickel hyperaccumulator plant Alyssum bertolonii. Microb Ecol 53:306–316. https://doi.org/10.1007/s00248-006-9164-3

Basak BB, Biswas DR (2009) Influence of potassium solubilizing microorganism (Bacillus mucilaginosus) and waste mica on potassium uptake dynamics by sudan grass (Sorghum vulgare Pers.) grown under two Alfisols. Plant and Soil 317:235–255. https://doi.org/10.1007/s11104-008-9805-z

Basak B, Biswas D (2012) Modification of waste mica for alternative source of potassium: evaluation of potassium release in soil from waste mica treated with potassium solubilizing bacteria (KSB). LAP LAMBERT Academic Publishing, Riga

Beattie GA (2007) Plant-associated bacteria: survey, molecular phylogeny, genomics and recent advances. In: Plant-associated bacteria. Springer, Dordrecht, pp 1–56

Becerra-Castro C, Prieto-Fernández A, Álvarez-López V, Monterroso C, Cabello-Conejo M, Acea M, Kidd P (2011) Nickel solubilizing capacity and characterization of rhizobacteria isolated from hyperaccumulating and non-hyperaccumulating subspecies of Alyssum serpyllifolium. Int J Phytoremediation 13:229–244

Belimov AA, Dodd IC, Hontzeas N, Theobald JC, Safronova VI, Davies WJ (2009) Rhizosphere bacteria containing 1-aminocyclopropane-1-carboxylate deaminase increase yield of plants grown in drying soil via both local and systemic hormone signalling. New Phytol 181:413–423. https://doi.org/10.1111/j.1469-8137.2008.02657.x

Bennett P, Choi W, Rogera J (1998) Microbial destruction of feldspars. Miner Manage 8:149–150

Bhattacharyya PN, Jha DK (2012) Plant growth-promoting rhizobacteria (PGPR): emergence in agriculture. World J Microbiol Biotechnol 28:1327–1350

Borch K, Bouma T, Lynch J, Brown K (1999) Ethylene: a regulator of root architectural responses to soil phosphorus availability. Plant Cell Environ 22:425–431

Boukhalfa H, Crumbliss AL (2002) Chemical aspects of siderophore mediated iron transport. Biometals 15:325–339. https://doi.org/10.1023/A:1020218608266

Braud A, Jezequel K, Bazot S, Lebeau T (2009) Enhanced phytoextraction of an agricultural Cr- and Pb-contaminated soil by bioaugmentation with siderophore-producing bacteria. Chemosphere 74:280–286. https://doi.org/10.1016/j.chemosphere.2008.09.013

Bünemann E (2008) Enzyme additions as a tool to assess the potential bioavailability of organically bound nutrients. Soil Biol Biochem 40:2116–2129

Burd GI, Dixon DG, Glick BR (2000) Plant growth-promoting bacteria that decrease heavy metal toxicity in plants. Can J Microbiol 46:237–245

Butterly C, Bünemann E, McNeill A, Baldock J, Marschner P (2009) Carbon pulses but not phosphorus pulses are related to decreases in microbial biomass during repeated drying and rewetting of soils. Soil Biol Biochem 41:1406–1416

Caballero-Mellado J, Onofre-Lemus J, Estrada-De Los Santos P, Martínez-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

Calvaruso C, Turpault M-P, Leclerc E, Frey-Klett P (2007) Impact of ectomycorrhizosphere on the functional diversity of soil bacterial and fungal communities from a forest stand in relation to nutrient mobilization processes. Microb Ecol 54:567–577

Carrillo-Castañeda G, Juárez Muñoz J, Ramón Peralta-Videa J, Gomez E, Gardea-Torresdey JL (2002) Plant growth-promoting bacteria promote copper and iron translocation from root to shoot in Alfalfa seedlings. J Plant Nutr 26:1801–1814

Chaintreuil C et al (2000) Photosynthetic bradyrhizobia are natural endophytes of the African wild rice *Oryza breviligulata*. Appl Environ Microbiol 66:5437–5447

Chamam A et al (2013) Plant secondary metabolite profiling evidences strain-dependent effect in the Azospirillum–Oryza sativa association. Phytochemistry 87:65–77

Charlson DV, Shoemaker RC (2006) Evolution of iron acquisition in higher plants. J Plant Nutr 29:1109–1125. https://doi.org/10.1080/01904160600689266

Chen L, Dodd IC, Theobald JC, Belimov AA, Davies WJ (2013) The rhizobacterium Variovorax paradoxus 5C-2, containing ACC deaminase, promotes growth and development of Arabidopsis thaliana via an ethylene-dependent pathway. J Exp Bot 64(6):1565–1673

Chi F, Shen SH, Cheng HP, Jing YX, Yanni YG, Dazzo FB (2005) Ascending migration of endophytic *rhizobia*, from roots to leaves, inside rice plants and assessment of benefits to rice growth physiology. Appl Environ Microbiol 71:7271–7278

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

Combes-Meynet E, Pothier JF, Moënne-Loccoz Y, Prigent-Combaret C (2011) The Pseudomonas secondary metabolite 2, 4-diacetylphloroglucinol is a signal inducing rhizoplane expression of Azospirillum genes involved in plant-growth promotion. Mol Plant Microbe Interact 24:271–284

Compant S, Clément C, Sessitsch A (2010) Plant growth-promoting bacteria in the rhizo- and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. Soil Biol Biochem 42:669–678. https://doi.org/10.1016/j.soilbio.2009.11.024

Contesto C et al (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

Criquet S, Ferre E, Farnet A (2004) Annual dynamics of phosphatase activities in an evergreen oak litter: influence of biotic and abiotic factors. Soil Biol Biochem 36:1111–1118

Crowley DE (2006) Microbial siderophores in the plant rhizosphere. In: Iron nutrition in plants and rhizospheric microorganisms. Springer, Dordrecht, pp 169–198

Curie C, Panaviene Z, Loulergue C, Dellaporta SL, Briat JF, Walker EL (2001) Maize yellow stripe1 encodes a membrane protein directly involved in Fe(III) uptake. Nature 409:346–349. https://doi.org/10.1038/35053080

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

Dalla Santa OR, Hernández RF, Alvarez GLM, Ronzelli Junior P, Soccol CR (2004) Azospirillum sp. inoculation in wheat, barley and oats seeds greenhouse experiments. Brazilian Arch Biol Technol 47:843–850

Dalton DA, Kramer S (2006) Nitrogen-fixing bacteria in non-legumes. In: Gnanamanickam SS (ed) Plant-associated bacteria. Springer Netherlands, Dordrecht, pp 105–130. https://doi.org/10.1007/978-1-4020-4538-7_3

Das I, Pradhan M (2016) Potassium-solubilizing microorganisms and their role in enhancing soil fertility and health. In: Potassium solubilizing microorganisms for sustainable agriculture. Springer, New Delhi, pp 281–291

Datta M, Banik S, Gupta R (1982) Studies on the efficacy of a phytohormone producing phosphate solubilizing Bacillus firmus in augmenting paddy yield in acid soils of Nagaland. Plant Soil 69:365–373

Desai A, Archana G (2011) Role of siderophores in crop improvement. In: Maheshwari KD (ed) Bacteria in agrobiology: plant nutrient management. Springer, Berlin, Heidelberg, pp 109–139. https://doi.org/10.1007/978-3-642-21061-7_6

Diby P, Bharathkumar S, Sudha N (2005) Osmotolerance in biocontrol strain of pseudomonas pseudoalcaligenes MSP-538: a study using osmolyte, protein and gene expression profiling. Ann Microbiol 55:243–247

Diby P, Sarma YR, Srinivasan V, Anandaraj M (2005b) Pseudomonas fluorescens mediated vigour in black pepper (*Piper nigrum* L.) under green house cultivation. Ann Microbiol 55:171–174

Diep CN, Hieu TN (2013) Phosphate and potassium solubilizing bacteria from weathered materials of denatured rock mountain, Ha Tien, Kiên Giang province Vietnam. Am J Life Sci 1:88–92

Dighton J, Boddy L (1989) Role of fungi in nitrogen, phosphorus and sulphur cycling in temperate forest ecosystems.

Dimkpa C, Weinand T, Asch F (2009) Plant-rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell Environ 32:1682–1694. https://doi.org/10.1111/j.1365-3040.2009.02028.x

Dobbelaere S, Croonenborghs A, Thys A, Broek AV, Vanderleyden J (1999) Phytostimulatory effect of Azospirillum brasilense wild type and mutant strains altered in IAA production on wheat. Plant and Soil 212:153–162

Dobbelaere S, Vanderleyden J, Okon Y (2003) Plant growth-promoting effects of diazotrophs in the rhizosphere. Crit Rev Plant Sci 22:107–149

Döbereiner J, Day J, Dart P (1972) Nitrogenase activity and oxygen sensitivity of the Paspalum notatum-Azotobacter paspali association. Microbiology 71:103–116

Drogue B, Combes-Meynet E, Moënne-Loccoz Y, Wisniewski-Dyé F, Prigent-Combaret C (2013) Control of the cooperation between plant growth-promoting Rhizobacteria and crops by rhizosphere signals. Mol Microbial Ecol Rhizosphere 1 and 2:279–293

Dutta S, Podile AR (2010) Plant growth promoting rhizobacteria (PGPR): the bugs to debug the root zone. Crit Rev Microbiol 36:232–244

Egamberdieva D (2009) Alleviation of salt stress by plant growth regulators and IAA producing bacteria in wheat. Acta Physiol Plantarum 31:861–864

Egamberdieva D, Kucharova Z (2009) Selection for root colonising bacteria stimulating wheat growth in saline soils. Biol Fertil Soils 45:563–571. https://doi.org/10.1007/s00374-009-0366-y

El Zemrany H et al (2006) Field survival of the phytostimulator Azospirillum lipoferum CRT1 and functional impact on maize crop, biodegradation of crop residues, and soil faunal indicators in a context of decreasing nitrogen fertilisation. Soil Biol Biochem 38:1712–1726

El-Khawas H, Adachi K (1999) Identification and quantification of auxins in culture media of Azospirillum and Klebsiella and their effect on rice roots. Biol Fertil Soils 28:377–381

Esquivel-Cote R, Ramírez-Gama RM, Tsuzuki-Reyes G, Orozco-Segovia A, Huante P (2010) Azospirillum lipoferum strain AZm5 containing 1-aminocyclopropane-1-carboxylic acid deaminase improves early growth of tomato seedlings under nitrogen deficiency. Plant and Soil 337:65–75. https://doi.org/10.1007/s11104-010-0499-7

Etesami H, Hosseini H, Alikhani H, Mohammadi L (2014) Bacterial biosynthesis of 1-aminocyclopropane-1-carboxylate (ACC) deaminase and indole-3-acetic acid (IAA) as endophytic preferential selection traits by rice plant seedlings. J Plant Growth Regul 33:654–670. https://doi.org/10.1007/s00344-014-9415-3

Etesami H, Alikhani H, Mirseyed Hosseini H (2015a) Indole-3-acetic acid and 1-aminocyclopropane-1-carboxylate deaminase: bacterial traits required in rhizosphere, rhizoplane and/or endophytic competence by beneficial bacteria. In: Maheshwari DK (ed) Bacterial metabolites in sustainable agroecosystem. Sustainable development and biodiversity, vol vol. 12. Springer International Publishing, Cham, pp 183–258. https://doi.org/10.1007/978-3-319-24654-3_8

Etesami H, Alikhani HA, Hosseini HM (2015b) Indole-3-acetic acid (IAA) production trait, a useful screening to select endophytic and rhizosphere competent bacteria for rice growth promoting agents. MethodsX 2:72–78. https://doi.org/10.1016/j.mex.2015.02.008

Fernández V, Winkelmann G (2005) The determination of ferric iron in plants by HPLC using the microbial iron chelator desferrioxamine E. Biometals 18:53–62. https://doi.org/10.1007/s10534-004-5773-9

Fowler D, Smith R, Muller J, Hayman G, Vincent K (2005) Changes in the atmospheric deposition of acidifying compounds in the UK between 1986 and 2001. Environ Pollut 137:15–25

Franche C, Lindström K, Elmerich C (2009) Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant and Soil 321:35–59

Gahan J, Schmalenberger A (2014) The role of bacteria and mycorrhiza in plant sulfur supply. Front Plant Sci 5

Gahoonia TS, Care D, Nielsen NE (1997) Root hairs and phosphorus acquisition of wheat and barley cultivars. Plant and Soil 191:181–188

Gamalero E, Glick BR (2012) Plant growth-promoting bacteria and metals phytoremediation phytotechnologies. In: Remediation of environmental contaminants. CRC Press, Boca Raton, pp 361–376

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

Garcia NS, Fu F, Sedwick PN, Hutchins DA (2015) Iron deficiency increases growth and nitrogen-fixation rates of phosphorus-deficient marine cyanobacteria. ISME J 9:238–245. https://doi.org/10.1038/ismej.2014.104

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

Glick BR (2012a) Plant growth-promoting bacteria: mechanisms and applications. Scientifica:1–15

Glick BR (2012b) Plant growth-promoting bacteria: mechanisms and applications. Scientifica:963401. 15 p.

Glick BR (2014) Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiol Res 169:30–39. https://doi.org/10.1016/j.micres.2013.09.009

Glick BR (2015a) Introduction to plant growth-promoting bacteria. In: Glick RB (ed) Beneficial plant-bacterial interactions. Springer International Publishing, Cham, pp 1–28. https://doi.org/10.1007/978-3-319-13921-0_1

Glick BR (2015b) Issues regarding the use of PGPB. In: Glick RB (ed) Beneficial plant-bacterial interactions. Springer International Publishing, Cham, pp 223–243. https://doi.org/10.1007/978-3-319-13921-0_8

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

Goldstein AH (1994) Involvement of the quinoprotein glucose dehydrogenase in the solubilization of exogenous phosphates by Gram-negative bacteria phosphate in microorganisms: cellular and molecular biology. ASM Press, Washington, DC, pp 197–203

Goswami D, Thakker JN, Dhandhukia PC (2016) Portraying mechanics of plant growth promoting rhizobacteria (PGPR): a review. Cogent Food Agric 2:1127500

Gray EJ, Smith DL (2005) Intracellular and extracellular PGPR: commonalities and distinctions in the plant-bacterium signaling processes. Soil Biol Biochem 37:395–412. https://doi.org/10.1016/j.soilbio.2004.08.030

Groudev SN (1987) Use of heterotrophic microorganisms in mineral biotechnology. Eng Life Sci 7:299–306

Guerinot ML (2010) Iron. In: Hell R, Mendel R-R (eds) Cell biology of metals and nutrients. Springer, Berlin, Heidelberg, pp 75–94. https://doi.org/10.1007/978-3-642-10613-2_4

Gundala PB, Chinthala P, Sreenivasulu B (2013) A new facultative alkaliphilic, potassium solubilizing, Bacillus Sp. SVUNM9 isolated from mica cores of Nellore District, Andhra Pradesh, India. Res Rev J Microbiol Biotechnol 2:1–7

Gyaneshwar P, Kumar GN, Parekh L, Poole P (2002) Role of soil microorganisms in improving P nutrition of plants. In: Food security in nutrient-stressed environments: exploiting plants' genetic capabilities. Springer, Dordrecht, pp 133–143

Hamdali H, Bouizgarne B, Hafidi M, Lebrihi A, Virolle MJ, Ouhdouch Y (2008) Screening for rock phosphate solubilizing Actinomycetes from Moroccan phosphate mines. Appl Soil Ecol 38:12–19

Han HS, Lee KD (2005) Phosphate and potassium solubilizing bacteria effect on mineral uptake, soil availability and growth of egg plant. Res J Agric Biol Sci 1:176–180

Hartmann A, Schmid M, Dv T, Berg G (2008) Plant-driven selection of microbes. Plant and Soil 321:235–257. https://doi.org/10.1007/s11104-008-9814-y

Harvey P, Warren R, Wakelin S (2009) Potential to improve root access to phosphorus: the role of non-symbiotic microbial inoculants in the rhizosphere. Crop Pasture Sci 60:144–151

Hayat R, Ali S, Amara U, Khalid R, Ahmed I (2010) Soil beneficial bacteria and their role in plant growth promotion: a review. Ann Microbiol 60:579–598. https://doi.org/10.1007/s13213-010-0117-1

He LY, Zhang YF, Ma HY, Chen ZJ, Wang QY, Qian M, Sheng XF (2010) Characterization of copper-resistant bacteria and assessment of bacterial communities in rhizosphere soils of copper-tolerant plants. Appl Soil Ecol 44:49–55

He H et al (2013) Characterization of endophytic Rahnella sp. JN6 from Polygonum pubescens and its potential in promoting growth and Cd, Pb, Zn uptake by Brassica napus. Chemosphere 90:1960–1965

Hong YF et al (2008) The sweet potato sporamin promoter confers high-level phytase expression and improves organic phosphorus acquisition and tuber yield of transgenic potato. Plant Mol Biol 67:347–361. https://doi.org/10.1007/s11103-008-9324-6

Hue NV, Vega S, Silva JA (2001) Manganese toxicity in a Hawaiian Oxisol affected by soil pH and organic amendments. Soil Sci Soc Am J 65:153–160

Hummerjohann J, Laudenbach S, Rétey J, Leisinger T, Kertesz MA (2000) The sulfur-regulated arylsulfatase gene cluster of Pseudomonas aeruginosa, a New Member of thecys Regulon. J Bacteriol 182:2055–2058

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

Illmer P, Barbato A, Schinner F (1995) Solubilization of hardly-soluble AlPO 4 with P-solubilizing microorganisms. Soil Biol Biochem 27:265–270

Iniguez AL, Dong Y, Carter HD, Ahmer BM, Stone JM, Triplett EW (2005) Regulation of enteric endophytic bacterial colonization by plant defenses. Mol Plant-Microbe Interact 18:169–178. https://doi.org/10.1094/mpmi-18-0169

Irwin JG, Campbell G, Vincent K (2002) Trends in sulphate and nitrate wet deposition over the United Kingdom: 1986–1999. Atmos Environ 36:2867–2879

Jain A et al (2007) Differential effects of sucrose and auxin on localized phosphate deficiency-induced modulation of different traits of root system architecture in Arabidopsis. Plant Physiol 144:232–247

Jakobsen I, Leggett ME, Richardson AE, Sims J, Sharpley A (2005) Rhizosphere microorganisms and plant phosphorus uptake. Phosphorus: Agric Environ:437–494

James EK et al (2002) Infection and colonization of rice seedlings by the plant growth-promoting bacterium *Herbaspirillum seropedicae* Z 67. Mol Plant Microbiol Interact 15:894–906

Janssen A, Meijer S, Bontsema J, Lettinga G (1998) Application of the redox potential for controlling a sulfide oxidizing bioreactor. Biotechnol Bioeng 60:147–155

Jewell MC, Campbell BC, Godwin ID (2010) Transgenic plants for abiotic stress resistance. In: Kole C, Michler CH, Abbott AG, Hall TC (eds) Transgenic crop plants. Springer, Berlin, Heidelberg, pp 67–132. https://doi.org/10.1007/978-3-642-04812-8_2

Jiang C-Y, Sheng X-F, Qian M, Wang Q-Y (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

Jin CW, He YF, Tang CX, Wu P, Zheng SJ (2006) Mechanisms of microbially enhanced Fe acquisition in red clover (Trifolium pratense L.). Plant. Cell Environ 29:888–897

Johnson GV, Lopez A, La Valle FN (2002) Reduction and transport of Fe from siderophores. Plant and Soil 241:27–33. https://doi.org/10.1023/A:1016007708926

Jones D, Dennis P, Owen A, Van Hees P (2003) Organic acid behavior in soils–misconceptions and knowledge gaps. Plant and Soil 248:31–41

Jorquera MA, Hernández MT, Rengel Z, Marschner P, de la Luz MM (2008) Isolation of culturable phosphobacteria with both phytate-mineralization and phosphate-solubilization activity from the rhizosphere of plants grown in a volcanic soil. Biol Fertil Soils 44:1025–1034

Jungk A (2001) Root hairs and the acquisition of plant nutrients from soil. J Plant Nutr Soil Sci 164:121–129

Karuppiah P, Rajaram S (2011) Exploring the potential of chromium reducing Bacillus sp. and there plant growth promoting activities. J Microbiol Res 1:17–23

Kertesz MA (2000) Riding the sulfur cycle–metabolism of sulfonates and sulfate esters in Gram-negative bacteria. FEMS Microbiol Rev 24:135–175

Kertesz MA, Mirleau P (2004) The role of soil microbes in plant sulphur nutrition. J Exp Bot 55:1939–1945. https://doi.org/10.1093/jxb/erh176

Kertesz MA, Fellows E, Schmalenberger A (2007) Rhizobacteria and plant sulfur supply. Adv Appl Microbiol 62:235–268

Keshavarz Zarjani J, Aliasgharzad N, Oustan S, Emadi M, Ahmadi A (2013) Isolation and characterization of potassium solubilizing bacteria in some Iranian soils. Arch Agron Soil Sci 59:1713–1723

Khalid A, Arshad M, Zahir ZA (2004) Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. J Appl Microbiol 96:473–480

Khalid A, Akhtar M, Mahmood M, Arshad M (2006) Effect of substrate-dependent microbial ethylene production on plant growth. Microbiology 75:231–236

Khalid A, Arshad M, Shaharoona B, Mahmood T (2009) Plant growth promoting rhizobacteria and sustainable agriculture. In: Microbial strategies for crop improvement. Springer, Berlin, Heidelberg, pp 133–160

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

Khan MS, Zaidi A, Wani PA (2007b) Role of phosphate-solubilizing microorganisms in sustainable agriculture—a review. Agron Sustain Dev 27:29–43. https://doi.org/10.1051/agro:2006011

Khan AA, Jilani G, Akhtar MS, Naqvi SMS, Rasheed M (2009a) Phosphorus solubilizing bacteria: occurrence, mechanisms and their role in crop production. J Agric Biol Sci 1:48–58

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

Kim K, McDonald G, Jordan D (1997) Solubilization of hydroxyapatite by Enterobacter agglomerans and cloned Escherichia coli in culture medium. Biol Fertil Soils 24:347–352

Kim KY, Jordan D, McDonald G (1998) Enterobacter agglomerans, phosphate solubilizing bacteria, and microbial activity in soil: effect of carbon sources. Soil Biol Biochem 30:995–1003

Konhauser K, Ferris F (1996) Diversity of iron and silica precipitation by microbial mats in hydrothermal waters, Iceland: Implications for Precambrian iron formations. Geology 24:323–326

Kumar A, Bahadur I, Maurya B, Raghuwanshi R, Meena V, Singh D, Dixit J (2015) Does a plant growth promoting rhizobacteria enhance agricultural sustainability. J Pure Appl Microbiol 9:715–724

Kumar KV, Singh N, Behl H, 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

Kumar KV, Srivastava S, Singh N, Behl H (2009) Role of metal resistant plant growth promoting bacteria in ameliorating fly ash to the growth of Brassica juncea. J Hazard Mater 170:51–57

Kundu BS, Sangwan P, Sharma PK, Nandwal AS (1997) Response of pearl millet to phytohormones produced by Azospirillum brasilense. Indian J Plant Physiol 2:101–104

Kuzyakov Y, Xu X (2013) Competition between roots and microorganisms for nitrogen: mechanisms and ecological relevance. New Phytol 198(3):656–669

Lambrecht M, Okon Y, Broek AV, Vanderleyden J (2000) Indole-3-acetic acid: a reciprocal signalling molecule in bacteria–plant interactions. Trends Microbiol 8:298–300

Latour X, Delorme S, Mirleau P, Lemanceau P (2009) Identification of traits implicated in the rhizosphere competence of fluorescent pseudomonads: description of a strategy based on population and model strain studies. In: Lichtfouse E, Navarrete M, Debaeke P, Véronique S, Alberola C (eds) Sustainable agriculture. Springer Netherlands, Dordrecht, pp 285–296. https://doi.org/10.1007/978-90-481-2666-8_19

Lemanceau P, Expert D, Gaymard F, Bakker PAHM, Briat JF (2009) Chapter 12 Role of iron in plant–microbe interactions. In: Advances in botanical research, vol vol. 51. Academic Press, pp 491–549. https://doi.org/10.1016/S0065-2296(09)51012-9

Leustek T, Martin MN, Bick J-A, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Biol 51:141–165

Leyval C, Berthelin J (1989) Interactions between Laccaria laccata, Agrobacterium radiobacter and beech roots: Influence on P, K, Mg, and Fe mobilization from minerals and plant growth. Plant and Soil 117:103–110

Li K, Ramakrishna W (2011) Effect of multiple metal resistant bacteria from contaminated lake sediments on metal accumulation and plant growth. J Hazard Mater 189:531–539

Li F, Li S, Yang Y, Cheng L (2006) Advances in the study of weathering products of primary silicate minerals, exemplified by mica and feldspar. Acta Petrol Mineral 25:440–448

Lian B, Fu P, Mo D, Liu C (2002) A comprehensive review of the mechanism of potassium releasing by silicate bacteria. Acta Mineral Sin 22:179–183

Liermann LJ, Kalinowski BE, Brantley SL, Ferry JG (2000) Role of bacterial siderophores in dissolution of hornblende. Geochim Cosmochim Acta 64:587–602

Lin Q-M, Rao Z-H, Sun Y-X, Yao J, Xing L-J (2002) Identification and practical application of silicate-dissolving bacteria. Agric Sci China 1:81–85

Liu D, Lian B, Dong H (2012) Isolation of Paenibacillus sp. and assessment of its potential for enhancing mineral weathering. Geomicrobiol J 29:413–421

Liu W, Xu X, Wu X, Yang Q, Luo Y, Christie P (2006) Decomposition of silicate minerals by Bacillus mucilaginosus in liquid culture. Environ Geochem Health 28:133–140

Lugtenberg B (2015) Life of microbes in the rhizosphere. In: Lugtenberg B (ed) Principles of plant-microbe interactions: microbes for sustainable agriculture. Springer International Publishing, Cham, pp 7–15. https://doi.org/10.1007/978-3-319-08575-3_3

Lugtenberg B, Kamilova F (2009) Plant-growth-promoting rhizobacteria. Annu Rev Microbiol 63:541–556

Lynch JP (2007) Turner review no. 14. Roots of the second green revolution. Aust J Bot 55:493–512

Ma Z, Baskin TI, Brown KM, Lynch JP (2003) Regulation of root elongation under phosphorus stress involves changes in ethylene responsiveness. Plant Physiol 131:1381–1390

Ma Y, Rajkumar M, Vicente J, Freitas H (2010) Inoculation of Ni-resistant plant growth promoting bacterium Psychrobacter sp. strain SRS8 for the improvement of nickel phytoextraction by energy crops. Int J Phytoremediation 13:126–139

Marschner H, Rimmington G (1988) Mineral nutrition of higher plants. Plant Cell Environ 11:147–148

Masciarelli O, Llanes A, Luna V (2014) A new PGPR co-inoculated with Bradyrhizobium japonicum enhances soybean nodulation. Microbiol Res 169:609–615

Maurya B, Meena VS, Meena O (2014) Influence of Inceptisol and Alfisol's potassium solubilizing bacteria (KSB) isolates on release of K from Waste mica. Vegetos 27:181–187

Mayak S, Tirosh T, Glick BR (2004) Plant growth-promoting bacteria that confer resistance to water stress in tomatoes and peppers. Plant Sci 166:525–530. https://doi.org/10.1016/j.plantsci.2003.10.025

Meena VS, Maurya BR, Verma JP (2014) Does a rhizospheric microorganism enhance K+ availability in agricultural soils? Microbiol Res 169:337–347

Meena VS, Maurya BR, Bahadur I (2015) Potassium solubilization by bacterial strain in waste mica. Bangladesh J Bot 43:235–237

Meena VS, Bahadur I, Maurya BR, Kumar A, Meena RK, Meena SK, Verma JP (2016) Potassium-solubilizing microorganism in evergreen agriculture: an overview. In: Potassium solubilizing microorganisms for sustainable agriculture. Springer, pp 1–20
Mengel K, Kirkby E (2001) Principles of plant nutrition, 5th edn. Kluwer Academic Publishers, Dordrecht, The Netherlands
Mills HAJJ et al. (1996) Plant analysis handbook II: a practical preparation, analysis, and interpretation guide. Potash and Phosphate Institute
Miransari M (2011) Hyperaccumulators, arbuscular mycorrhizal fungi and stress of heavy metals. Biotechnol Adv 29:645–653
Misra N, Gupta G, Jha PN (2012) Assessment of mineral phosphate-solubilizing properties and molecular characterization of zinc-tolerant bacteria. J Basic Microbiol 52:549–558
Molla AH, Shamsuddin ZH, Halimi MS, Morziah M, Puteh AB (2001) Potential for enhancement of root growth and nodulation of soybean co-inoculated with Azospirillum and Bradyrhizobium in laboratory systems. Soil Biol Biochem 33:457–463
Mostajeran A, Amooaghaie R, Emtiazi G (2002) Root hair density and deformation of inoculated roots of wheat cultivars by Azospirillum brasilense Azospirillum/Trichoderma: the effects on dry bean and role of IAA in this phenomenon. Iranian Biol J 13:18–28
Nadeem SM, Zahir ZA, Naveed M, Arshad M (2009) Rhizobacteria containing ACC-deaminase confer salt tolerance in maize grown on salt-affected fields. Can J Microbiol 55:1302–1309
Nair A, Juwarkar AA, Singh SK (2007) Production and characterization of siderophores and its application in arsenic removal from contaminated soil. Water Air Soil Pollut 180:199–212
Nannipieri P, Giagnoni L, Landi L, Renella G (2011) Role of phosphatase enzymes in soil. In: Phosphorus in action. Springer, Heidelberg, pp 215–243
Niu YF, Chai RS, Jin GL, Wang H, Tang CX, Zhang YS (2013) Responses of root architecture development to low phosphorus availability: a review. Ann Bot 112:391–408. https://doi.org/10.1093/aob/mcs285
Okon Y, Itzigsohn R (1995) The development of Azospirillum as a commercial inoculant for improving crop yields. Biotechnol Adv 13:415–424
Olander LP, Vitousek PM (2004) Biological and geochemical sinks for phosphorus in soil from a wet tropical forest. Ecosystems 7:404–419
Ortíz-Castro R, Contreras-Cornejo HA, Macías-Rodríguez L, López-Bucio J (2009) The role of microbial signals in plant growth and development. Plant Signal Behav 4:701–712
Osmont KS, Sibout R, Hardtke CS (2007) Hidden branches: developments in root system architecture. Annu Rev Plant Biol 58:93–113
Osorio Vega NW (2007) A review on beneficial effects of rhizosphere bacteria on soil nutrient availability and plant nutrient uptake Revista Facultad Nacional de Agronomia. Medellin 60:3621–3643
O'Sullivan DJ, O'Gara F (1992) Traits of fluorescent Pseudomonas spp. involved in suppression of plant root pathogens. Microbiol Rev 56:662–676
Oves M, Khan MS, Zaidi A (2013) Chromium reducing and plant growth promoting novel strain Pseudomonas aeruginosa OSG41 enhance chickpea growth in chromium amended soils. Eur J Soil Biol 56:72–83
Pagella C, De Faveri D (2000) H 2 S gas treatment by iron bioprocess. Chem Eng Sci 55:2185–2194
Panhwar QA, Jusop S, Naher UA, Othman R, Razi MI (2013) Application of potential phosphate-solubilizing bacteria and organic acids on phosphate solubilization from phosphate rock in aerobic rice. Scientific World Journal 2013
Park MR, Baek S-H, Reyes BG, Yun SJ (2007) Overexpression of a high-affinity phosphate transporter gene from tobacco (NtPT1) enhances phosphate uptake and accumulation in transgenic rice plants. Plant and Soil 292:259–269. https://doi.org/10.1007/s11104-007-9222-8
Parker DR, Reichman SM, Crowley DE (2005) Metal chelation in the rhizosphere. Agronomy 48:57
Parks EJ, Olson GJ, Brinckman FE, Baldi F (1990) Characterization by high performance liquid chromatography (HPLC) of the solubilization of phosphorus in iron ore by a fungus. J Ind Microbiol 5:183–189

Parmar N, Dadarwal KR (1999) Stimulation of nitrogen fixation and induction of flavonoid-like compounds by rhizobacteria. J Appl Microbiol 86:36–44

Parmar P, Sindhu S (2013) Potassium solubilization by rhizosphere bacteria: influence of nutritional and environmental conditions. J Microbiol Res 3:25–31

Paul D (2012) Osmotic stress adaptations in rhizobacteria. J Basic Microbiol 52:1–10

Paul D, Sarma YR (2006) Plant growth promoting rhizobacteria (PGPR)-mediated root proliferation in black pepper (Piper nigrum L.) as evidenced through GS Root software. Arch Phytopathol Plant Protect 39:311–314. https://doi.org/10.1080/03235400500301190

Pedraza RO, Ramírez-Mata A, Xiqui ML, Baca BE (2004) Aromatic amino acid aminotransferase activity and indole-3-acetic acid production by associative nitrogen-fixing bacteria. FEMS Microbiol Lett 233:15–21

Pereira PAA, Bliss FA (1989) Selection of common bean (Phaseolus vulgaris L.) for N2 fixation at different levels of available phosphorus under field and environmentally-controlled conditions. Plant and Soil 115:75–82

Pérez-Torres C-A, López-Bucio J, Cruz-Ramírez A, Ibarra-Laclette E, Dharmasiri S, Estelle M, Herrera-Estrella L (2008) Phosphate availability alters lateral root development in Arabidopsis by modulating auxin sensitivity via a mechanism involving the TIR1 auxin receptor. Plant Cell 20:3258–3272

Petrini LE, Petrini O, Laflamme G (1989) Recovery of endophytes of Abies balsamea from needles and galls of Paradiplosis tumifex. Phytoprotection 70:97–103

Pikovskaya R (1948) Mobilization of phosphorus in soil in connection with vital activity of some microbial species. Mikrobiologiya 17:e370

Pothier JF, Wisniewski-Dye F, Weiss-Gayet M, Moenne-Loccoz Y, Prigent-Combaret C (2007) Promoter-trap identification of wheat seed extract-induced genes in the plant-growth-promoting rhizobacterium Azospirillum brasilense Sp245. Microbiology 153:3608–3622

Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MAK (2007) Stress-induced morphogenic responses: growing out of trouble? Trends Plant Sci 12:98–105

Potters G, Pasternak TP, Guisez Y, Jansen MAK (2009) Different stresses, similar morphogenic responses: integrating a plethora of pathways. Plant Cell Environ 32:158–169

Prajapati K, Modi H (2012) Isolation and characterization of potassium solubilizing bacteria from ceramic industry soil. CIBTech J Microbiol 1:8–14

Prajapati K, Sharma M, Modi H (2012) Isolation of two potassium solubilizing fungi from ceramic industry soils. Life Sci Leaflets 5:71–75

Prajapati K, Sharma MC, Modi HA (2013) Growth promoting effect of potassium solubilizing microorganisms on okra (Abelmoscus Esculantus). Int J Agric Sci Res (IJASR) 1:181–188

Rajawat M, Singh S, Singh G, Saxena A (2012) Isolation and characterization of K-solubilizing bacteria isolated from different rhizospheric soil. In: Proceeding of 53rd annual conference of association of microbiologists of India, p 124

Rajkumar M, Ae N, Prasad MNV, Freitas H (2010) Potential of siderophore-producing bacteria for improving heavy metal phytoextraction. Trends Biotechnol 28:142–149

Ramaekers L, Remans R, Rao IM, Blair MW, Vanderleyden J (2010) Strategies for improving phosphorus acquisition efficiency of crop plants. Field Crops Res 117:169–176

Ramos-Solano B, Lucas García JA, Garcia-Villaraco A, Algar E, Garcia-Cristobal J, Gutierrez Mañero FJ (2010) Siderophore and chitinase producing isolates from the rhizosphere of Nicotiana glauca Graham enhance growth and induce systemic resistance in Solanum lycopersicum L. Plant and Soil 334:189–197. https://doi.org/10.1007/s11104-010-0371-9

Rashid M, Khalil S, Ayub N, Alam S, Latif F (2004a) Organic acids production and phosphate solubilization by phosphate solubilizing microorganisms (PSM) under in vitro conditions. Pak J Biol Sci 7:187–196

Rashid N, Imanaka H, Fukui T, Atomi H, Imanaka T (2004b) Presence of a novel phosphopentomutase and a 2-deoxyribose 5-phosphate aldolase reveals a metabolic link between pentoses and central carbon metabolism in the hyperthermophilic archaeon Thermococcus kodakaraensis. J Bacteriol 186:4185–4191. https://doi.org/10.1128/jb.186.13.4185-4191.2004

Rasouli-Sadaghiani M, Malakouti MJ, Khavazi K, Miransari M (2014) Siderophore Efficacy of Fluorescent Pseudomonades Affecting Labeled Iron (59Fe) Uptake by Wheat (Triticum aestivum L.) Genotypes Differing in Fe Efficiency. In: Miransari M (ed) Use of microbes for the alleviation of soil stresses: Volume 2: Alleviation of soil stress by PGPR and Mycorrhizal fungi. Springer New York, New York, NY, pp 121–132. https://doi.org/10.1007/978-1-4939-0721-2_7

Renella G, Egamberdiyeva D, Landi L, Mench M, Nannipieri P (2006) Microbial activity and hydrolase activities during decomposition of root exudates released by an artificial root surface in Cd-contaminated soils. Soil Biol Biochem 38:702–708

Richardson AE, Simpson RJ (2011) Soil microorganisms mediating phosphorus availability update on microbial phosphorus. Plant Physiol 156:989–996

Richardson A, Pankhurst C, Doube B, Gupta V, Grace P (1994) Soil microorganisms and phosphorus availability Soil biota: management in sustainable farming systems, 50–62

Richardson AE, Barea J-M, McNeill AM, Prigent-Combaret C (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant and Soil 321:305–339

Robertson LA, Kuenen JG (2006) The colorless sulfur bacteria. In: The prokaryotes. Springer, New York, pp 985–1011

Robin A, Vansuyt G, Hinsinger P, Meyer JM, Briat J-F, Lemanceau P (2008) Iron dynamics in the rhizosphere: consequences for plant health and nutrition. Adv Agron 99:183–225

Rodriguez H, Gonzalez T, Goire I, Bashan Y (2004) Gluconic acid production and phosphate solubilization by the plant growth-promoting bacterium Azospirillum spp. Naturwissenschaften 91:552–555

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

Römheld V, Kirkby EA (2010) Research on potassium in agriculture: needs and prospects. Plant and Soil 335:155–180

Rosenqvist J, Kilpatrick AD, Yardley BW (2014) Rochelle CA dissolution of K-feldspar at CO_2-saturated conditions. In: EGU General Assembly Conference Abstracts, p 10909

Ryan RP, Germaine K, Franks A, Ryan DJ, Dowling DN (2008) Bacterial endophytes: recent developments and applications. FEMS Microbiol Lett 278:1–9

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

Saharan B (2011) Plant growth promoting rhizobacteria: a critical review. Life Sciences and Medicine Research

Saleem M, Arshad M, Hussain S, Bhatti AS (2007) Perspective of plant growth promoting rhizobacteria (PGPR) containing ACC deaminase in stress agriculture. J Ind Microbiol Biotechnol 34:635–648. https://doi.org/10.1007/s10295-007-0240-6

Sandip B, Subrata P, Swati RG (2011) Isolation and characterization of plant growth promoting Bacillus Thuringiensis from agricultural soil of West Bengal. Res J Biotechnol 6:9–13

Sangeeth KP, Bhai RS, Srinivasan V (2012) Paenibacillus glucanolyticus, a promising potassium solubilizing bacterium isolated from black pepper (Piper nigrum L.) rhizosphere. J Spices Aromat Crops 21

Santi C, Bogusz D, Franche C (2013) Biological nitrogen fixation in non-legume plants. Ann Bot 111:743–767. https://doi.org/10.1093/aob/mct048

Saravanan V, Madhaiyan M, Thangaraju M (2007) Solubilization of zinc compounds by the diazotrophic, plant growth promoting bacterium Gluconacetobacter diazotrophicus. Chemosphere 66:1794–1798

Saxena A, Tilak K (1998) Free-living nitrogen fixers: Its role in crop production Microbes for Health, Wealth and Sustainable Environment, Malhotra Publ Co, New Delhi Edited by Verma AK: 25-64

Schalk IJ, Hannauer M, Braud A (2011) New roles for bacterial siderophores in metal transport and tolerance. Environ Microbiol 13:2844–2854

Schmidt W (2003) Iron solutions: acquisition strategies and signaling pathways in plants. Trends Plant Sci 8:188–193. https://doi.org/10.1016/S1360-1385(03)00048-7

Seeling B, Zasoski RJ (1993) Microbial effects in maintaining organic and inorganic solution phosphorus concentrations in a grassland topsoil. Plant and Soil 148:277–284

Sevilla M, Gunapala N, Burris R, Kennedy C (2001) Comparison of benefit to sugarcane plant growth and 15N2 incorporation following inoculation of sterile plants with Acetobacter diazotrophicus wild-type and nif mutant strains. Mol Plant Microbe Interact 14:358–366

Shaharoona B, Jamro G, Zahir Z, Arshad M, Memon K (2007) Effectiveness of Various Pseudomonas spp. and Burkholderia caryophylli Containing ACC-Deaminase for Improving Growth and Yield of Wheat (Triticum aestivum I.). J Microbiol Biotechnol 17:1300

Sharma A, Johri BN, Sharma AK, Glick BR (2003) Plant growth-promoting bacterium Pseudomonas sp. strain GRP 3 influences iron acquisition in mung bean (Vigna radiata L. Wilczek). Soil Biol Biochem 35:887–894

Sharma SB, Sayyed RZ, Trivedi MH, Gobi TA (2013) Phosphate solubilizing microbes: sustainable approach for managing phosphorus deficiency in agricultural soils. SpringerPlus 2:1

Sharma A, Shankhdhar D, Shankhdhar S (2016) Potassium-solubilizing microorganisms: mechanism and their role in potassium solubilization and uptake. In: Potassium solubilizing microorganisms for sustainable agriculture. Springer, Switzerland, pp 203–219

Shelobolina E, Xu H, Konishi H, Kukkadapu R, Wu T, Blöthe M, Roden E (2012) Microbial lithotrophic oxidation of structural Fe(II) in biotite. Appl Environ Microbiol 78:5746–5752. https://doi.org/10.1128/AEM.01034-12

Sheng XF (2005) Growth promotion and increased potassium uptake of cotton and rape by a potassium releasing strain of Bacillus edaphicus. Soil Biol Biochem 37:1918–1922

Sheng XF, He LY (2006) Solubilization of potassium-bearing minerals by a wild-type strain of Bacillus edaphicus and its mutants and increased potassium uptake by wheat. Can J Microbiol 52:66–72

Sheng X, Huang W (2001) Mechanism of potassium release from feldspar affected by the sprain Nbt of silicate bacterium. Acta Pedol Sin 39:863–871

Sheng XF, Zhao F, He LY, Qiu G, Chen L (2008) Isolation and characterization of silicate mineral-solubilizing Bacillus globisporus Q12 from the surfaces of weathered feldspar. Can J Microbiol 54:1064–1068

Shweta B, Maheshwari DK, Dubey RC, Arora DS, Bajpai VK, Kang SC (2008) Beneficial effects of fluorescent pseudomonads on seed germination, growth promotion, and suppression of charcoal rot in groundnut (Arachis hypogea L.). J Microbiol Biotechnol 18:1578–1583

Siebner-Freibach H, Hadar Y, Chen Y (2003) Siderophores sorbed on Ca-montmorillonite as an iron source for plants. Plant and Soil 251:115–124

Sindhu SS, Parmar P, Phour M (2014a) Nutrient cycling: potassium solubilization by microorganisms and improvement of crop growth. In: Geomicrobiology and biogeochemistry. Springer, Berlin, pp 175–198

Sindhu SS, Phour M, Choudhary SR, Chaudhary D (2014b) Phosphorus cycling: prospects of using rhizosphere microorganisms for improving phosphorus nutrition of plants. In: Geomicrobiology and biogeochemistry. Springer, Berlin, pp 199–237

Sindhu SS, Parmar P, Phour M, Sehrawat A (2016) Potassium-solubilizing microorganisms (KSMs) and its effect on plant growth improvement. In: Potassium solubilizing microorganisms for sustainable agriculture. Springer, New Delhi, pp 171–185

Singh AK, Cameotra SS (2013) Rhamnolipids production by multi-metal-resistant and plant-growth-promoting rhizobacteria. Appl Biochem Biotechnol 170:1038–1056

Singh JS, Pandey VC, Singh D (2011) Efficient soil microorganisms: a new dimension for sustainable agriculture and environmental development Agriculture. Ecosyst Environ 140:339–353

Singh Y, Ramteke P, Shukla PK (2013) Isolation and characterization of heavy metal resistant Pseudomonas spp. and their plant growth promoting activities. Adv Appl Sci Res 4:269–272

Singh NP, Singh RK, Meena VS, Meena RK (2015) Can we use Maize (Zea mays) Rhizobacteria as Plant Growth Promoter? Vegetos-An Int J Plant Res 28:86–99

Spaepen S, Vanderleyden J, Remans R (2007) Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol Rev 31:425–448

Spaepen S, Dobbelaere S, Croonenborghs A, Vanderleyden J (2008) Effects of Azospirillum brasilense indole-3-acetic acid production on inoculated wheat plants. Plant Soil 312:15–23. https://doi.org/10.1007/s11104-008-9560-1

Sparks DL (1987) Potassium dynamics in soils. In: Advances in soil science. Springer, New York, pp 1–63

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

Stamford NP, Santos PRD, Moura AMMFD, Freitas ADSS (2003) Biofertilizers with natural phosphate, sulphur and Acidithiobacillus in a soil with low available P. Sci Agricola 60:767–773

Stearns JC, Shah S, Greenberg BM, Dixon DG, Glick BR (2005) Tolerance of transgenic canola expressing 1-aminocyclopropane-1-carboxylic acid deaminase to growth inhibition by nickel. Plant Physiol Biochem 43:701–708

Steenhoudt O, Vanderleyden J (2000) Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol Rev 24:487–506

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

Štyriakova I, Štyriak I, Galko I, Hradil D, Bezdicka P (2003) The release of iron-bearing minerals and dissolution of feldspars by heterotrophic bacteria of Bacillus species. Ceram Silik 47:20–26

SubbaRao NS (1982) Advances in agricultural microbiology Oxford and IBH Publications Company, India: 229–305

Subhashini D, Kumar A (2014) Phosphate solubilising Streptomyces spp. obtained from the rhizosphere of Ceriops decandra of Coringa mangroves. Indian J Agric Sci:84

Sugawara M, Okazaki S, Nukui N, Ezura H, Mitsui H, Minamisawa K (2006) Rhizobitoxine modulates plant-microbe interactions by ethylene inhibition. Biotechnol Adv 24:382–388. https://doi.org/10.1016/j.biotechadv.2006.01.004

Sutherland IW (2001) Biofilm exopolysaccharides: a strong and sticky framework. Microbiology 147:3–9

Swaby R, Sperber J (1958) Phosphate dissolving micro-organisms in the rhizosphere of legumes Nutrition of the legumes (EG Hollworth, ed):289-294

Tang C, Robson AD, Dilworth MJ (1990) A split-root experiment shows that iron is required for nodule initiation in Lupinus angustifolius L. New Phytol 115:61–67

Tang K, Baskaran V, Nemati M (2009) Bacteria of the sulphur cycle: an overview of microbiology, biokinetics and their role in petroleum and mining industries. Biochem Eng J 44:73–94

Tank N, Saraf M (2009) Enhancement of plant growth and decontamination of nickel-spiked soil using PGPR. J Basic Microbiol 49:195–204

Tarafdar JC, Yadav RS, Meena SC (2001) Comparative efficiency of acid phosphatase originated from plant and fungal sources. J Plant Nutr Soil Sci 164:279–282

Taurian T et al (2010) Phosphate-solubilizing peanut associated bacteria: screening for plant growth-promoting activities. Plant and Soil 329:421–431

Theunis M (2005) IAA biosynthesis in rhizobia and its potential role in symbiosis PhD thesis, Universiteit Antwerpen

Trolove S, Hedley M, Kirk G, Bolan N, Loganathan P (2003) Progress in selected areas of rhizosphere research on P acquisition. Soil Res 41:471–499

Tsavkelova EA, Klimova SY, Cherdyntseva TA, Netrusov AI (2006) Microbial producers of plant growth stimulators and their practical use: A review. Appl Biochem Microbiol 42:117–126. https://doi.org/10.1134/S0003683806020013

Turner BL (2006) Inositol phosphates in soil: amounts, forms and significance of the phosphorylated inositol stereoisomers. In: Inositol phosphates: linking agriculture and the environment, vol 4. CAB International, Wallingford, UK, p 186

Ullman WJ, Welch SA (2002) Organic ligands and feldspar dissolution water–rock interactions, ore deposits, and environmental geochemistry: A tribute to David Crerar. Special Publication 7:3–35

Upadhyay A, Srivastava S (2010) Evaluation of multiple plant growth promoting traits of an isolate of Pseudomonas fluorescens strain Psd

Uroz S, Calvaruso C, Turpault M-P, Pierrat JC, Mustin C, Frey-Klett P (2007) Effect of the mycorrhizosphere on the genotypic and metabolic diversity of the bacterial communities involved in mineral weathering in a forest soil. Appl Environ Microbiol 73:3019–3027

Uroz S, Calvaruso C, Turpault M-P, Frey-Klett P (2009) Mineral weathering by bacteria: ecology, actors and mechanisms. Trends Microbiol 17:378–387

Urrutia MM, Beveridge TJ (1994) Formation of fine-grained metal and silicate precipitates on a bacterial surface (Bacillus subtilis). Chem Geol 116:261–280

Van Der Heijden MG, Bardgett RD, Van Straalen NM (2008) The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. Ecol Lett 11:296–310

Vandevivere P, Welch SA, Ullman WJ, Kirchman DL (1994) Enhanced dissolution of silicate minerals by bacteria at near-neutral pH. Microb Ecol 27:241–251

Vansuyt G, Robin A, Briat J-F, Curie C, Lemanceau P (2007) Iron acquisition from Fe-pyoverdine by Arabidopsis thaliana. Mol Plant Microbe Interact 20:441–447

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

Velázquez E, Silva LR, Ramírez-Bahena M-H, Peix A (2016) Diversity of Potassium-Solubilizing Microorganisms and Their Interactions with Plants. In: Meena SV, Maurya RB, Verma PJ, Meena SR (eds) Potassium solubilizing microorganisms for sustainable agriculture. Springer India, New Delhi, pp 99–110. https://doi.org/10.1007/978-81-322-2776-2_7

Veresoglou SD, Menexes G (2010) Impact of inoculation with Azospirillum spp. on growth properties and seed yield of wheat: a meta-analysis of studies in the ISI Web of Science from 1981 to 2008. Plant and Soil 337:469–480

Verma JP, Yadav J, Tiwari KN, Jaiswal DK (2014) Evaluation of plant growth promoting activities of microbial strains and their effect on growth and yield of chickpea (Cicer arietinum L.) in India. Soil Biol Biochem 70:33–37

Vessey JK (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant and Soil 255:571–586

Wainwright M (1984) Sulfur oxidation in soils. Adv Agron 37:349–396

Walker V et al (2012) Variation of secondary metabolite levels in maize seedling roots induced by inoculation with Azospirillum, Pseudomonas and Glomus consortium under field conditions. Plant and Soil 356:151–163

Wani PA, Khan MS (2010) Bacillus species enhance growth parameters of chickpea (Cicer arietinum L.) in chromium stressed soils. Food Chem Toxicol 48:3262–3267

Wang RR, Wang Q, He LY, Qiu G, Sheng XF (2015) Isolation and the interaction between a mineral-weathering Rhizobium tropici Q34 and silicate minerals. World J Microbiol Biotechnol 31:747–753

Welch S, Taunton A, Banfield J (2002) Effect of microorganisms and microbial metabolites on apatite dissolution. Geomicrobiol J 19:343–367

Wen F, VanEtten HD, Tsaprailis G, Hawes MC (2007) Extracellular proteins in pea root tip and border cell exudates. Plant Physiol 143:773–783

Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J Exp Bot 52:487–511

Whitelaw MA (1999) Growth promotion of plants inoculated with phosphate-solubilizing fungi. Adv Agron 69:99–151

Wintergerst ES, Maggini S, Hornig DH (2007) Contribution of selected vitamins and trace elements to immune function. Ann Nutr Metab 51:301–323. https://doi.org/10.1159/000107673

Wu SC, Cao ZH, Li ZG, Cheung KC, Wong 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

Xiafang S, Weiyi H (2002) Study on the conditions of potassium release by strain NBT of silicate bacteria Scientia Agricultural Sinica (China)

Xiao B, Huang Y, Tang N, Xiong L (2007) Over-expression of a LEA gene in rice improves drought resistance under the field conditions TAG Theoretical and applied genetics Theoretische und angewandte. Genetik 115:35–46. https://doi.org/10.1007/s00122-007-0538-9

Yehuda Z, Shenker M, Romheld V, Marschner H, Hadar Y, Chen Y (1996) The Role of Ligand Exchange in the Uptake of Iron from Microbial Siderophores by Gramineous Plants. Plant Physiol 112:1273–1280

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

Zahedi H (2016) Growth-promoting effect of potassium-solubilizing microorganisms on some crop species. In: Potassium solubilizing microorganisms for sustainable agriculture. Springer, New Delhi, pp 31–42

Zahir ZA, Ghani U, Naveed M, Nadeem SM, Asghar HN (2009) Comparative effectiveness of Pseudomonas and Serratia sp. containing ACC-deaminase for improving growth and yield of wheat (Triticum aestivum L.) under salt-stressed conditions. Arch Microbiol 191:415–424

Zaidi A, Khan MS, Ahemad M, Oves M, Wani P (2009) Recent advances in plant growth promotion by phosphate-solubilizing microbes. In: Microbial strategies for crop improvement. Springer, pp 23–50

Zeng X, Liu X, Tang J, Hu S, Jiang P, Li W, Xu L (2012) Characterization and Potassium-Solubilizing Ability of Bacillus Circulans Z 1–3. Adv Sci Lett 10:173–176

Zhang C, Kong F (2014) Isolation and identification of potassium-solubilizing bacteria from tobacco rhizospheric soil and their effect on tobacco plants. Appl Soil Ecol 82:18–25

Zhang A-M, Zhao G-Y, Gao T-G, Wang W, Li J, Zhang S-F, Zhu B-C (2013) Solubilization of insoluble potassium and phosphate by Paenibacillus kribensis CX-7: a soil microorganism with biological control potential. Afr J Microbiol Res 7:41–47

Zhao F, X-f S, Huang Z, He L (2008) Isolation of mineral potassium-solubilizing bacterial strains from agricultural soils in Shandong Province. Biodivers Sci 16:593–600

Zheng C-J, Tu G-q (2005) Study on the potassium dissolving ability of silicate bacteria. J Shaoguan Univ (Soc Sci) 6:025

Zhu D, Kwon S, Pignatello JJ (2005) Adsorption of single-ring organic compounds to wood charcoals prepared under different thermochemical conditions. Environ Sci Tech 39:3990–3998. https://doi.org/10.1021/es050129e

Zörb C, Senbayram M, Peiter E (2014) Potassium in agriculture–status and perspectives. J Plant Physiol 171:656–669

Zuo Y, Zhang F (2010) Soil and crop management strategies to prevent iron deficiency in crops. Plant and Soil 339:83–95. https://doi.org/10.1007/s11104-010-0566-0

10 Plant Growth and Development Under Suboptimal Light Conditions

Ravinderjit Kaur, Gaganpreet Kaur, Kashmir Singh, and Baljinder Singh

Abstract

Light regulates various processes throughout the plant life from seed germination to flowering. Photoreceptors (phytochromes and cryptochromes) sense the changes in light conditions that trigger various signaling mechanisms resulting in upregulation and downregulation of several genes and transcription factors. Therefore, genetic and physiological responses, i.e., seedling growth and development, skotomorphogenesis, photomorphogenesis, shade avoidance, and flowering, are regulated by the changes in gene expression mediated by light. Phytohormones are also involved in controlling these developmental changes. Light also plays an important role in plant defense against various pathogens by inducing the jasmonic acid and salicylic acid pathways that trigger SAR (systemic acquired response). Once the plant becomes reproductively competent, light regulates the complex process of floral initiation by activation of floral genes and flowering hormones.

Keywords

Photoreceptors · Cryptochromes · Skotomorphogenesis · Jasmonic acid

10.1 Introduction

Plants being immobile have acquired intrinsic properties to adapt themselves according to the changing environmental conditions for their survival. Various abiotic factors like wind, water, temperature, and light influence the growth and development of

Ravinderjit Kaur and Gaganpreet Kaur contributed equally to this work.

R. Kaur · G. Kaur · K. Singh · B. Singh (✉)
Department of Biotechnology, Panjab University, Chandigarh, India
e-mail: sbaljinder@pu.ac.in

© Springer Nature Singapore Pte Ltd. 2020
M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_10

plants. Light being a major environmental factor plays a vital role in overall growth and development of plants. It is responsible for induction of massive reprogramming of gene expression in plants (Petrillo et al. 2014). The change in the quantity, quality, and duration of light leads to alteration in various basic processes in plants like seed germination, photomorphogenesis, and transition to flowering which are regulated by expression of various genes.

10.1.1 Light Quantity, Quality, and Duration

Light quantity is the intensity or concentration of sunlight which increases or decreases according to seasonal variations. Basically, light quality is the color or wavelength of light that reaches to the plant surface. Light duration is the amount of time that a plant is exposed to sunlight, i.e., photoperiodism. It mainly controls the floral development. Photoperiodic regulation is controlled by various genes which are either activated or inhibited depending upon the duration of exposure to light (Thomas 2006).

10.1.2 Phytohormones

Plant hormones like auxins, cytokinins, gibberellins, abscisic acid, ethylene, and brassinosteroids regulate the developmental translations and are crucial for growth regulations. In plants, various light responses control the changes in hormonal metabolism and distribution. Phytohormones like abscisic acid, gibberellic acid, and ethylene respond to varying light duration and thus regulate the process of seed germination (de Wit et al. 2016).

Global gene expression is altered in response to changes in light conditions. Light is perceived by photoreceptors triggering many of the biological processes in plants by affecting gene expression (Rossel et al. 2002). Cryptochromes and phytochromes are the main photoreceptors that can localize in the nucleus and control light-regulated nuclear gene expression by transducing these signals to chromatin, influencing the process of transcription, post-transcription, alternative splicing, and translation that ultimately leads to adaptive changes at the cellular and organismic levels (Petrillo et al. 2014). One of the important organelle in plants, chloroplast, can also act as a light sensor and is involved primarily in the process of photosynthesis (Godoy Herz et al. 2014).

10.2 Seed Germination

The sprouting of a seedling from a seed is the beginning of a plant life which is regulated by two major environmental factors, i.e., water and light. During unfavorable conditions, seeds are present in a dormant state where metabolic activity is very low. When ample amount of water is present in the surroundings, the seed uptakes

water by the process of imbibition and gets filled with water, which plays a vital role in the activation of various proteins and enzymes that are involved in the process of plant growth.

The seedling establishment and its further growth are regulated by phytochromes (photochromic proteins) which are light-sensitive proteins called photoreceptors. Various photoreceptor proteins of different families perceive the light spectrum ranging from near UV-B (280–315 nm) to far-red (750 nm) light (de Wit et al. 2016).

10.2.1 Phytochromes and Their Role in Seed Germination

Phytochromes are cytoplasmic serine/threonine kinases which sense both red and far-red light. It is a homodimer (MW 250 kDa) consisting of a polypeptide chain called apoprotein (MW 124 kDa) with a covalently attached linear tetrapyrrole light-absorbing pigment molecule called chromophore also called phytochromobilin via a thioether linkage to an invariant cysteine residue (Rockwell et al. 2006).

The five members of phytochrome gene family are PHYA, PHYB, PHYC, PHYD, and PHYE (Devlin and Kay 2000). These phytochromes are divided into two categories: TYPE I which is light labile and TYPE II which is light stable. TYPE I includes PHYA, which encodes for the protein phytochromeA (phyA) which mainly perceives far-red light, whereas TYPE 2 includes the PHYB, PHYC, PHYD, and PHYE, and this family of phytochromes perceive red light (Neff et al. 2000). The blue light and UV-A wavelength of the spectrum is sensed by another group of photoreceptors called cryptochromes and phototropins, whereas UVR8 photoreceptor receives UV-B light (Petrillo et al. 2014).

10.2.2 Photoreversibility

In etiolated seedlings, phytochromes are present in a red-light-absorbing form, Pr (λ_{max} = 660 nm) which upon exposure to red light gets photoconverted into far-red-light-absorbing form, Pfr (λ_{max} = 730 nm). When Pfr is exposed to far-red light, it is photoconverted to Pr. This process is known as photoreversibility. During the conversion of Pr to Pfr, both chromophore and protein moieties undergo conformational changes. The Pr chromophore undergoes a cis-trans isomerization of the double bond between C15 and C16 and rotation of C14–C15 single bond (Fig. 10.1).

Plant hormones like gibberellic acid and abscisic acid play an important role in seed germination. During seed germination and seedling establishment, gibberellins stimulate the production of α-amylase and other hydrolytic enzymes in the aleurone layer surrounding the endosperms of grains which help in the breakdown of complex food resources. It also plays a major role in stem elongation. Abscisic acid acts as an antagonist to gibberellins during seed germination thus inhibiting this process. This phytohormone also plays an important role during stress conditions and promotes the process of seed dormancy.

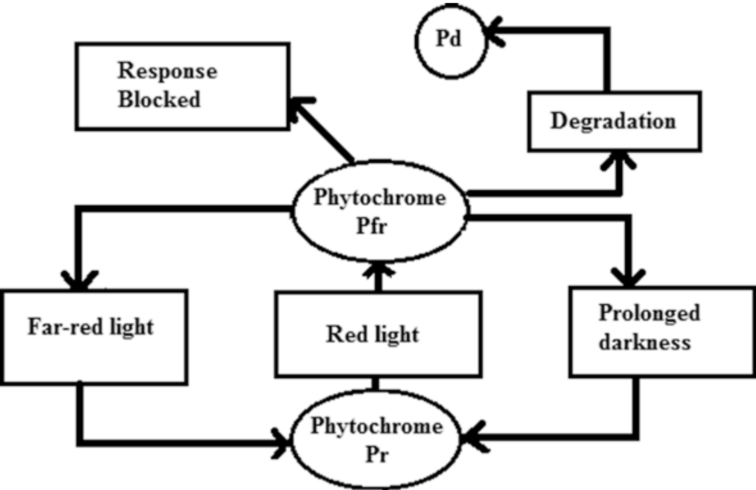


Fig. 10.1 Photoreversibility. Pr (inactive) changes to Pfr (active) upon exposure to red light, and Pfr reverts back to Pr form when exposed to far-red light. Prolonged darkness can also convert Pfr to Pr or mediate its degradation. The degraded product is referred as Pd

10.2.3 Gene Signaling Mechanism During Seed Germination

In model plant *Arabidopsis thaliana*, light-regulated germination signaling process is well understood. This process is regulated by abscisic acid that inhibits germination, whereas gibberellins promote the process of seed germination. In dark conditions, phyB resides in the cytosol in an inactivated form; thus PIF1 (phytochrome-interacting factor 1) which is a transcription factor gets accumulated in the nucleus and regulates transcription of various genes that leads to accumulation of abscisic acid, whereas the biosynthesis of gibberellic acid is inhibited.

In contrast, red light activates phyB, stabilizing its Pfr form which migrates into the nucleus. PIF1 a transcription factor interacts with phytochrome in the nucleus where its phosphorylation and degradation occur via ubiquitin ligase. Further its activity is inhibited by the heterodimer formation along with long hypocotyl in far-red 1 (HFR1). Hence, the genes involved in the process of gibberellic acid biosynthesis are activated (GA3 oxidase1 and GA3 oxidase2). Also the *CYP707A2* gene involved in abscisic acid catabolism is activated. Thus, all the events during light conditions lead to the accumulation of gibberellic acid and inhibition of abscisic acid which triggers the process of seed germination (de Wit et al. 2016) (Fig. 10.2 and Table 10.1).

Therefore, it is the light quality (red and far-red light) that regulates seed germination. We conclude that the phenomenon of seed germination is affected by varying light conditions. It is a highly complex process which is governed by the plant pigment phytochrome within the seed. Red light sensed by phytochrome induces seed germination, whereas far-red and blue light inhibits the growth and its germination. So, the amount of light either continuous or brief exposure effects the process of seed germination.

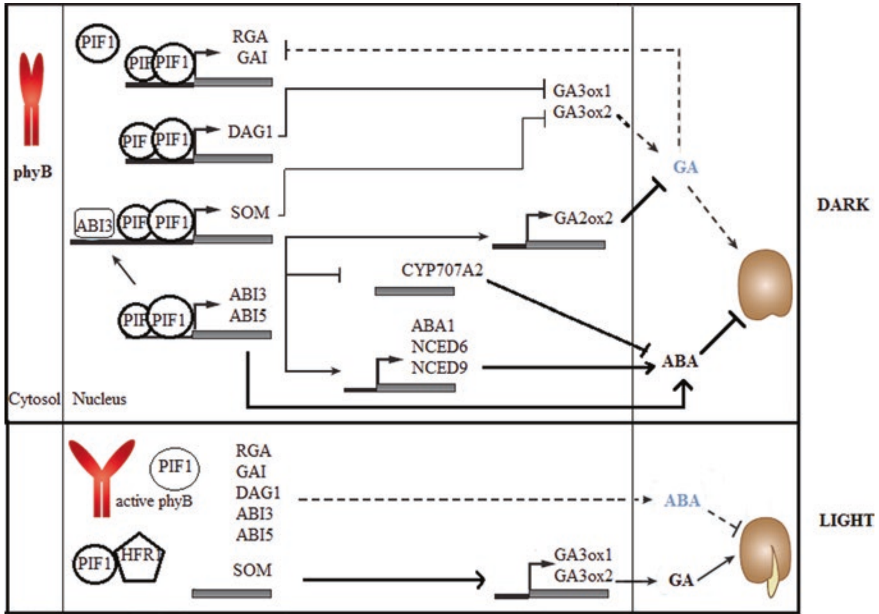


Fig. 10.2 In dark conditions, inactive phyB resides in the cytosol, due to which PIF1 is accumulated in the nucleus. Thus, genes RGA, GAI, DAG1, SOM, ABI3, and ABI5 are transcribed, as a result of which ABA is accumulated and GA biosynthesis is inhibited. Hence, the seed does not germinate in dark. In contrast, red light activates phyB and translocated into the nucleus and mediates the degradation PIF1. HFR1 forms a heterodimer with PIF1, which lowers down its activity further lowering the levels of ABA concentration and accumulation of GA concentrations. Hence, seed germination takes place in light

10.3 Seedling Growth and Development

The growth and development of the seedling start with the process of skotomorphogenesis followed by photomorphogenesis. During these developmental changes, seedling emerges from the seed and reaches the soil surface.

10.3.1 Skotomorphogenesis

After the process of seed germination has occurred, the process of etiolated growth of the seedling, i.e., skotomorphogenesis (growth in the dark), begins from the buried seed towards the soil surface in the upward direction (Toledo-Ortiz et al. 2010). In this process, hypocotyl elongation occurs in the dark at a very fast rate, such that its tip reaches the soil surface where it is exposed to light before the seed resources are exhausted. The cotyledons in the dark are tightly closed and are underdeveloped. The energy is derived from the reserve food material present in the seed as the photosynthetic machinery is inactive in this stage. The etioplasts are undifferentiated in

Table 10.1 Genes involved in seed germination in *Arabidopsis thaliana* induced by light via phytochrome B (phyB)

Genes involved in seed germination in *Arabidopsis*	Hormone effected	Gene function
RGA (Repressor of GAI-3)	Gibberellic acid (GA)	Inhibition of GA biosynthesis
GAI (Gibberellic acid insensitive)	Gibberellic acid (GA)	Inhibition of GA biosynthesis
DAG (DOF affected germination)	Gibberellic acid (GA)	Inhibition of GA3ox1 and GAox2
SOM (Somnus)	Gibberellic acid (GA)	Inhibition of GA3ox1 and GAox2
GA3ox1 (Gibberellin3-oxidase 1)	Gibberellic acid (GA)	Activation of GA biosynthesis
GA3ox2 (Gibberellin3-oxidase 2)	Gibberellic acid (GA)	Activation of GA biosynthesis
GA2ox2 (Gibberellin2-oxidase 2)	Gibberellic acid (GA)	Inhibition of GA biosynthesis
ABI3 (Abscisic acid insensitive 3)	Abscisic acid (ABA)	Activation of ABA biosynthesis
ABI5 (Abscisic acid insensitive 5)	Abscisic acid (ABA)	Activation of ABA biosynthesis
ABA1 (Abscisic acid 1)	Abscisic acid (ABA)	Activation of ABA biosynthesis
NCED6 (9-cis-epoxycarotenoid dioxygenase 6)	Abscisic acid (ABA)	Activation of ABA biosynthesis
NCED9 (9-cis-epoxycarotenoid dioxygenase 9)	Abscisic acid (ABA)	Activation of ABA biosynthesis
CYP707A2	Abscisic acid (ABA)	Inhibition of ABA synthesis (catabolism of ABA)

which the precursor of chlorophyll, protochlorophyllide, accumulates which gives it a yellowish appearance.

This type of etiolated growth, i.e., growth in the absence of light, is governed by various transcription regulators which regulate the expression of various genes. Most of them are bHLH TFs. Among these, HY5 plays an important role in hypocotyl elongation. In the absence of light, HY5 is degraded by ubiquitinylation via COP1 as HY5 is a negative regulator of hypocotyl elongation; as a result, the absence of HY5 leads to hypocotyl elongation. COP1 also degrades HFR1 (long hypocotyl in far-red 1) in the dark, which is an atypical bHLH TF and is involved in phytochrome- and cryptochrome-dependent signal transduction (Mancini et al. 2016; Toledo-Ortiz et al. 2014).

PIFs (phytochrome-interacting factors) on the other hand indirectly regulate hypocotyl elongation by degrading PhyB. Hormones involved in etiolated growth conditions are auxins, cytokinins, ethylene, gibberellins, and brassinosteroids. Gibberellins destabilize DELLA protein (growth repressing transcription regulator) and thus lead to hypocotyl elongation (Achard et al. 2004). Thus, the process of

hypocotyl elongation is a characteristic of skotomorphogenesis, i.e., the growth of a seedling under the soil in the dark conditions.

10.3.2 Photomorphogenesis

Once the seedling has emerged out from the soil, further development is mediated by light which has been termed as photomorphogenesis (Godoy Herz et al. 2014; Toledo-Ortiz et al. 2010; Arsovski et al. 2012). After germination, the light-grown seedlings have a different morphology than those grown in the dark, as seedlings in the dark conditions do not express light-inducible genes. Upon exposure to light, the light-responsive genes are induced; therefore the seedling undergoes rapid light-mediated morphological changes.

In the presence of light, transcription regulators HY5 and HFR1 are accumulated in the nucleus because their degradation by COP1-mediated ubiquitin ligase is prevented, as a result of which the elongation of hypocotyls is inhibited. Upon exposure to light, the underdeveloped cotyledons start expanding; thus the light capturing surface is increased. The process of de-etiolation begins with the greening of cotyledons in which chloroplasts start accumulating the chlorophyll pigment. Hence, the green chloroplasts are photosynthetically active, and therefore now the energy is derived by the process of photosynthesis which is utilized for further growth and development.

10.4 Shade Avoidance

Once the seedling becomes a photoautotroph, both the external and the internal environments regulate the plant growth and help it to enter in the juvenile phase. Many factors like pathogens and shade due to neighboring vegetation (canopy) act as obstacles during this phase transition. Light is often a limiting factor in dense forests where canopy blocks the light from reaching the plants growing below it. These plants hence respond by the phenomenon of shade avoidance, i.e., changes which occur in response to the enrichment of far-red light under a leaf canopy (Casal 2012; Franklin and Whitelam 2004). These varying light conditions are sensed by phytochromes, and such responses include elevation of leaf angles (hyponasty), enhanced hypocotyls and petiole growth, early flowering, abundant PIF4 and PIF5 proteins, degradation of DELLA proteins, etc. collectively known as shade avoidance syndrome (Leivar et al. 2008). The R:FR ratio decreases in the shade conditions leading to alterations in the growth of the plant.

10.5 Role of Light in Plant Defense

In the initial phase of growth, the plant utilizes most of the energy fixed by the process of photosynthesis for its growth. When surplus amounts of carbohydrates are available, they are converted into secondary metabolites like terpenes, alkaloids, nitrogen-containing compounds, etc., which play an important role in plant defense.

Thus, in growing plants, these compounds are produced in a very less amount because the major proportion of carbon fixed is diverted for its growth and development. Due to the lower levels of secondary metabolites in the new emerging seedling, the growing plant is vulnerable to attack by pathogens. Thus, in this phase of growth, the plant needs to grow at a very fast rate in order to compete with its neighboring plants and also needs to defend itself from the microbial pathogens (Ballare 2014). As the plant is still growing in its juvenile phase, the intact barrier consisting of the bark or waxy cuticle is not available as a first line of defense to protect the plant against the microbial attack, as a result of which, the pathogen can easily enter inside the plant body.

Light sensed by the phytochrome B (phyB) photoreceptor induces signal transduction pathways that lead to the production of various compounds like jasmonic acid, salicylic acid, etc., which are involved in plant defense mechanism. These compounds trigger the induction of **SAR** (systemic acquired response) (Bolton 2009; Ryals et al. 1996). Hence, if the plant is growing under suboptimal conditions, i.e., shade (low R:FR), it leads to inactivation of phyB that downregulates the jasmonic acid and salicylic acid signaling.

10.6 Light-Mediated Floral Induction

With time, various developmental changes cause alterations in the plant growth due to which the plant enters from juvenile stage to a mature adult plant. In juvenile phase, vegetative meristem does not respond to internal and external signals that initiate flowering, i.e., incompetence. Under internal optimal conditions, the developmental changes allow the plant to become reproductively competent. These conditions include factors like plant size, number of vegetative nodes, and amount of sucrose, the main energy source which fuels the plant to begin the complex process of floral initiation. Along with these factors, hormones such as gibberellic acid, cytokinins, and "florigen" also known as the flowering hormone play a major role in gaining internal competence (Corbesier and Coupland 2006). Once the plant becomes internally competent to reproduce, external environmental conditions like light (photoperiod, light quality, and quantity), temperature (vernalization, i.e., exposure to long cold conditions), nutrient, and water availability determine the process of floral induction.

10.6.1 Photoperiodism

Photoperiodism plays a crucial role in regulating floral development. Different plants respond to light conditions depending upon the duration of light exposure to that respective plant. Based on this condition, these plants are divided into three basic categories, namely short-day plants, long-day plants, and day-neutral plants. The short-day plants are those in which floral initiation takes place when exposure to light is less than the critical duration, for example chrysanthemum and soybeans. These plants usually flower in late summer. The long-day plants require exposure to light for a period more than a well-defined critical duration, i.e., these plants require a long duration of light exposure, for example lettuce and spinach. These plants normally flower in spring and early summer. The third category includes day-neutral plants in which there is no relation between photoperiodism and floral induction. Initiation of flowering occurs independent of light duration. For example, *Sorghastrum nutans* commonly known as Indian grass is a day-neutral plant (Lumsden 2002; Andreas and Coupland 2012).

The shoot apical meristem (SAM) in the initial phases of plant growth leads to the formation of vegetative organs like leaves, but once the plant becomes internally competent to reproduce and all the external conditions are favorable and optimal, SAM makes transition to the reproductive development, and the production of flower is initiated (Benlloch et al. 2007). These changes are mediated by signals that turn on various genes that lead to morphological changes specifying the location of floral organs like sepals, petals, stamens, and carpels.

10.6.2 Photoreceptor Proteins Regulating Floral Formation

Various photoreceptor proteins are present in the leaf of the plant which play a vital role in the process of gene activation that leads to the initiation of flowering. In *Arabidopsis thaliana*, a facultative long-day plant, the far-red and blue light is perceived by phytochrome (phyA) and cryptochrome (cry1 and cry2) (Zuo et al. 2011) that promote floral initiation, whereas the red light perceived by phyB inhibits flowering. Florigen (flowering hormone) production takes place in leaves which is regulated by the duration of light exposure, i.e., photoperiodism, and later on this signaling hormone is translocated via phloem to SAM where the process of flower formation is induced (Tsuji and Taoka 2014).

10.6.3 Genes Involved in Floral Formation

As a result of the abovementioned events, various genes are activated that initiate the process of flowering. These genes are divided into two groups, namely floral meristem identity genes that are responsible for the transition of vegetative meristem to floral meristem, and they include *AGAMOUS-LIKE 20, LEAFY, APETALA1 (AP1),* etc. The second group is floral organ identity genes or floral homeotic genes

that lead to the formation of the floral parts, and these include *APETALA2 (AP2), APETALA3 (AP3)/PISTILATA (PI),* and *AGAMOUS (AG).* These two groups of genes act in a sequential manner to initiate the process of flowering. The signaling hormone "florigen" is responsible for the activation of *CONSTANS* gene which is expressed in long days, and it encodes a transcription factor that further initiates the signaling cascade by activating *AGAMOUS-LIKE* 20, a floral meristem identity gene (Achard et al. 2007). It further activates *LEAFY* which is the most important gene for the production of flower. LEAFY in turn activates floral homeotic genes that are responsible for the formation of various floral organs (Benlloch et al. 2007; Balasubramanian et al. 2006). *AGAMOUS-LIKE* 20 is inhibited by another gene *FLOWERING LOCUS C*. Therefore, to initiate the process of floral induction, this inhibition needs to be relieved which is done upon exposure to low-temperature conditions, i.e., the process of vernalization (Figs. 10.3 and 10.4).

Along with low temperature, gibberellins also play an important role in controlling this complex process of flowering. Thus, in most of the plants, both light and temperature play a very important role in controlling the rate of flowering.

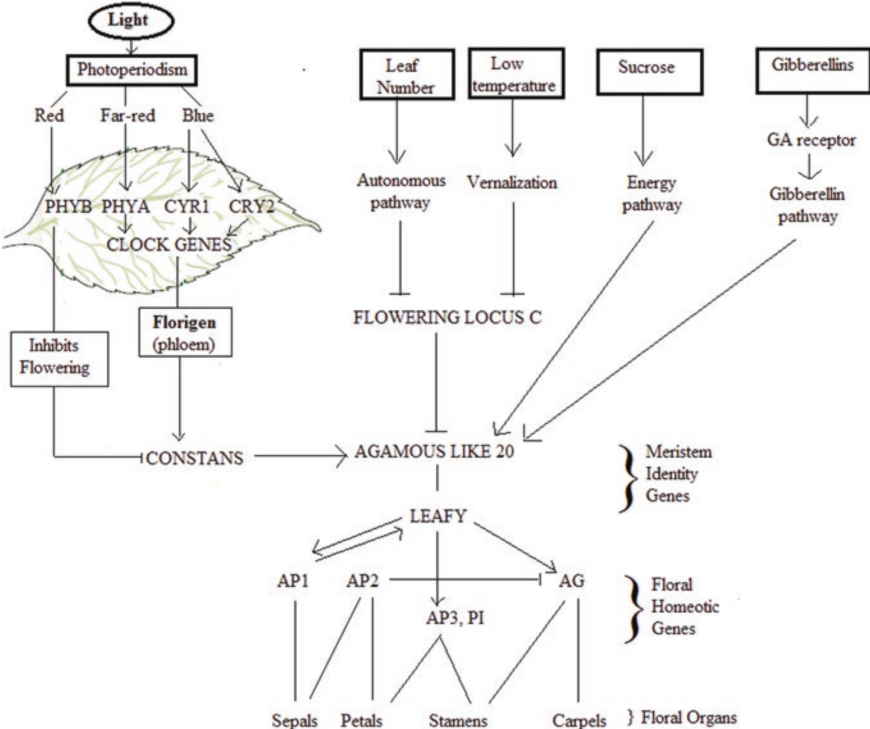


Fig. 10.3 Various genes are activated or inhibited by different floral developmental processes including photoperiodism, autonomous/vernalization pathway, energy pathway, and gibberellin pathway that eventually lead to the production of various floral organs

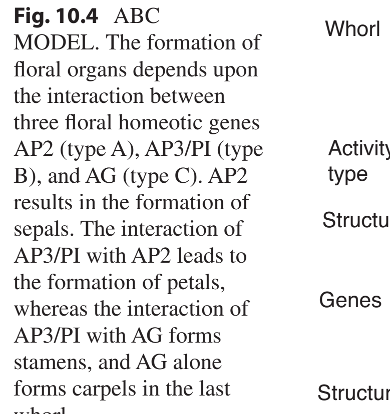
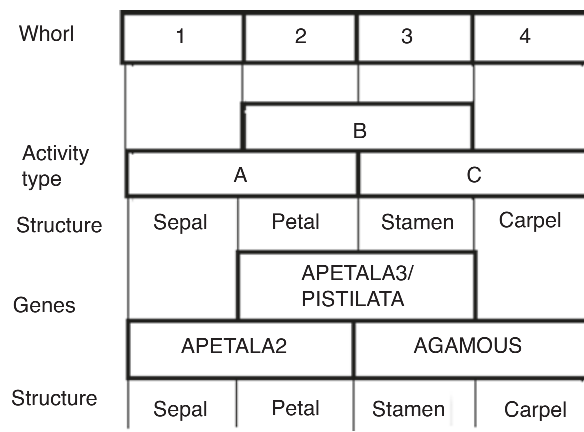


Fig. 10.4 ABC MODEL. The formation of floral organs depends upon the interaction between three floral homeotic genes AP2 (type A), AP3/PI (type B), and AG (type C). AP2 results in the formation of sepals. The interaction of AP3/PI with AP2 leads to the formation of petals, whereas the interaction of AP3/PI with AG forms stamens, and AG alone forms carpels in the last whorl



10.7 Summary

Light plays a very important role in overall growth and development of a plant starting from the seed germination till it becomes a full-fledged adult reproductive plant. Plants respond to the external light conditions via photoreceptors. Phytochromes and cryptochromes are the main photoreceptors that sense red/far-red and blue light, respectively, and trigger various signaling cascades, as a result of which, various metabolic processes are either initiated or repressed. The processes like seed germination, seedling growth and development, formation of secondary metabolites, and floral initiation are the most important events in the plant life. Light basically regulates various phytohormones like auxins, gibberellins, abscisic acid, ethylene, cytokinins, brassinosteroids, etc. which in turn regulate the overall growth and development of the plant. Photoperiodism and vernalization are the two main processes that control the process of flower formation in adult plants. Light perceived by photoreceptors upregulates and downregulates the transcription of various genes which are involved in the plant developmental process. Thus, without light, there is no life.

References

Achard P, Herr A, Baulcombe DC, Harberd NP (2004) Modulation of floral development by a gibberellins-regulated microRNA. Development 131:3357–3365. https://doi.org/10.1242/dev.01206

Achard P, Baghour M, Chapple A, Hedden P, Straeten DVD, Genschi KP, Moritz T, Harberd NP (2007) The plant stress hormone ethylene controls floral transition via DELLA-dependent regulation of floral meristem-identity genes. PNAS 104:6484–6489. https://doi.org/10.1073/pnas.0610717104

Andreas F, Coupland G (2012) The genetic basis of flowering responses to seasonal cues. Nat Rev Genet 13:627–639. https://doi.org/10.1038/nrg3291

Arsovski AA, Galstyan A, Guseman JM, Nemhauser JL (2012) Photomorphogenesis: January 31, 2012. The Arabidopsis book, doi: https://doi.org/10.1199/tab.0147. American Society of Plant Biologists

Balasubramanian S, Sureshkumar S, Lempe J, Weigel D (2006) Potent induction of *Arabidopsis thaliana* flowering by elevated growth temperature. PLoS Genet 2(7):e106. https://doi.org/10.1371/journal.pgen.0020106

Ballare CL (2014) Light regulation of plant defense. Annu Rev Plant Biol 65:335–363. https://doi.org/10.1146/annurev-arplant-050213-040145

Benlloch R, Berbel A, Serrano-Mislata A, Madueno F (2007) Floral initiation and inflorescence architecture: a comparative view. Ann Bot 100:659–676. https://doi.org/10.1093/aob/mcm146

Bolton MD (2009) Primary metabolism and plant defense—fuel for the fire. MPMI 22:487–497. https://doi.org/10.1097/MPMI-22-5-0487

Casal JJ (2012) Shade avoidance: January 19, 2012. The Arabidopsis Book. American Society of Plant Biologists. https://doi.org/10.1199/tab.0157

Corbesier L, Coupland G (2006) The quest for florigen: a review of recent progress. J Exp Bot 57:3395–3403. https://doi.org/10.1093/jxb/erl095

de Wit M, Galvao VC, Fankhauser C (2016) Light mediated hormonal regulation of plant growth and development. Annu Rev Plant Biol 67:513–537. https://doi.org/10.1146/annurev-arplant-043015-112252

Devlin PF, Kay SA (2000) Cryptochromes are required for phytochrome signaling to the circadian clock but not for rhythmicity. Plant Cell 12:2499–2509. https://doi.org/10.1105/tpc.12.12.2499

Franklin KA, Whitelam GC (2004) Phytochromes and shade avoidance responses in plants. Ann Bot 96:169–175. https://doi.org/10.1093/aob/mci165

Godoy Herz MA, Kornblihtt AR, Barta A, Kalyna M, Petrillo E (2014) Shedding light on the chloroplast as a remote control of nuclear gene expression. Plant Signal Behav 9(11):e976150. https://doi.org/10.4161/15592324.2014.976150

Leivar P, Monte E, Oka Y, Liu T, Carle C, Castillon A, Huq E, Quil M (2008) Multiple phytochrome- interacting bHLH transcription factors repress premature seedling photomorphogenesis in darkness. Curr Biol 18:1815–1823. https://doi.org/10.1016/j.cub.2008.10.058

Lumsden PJ (2002) Photoperiodism in plants. Biol Rhythms:181–191. https://doi.org/10.1007/978-3-662-06085-815

Mancini E, Sanchez SE, Romanowski A, Schlaen RG, Sanchez-Lamas M, Cerdan PD, Yanovsky MJ (2016) Acute effects of light on alternative splicing in light grown plants. Phytochem Photobiol 92:126–133. https://doi.org/10.1111/php.12550

Neff MM, Fankhauser C, Chory J (2000) Light: an indicator of time and place. Genes Dev 14:257–271. https://doi.org/10.1101/gad.14.3.257

Petrillo E, Godoy Herz MA, Barta A, Kalyna M, Kornblihtt AR (2014) Let there be light: regulation of gene expression in plants. RNA Biol 11(10):1215–1220. https://doi.org/10.4161/15476286.2014.972852

Rockwell NC, Su YS, Lagarias JC (2006) Phytochrome structure and signaling mechanisms. Annu Rev Plant Biol 57:837–858. https://doi.org/10.1146/annurev.arplant.56.032604.144208

Rossel JB, Wilson IW, Pogson BJ (2002) Global changes in gene expression in response to high light in Arabidopsis. Plant Physiol 130:1109–1120. https://doi.org/10.1104/pp.005595

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996) Systemic acquired resistance. Plant Cell 8:1809–1819

Thomas B (2006) Light signaling and flowering. J Exp Bot 57:3387–3393. https://doi.org/10.1093/jxb/erl071

Toledo-Ortiz G, Huq E, Rodriguez-Concepcion M (2010) Direct regulation of phytoene synthase gene expression and carotenoid biosynthesis by phytochrome interacting factors. PNAS 107:11626–11631. https://doi.org/10.1073/pnas.0914428107

Toledo-Ortiz G, Johannson H, Lee KP, Bou-Torrent J, Stewart K, Steel G, Rodriguez-Concepcion M, Halliday KJ (2014) The HY5-PIF regulatory module coordinates light and temperature control of photosynthetic gene transcription. PLoS Genet 10(6):e1004416. https://doi.org/10.1371/journal.pgen.1004416

Tsuji H, Taoka KI (2014) Florigen signaling. Enzymes 35:1874–6047. https://doi.org/10.1016/B978-0-12-801922-1.00005-1

Zuo Z, Liu H, Liu B, Liu X, Lin C (2011) Interaction of CRY2 with SPA1 regulates COP1 activity and floral initiation in Arabidopsis. Curr Biol 21:841–847. https://doi.org/10.1016/j.cub.2013.03.048

Microbial Biotechnology: A Key to Sustainable Agriculture

11

S. K. Gosal, Jaspreet Kaur, and Jupinder Kaur

Abstract

The exploitive and improper agricultural practices lead to degradative processes such as nutrient depletion, loss in soil fertility, and soil organic matter. These processes contribute to a serious decline in soil productivity. The degraded soils can be restored and rehabilitated by alternative agricultural practices such as use of potential microbial inoculants to provide favorable environment for optimum crop production and protection. The use of bioinoculant is one of the important components of integrated nutrient management as they facilitate a cost-effective renewable source of plant nutrients which supplement chemical fertilizers contributing to sustainable agriculture. Several microorganisms are currently being marketed commercially as biofertilizers for crop plants. Unfortunately, these microorganisms are not always as efficient in the field as they are in laboratory or greenhouse experiments. The use of microbial biotechnology has manipulated the microorganism at their genetic level which leads to increase in their survival and efficiency in soil. The genetically modified microorganisms can be used as potent bioinoculants in agriculture, but their undesirable effects and ethical implications still remain a major problem whether they should be accepted or not. The presence of antibiotic resistance gene, horizontal transfer of genes, and unstable vector in modified microorganism made them unsuitable for environmental application as these characteristics can get transferred to indigenous microorganisms which lead to mutations. More intense research is required to assess the stability of genetically modified microorganism and their effect on indigenous microflora. These studies can open the way to the production of more effective, stable, and reliable recombinant inoculants for maintaining sustainable agriculture.

S. K. Gosal (✉) · J. Kaur · J. Kaur
Department of Microbiology, Punjab Agricultural University, Ludhiana, India

© Springer Nature Singapore Pte Ltd. 2020

M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_11

Keywords
Bioinoculants · Green revolution · Microbial communities · Bioinoculants

11.1 Introduction

The intensive agricultural technologies led to a "green revolution" which resulted in enhanced global pollution, unfavorable climatic changes, and loss of biodiversity. The first two decades of the "green revolution" increased the productivity of crops immensely. Afterwards, the crop productivity began to decline due to the indiscriminate use of agrochemicals along with deficiencies of essential nutrients (major and minor nutrients) in the soil. In response to this particular situation, the development of sustainable agriculture is required that results in high productivities of crop with a minimal imbalance in the environment (Noble and Ruaysoongnern 2010). The most promising strategy to reach this goal is to substitute hazardous agrochemicals with environment-friendly microbes. This biological approach is based on exploiting the role of soil microbial communities for a sustainable and healthy crop production while preserving the biosphere. The microbial communities present in the soil can be used as potential bioinoculants. Their application can improve the soil health, soil fertility as well as protect crops from biotic (pathogens, pests), and abiotic (including pollution and climatic change) stresses. Use of these microbial inoculants facilitates nutrient availability to agricultural crops which in turn increases growth as well as crop productivity.

The efficiency of bioinoculants is affected by various environmental factors which lead to uncertainty in their performance under greenhouse and field condition. The remedy to this particular situation can be "microbial biotechnology" which is a synthetic research field to exploit the genetic information of microbes to generate more efficient microbial strains. This approach can increase reliability of the bioinoculants in different environmental conditions. The regular movement of microorganisms between plant, animal, and soilborne niches is analyzed to reconstruct the microbiota in natural and agricultural ecosystems, so that the genetic information about a particular gene can be used to improve the microorganism potential. Accordingly, several strategies have been designed for effective exploitation of beneficial microbial services for creating sustainable and environmental friendly agroecosystem. In this chapter, we elucidate the application of genetic engineered microbes on the improvement in potential of microorganism by genetic modification for the specific traits and their survival in soil.

11.2 Role of Microbes in Sustainable Agriculture

The present agricultural system has faced major challenges in recent years, which include overdependence on agrochemicals, global climate change, population explosion, and increased economic and environmental costs of nonrenewable

resources. The present agricultural practices used to get higher productivity lead to major problems associated with pollution of soil, surface, and groundwater due to enhanced usage of agrochemicals. Consequently, these agrochemicals have adverse impacts on the environment, safety, and quality of food. So, there is a need to reduce the excessive use of agrochemicals (fertilizers and pesticides) in food crop production. This leads to increased interest in sustainable agriculture, which can be defined as maintenance of biological diversity, productivity, regeneration capacity, vitality, and ability of agricultural ecosystem by managing and utilizing the indigenous biological resources. The concept of sustainable agriculture emphasizes on the use of microbial inoculants in shifting the soil microbial equilibrium to get higher productivity along with the protection of crops. Microorganisms can be harnessed to produce biofertilizers, to decompose organic wastes more efficiently and combat plant-pest diseases with greater efficiency than ever before. In agricultural ecosystem, microorganisms have a critical role in transformation, mineralization, and solubilization of nutrients. The population of microorganisms in their natural habitat is often scanty, and it can be enhanced by addition of microbial inoculants with specific properties. The microbial inoculants can be represented as potential "green" alternative to the intensive use of agrochemicals (artificial fertilizers and pesticides) in agricultural systems. A wide spectrum of preparations of diverse microbial species may be used as substituent nutrients to enhance crop production and disease suppression (Andrews et al. 2010). However, nutrient substitution by microbes is usually partial and only sometimes can be complete. The balance between microbial inoculants, organic manure, and inorganic nutrition may lead to improvement in sustainable agroecosystems (Hedin et al. 2009). This integrated approach is most promising in all agricultural systems as the strong correlations between the microorganism and crops. Presence of microbes increases the nutrient availability such as nitrogen fixation and phosphorus solubilization and mobilization hence increasing the nutrient availability. Microbes also secrete secondary metabolites which include plant growth-promoting hormones, metal chelators, antibiotics, and exopolysaccharides. These secondary metabolites increase the plant growth, suppress disease incidence, and improve metal scavenging as well as improve the soil texture by increasing soil organic carbon. The improved soil texture enhances the physiochemical properties of soil such as high water-holding capacity and pH buffering. So, microbes have an important role in soil nutrient transformation, soil physicochemical properties, and consequently in maintaining sustainability in agricultural ecosystem which is lacking in the case of conventional practices (Fig. 11.1).

11.3 Role of Biotechnology in Sustainable Agriculture

Biotechnology is defined as "any technological application that uses biological systems, living organisms, or derivatives thereof, to make or modify products or processes for specific use" (UN Convention on Biological Diversity, Art. 2, 2017). By implementing genetic engineering, it is possible to get specific properties in host that cannot be produced by means of traditional method. All of the means of genome

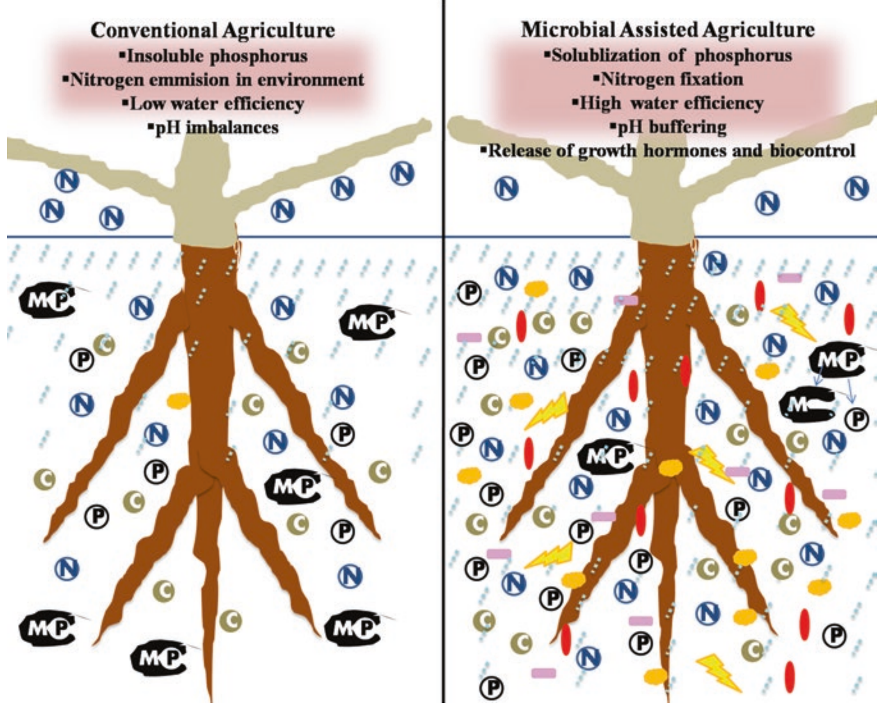


Fig. 11.1 Role of soil microorganism in nutrient transformation. ◯—available nitrogen, metal-bound insoluble phosphorus, ◯—available phosphorus, ●—organic carbon, —plant growth-promoting metabolites, and , , —rhizospheric microorganisms

manipulation used in biotechnology belong to genetic engineering and rDNA technology. The genetic engineering differs from genetic changes that occur in nature as in this it is possible to combine the characteristics of evolutionally different and unrelated species. In this way, molecular methods are replacing conventional genetic methods used in agriculture. The use of biotechnology increased food production as well as made agriculture more sustainable from an environmental point of view. Biotechnology has been contributing important role in sustainable agriculture through various ways as shown in Fig. 11.2.

Biotechnology contributes to sustainable agriculture by reducing the dependence on agrochemicals, particularly pesticides, through the employment of genetically modifications that promotes plant growth, improves nutrient availability, and increases resistance to biotic and abiotic stresses.

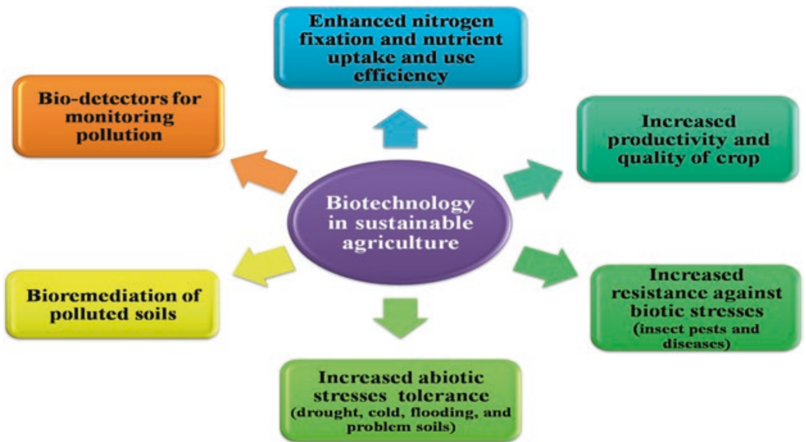


Fig. 11.2 Contribution of biotechnology in sustainable agriculture

11.4 Microbial Biotechnology and Agriculture

Soil microbes are composed of prokaryotic (eubacteria, actinomycetes, and archaea) and eukaryotic (fungi, yeasts, protozoa, and algae) organisms, whose populations vary from soil to soil. Their population in soil is influenced by variety of soil and environmental parameters including cultivar, soil texture, soil structure, soil pH, soil moisture content, soil temperature, etc. There are enormous ways in which microbes have been used over the past years. These include exploiting microbes for the benefits of agriculture, medicine industry, food industry, genetic engineering, and environmental protection. The uniqueness of microorganisms, their uncertain nature, and biosynthetic capabilities in particular set of conditions made them the potent candidates for solving various problems of agroecosystem. For increasing their biological capabilities and certainty, one of the technological advances is to increase the microbial potential by application of "biotechnology" which is an integral application of biochemistry, microbiology, and genetic engineering (Fig. 11.3).

Microbial biotechnology deals with the manipulation of microbes or their components to produce useful products for various applications in biological science. This technique permitted breakthroughs in agriculture to produce healthier foods, safer pesticides, innovative environmental technologies, and new energy sources. Microbial biotechnology can improve the agroecosystem by employing microorganisms to increase the soil nutrient content, enhance crop productivity, and enhance resistance to specific plant pathogens and to produce safer herbicides. Complete genome sequence of a microbe provides crucial information about its biology, which leads to the first step toward understanding a microbe's biological potentials and modifying them according to agricultural needs. The microbes which are genetically manipulated with gene of interest by using biotechnological techniques are referred to as genetically modified microorganism (GMM). Microbes are engineered to (1) improve expression of beneficial traits and (2) be traceable in plant and soil systems (Fig. 11.4).

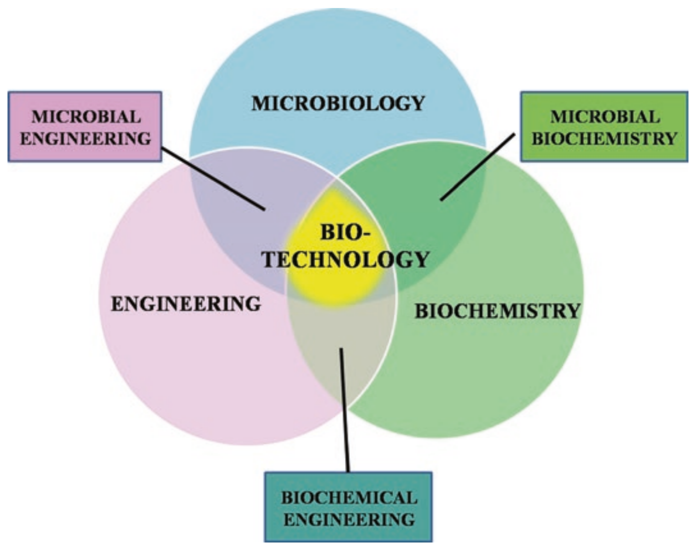


Fig. 11.3 Biotechnology of an integrated branch of various applied sciences

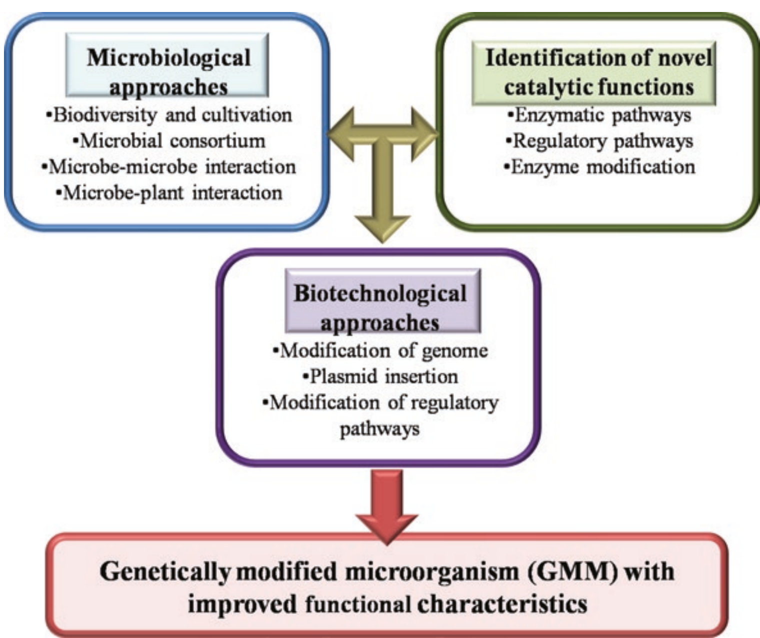


Fig. 11.4 Schematic representation to generate genetic modification in microorganism

Application of microbes in biotechnology (microbial biotechnology) allows the genomic studies, which lead to breakthrough in improved bacterial inoculants. These genetically modified bacterial inoculants are efficient in their functional characteristics such as biological control of pest, reduced virulence for pathogens, and bioremediation of environment. Therefore, generation of genetically modified microorganism by the application of biotechnology is getting better attention in advancing sustainable development of agroecosystem and environmental health.

11.5 Molecular Tools for Manipulation of Microorganisms

Genetic engineering is a modern technology which allows manipulation in genetic makeup of microorganisms to achieve planned and desired results to perform specific functions. This technique involves isolation, introduction, integration, and stable expression of the foreign gene into an unrelated organism. It offers opportunity to create artificial combination of desirable genes that do not exist together in nature. The microorganisms can be manipulated by engineering single gene or operon, modification in biochemical pathway, and alterations in the existing genes sequences (Dale and Park 2007). The microorganisms in which genetic manipulations are stable and fully expressed are referred to as genetically modified microorganisms (GMM). The steps for constructing genetically modified microorganisms are the following:

1. **Gene isolation and excision:** Insertion of exogenous gene into a non-native host requires selection and physical excision of desired DNA so that it can be transferred to host for proper gene expression. The isolation of desired DNA requires three basic steps:
   (a) **Opening the target cells:** The target cell will be disrupted using gentle methods, preferably utilizing enzymatic degradation of cell wall material (if present) and detergent-mediated lysis of cell membrane. Lysis of cells yields mixture of DNA, proteins, and lipids with other cell components.
   (b) **Separation and recovery of the DNA:** The lysed product obtained from the opening of cell will be treated with deproteinases and lipases following one or more extractions using phenol or phenol/chloroform mixtures. Then, subsequent centrifugation results into separation of the protein molecules into the phenol phase and nucleic acids in the aqueous phase. The nucleic acid (DNA and RNA) obtained in aqueous phase will be precipitated from solution using isopropanol or ethanol. For preparation of pure DNA, the nucleic acids will be further treated with enzyme ribonuclease (RNase) which digests the RNA in the preparation. If the target gene is located in the plasmid DNA, then further gradient centrifugation will be required. As an alternative to gradient centrifugation, size-exclusion chromatography (gel filtration) or similar techniques may be used.
   (c) **Excision of target gene:** Pure isolated DNA will be treated with restriction endonucleases (RE) type II to excise target gene. These enzymes hydrolyzed the sugar-phosphate backbone of DNA molecules at specific site; this

specific site is called as restriction site. Excision with the desired gene should be excised with its promoter sequences so that the genes can be expressed into the host cell.

2. **Vector preparation and ligation:** Cloning vector can be defined as "a DNA molecule originating from a virus, a plasmid, or the cell of other organism into which the desired DNA fragment of appropriate size can be integrated without the loss of its self-replication property." Vectors act as gene vehicle which introduces foreign gene into host cells, where it clones to reproduce in large quantities. There are various types of vectors which include plasmids, cosmids, yeast artificial chromosomes, transposons, etc. The selection of cloning vector depends upon various factors like the method of gene transfer, the desired outcome of the modification, and the application of the modified microorganisms (laboratory or environmental). For example, replicating vectors (plasmid vectors with high or low copy numbers) are commonly used to express the desired genes in heterologous hosts for manufacturing expressed proteins. These vectors are also used to increase the copies of gene in cell to enhance the production of the metabolite. Cosmid and yeast artificial chromosome vectors can accept DNA fragments as large as 100 kb. These are necessary to clone a large piece of DNA into host for high-level metabolite production (Sosio et al. 2000). Conjugal vectors are alternative approach to transfer the gene which is usually difficult to transform. These vectors can transfer the genes into indigenous microorganism by horizontal transfer. Many genes have been transferred by integrating the gene with transposons. Transposons can move from one genomic position to another by a cut-and-paste mechanism. They are powerful method for genetic change which can significantly modify many genomes. As genetic tools, DNA transposons can introduce a piece of foreign DNA into a genome. For example, plasmid-born catabolic genes for the degradation of toxic substances can be often located in transposons. It was reported that pUTK21 plasmid of *P. fluorescens* HK44 was made by transposon Tn*4431* insertion into NAH7 plasmid from *P. fluorescens* 5R. This transposon originated from *Vibrio fischeri* and carried *luxCDABE* gene cassette. This genes cassette was expressed under a common promoter which resulted in simultaneous degradation of naphthalene and luminescent signal.

   The desired gene can be ligated into selected vector DNA using specific enzymes called as DNA ligases. These are important cellular enzymes which seal discontinuities in the sugar-phosphate backbone chains that arise during joining the target DNA with vector DNA. The most commonly used T4 DNA ligase enzyme is purified from *E. coli* cells infected with bacteriophage T4. This enzyme works the best at 37 °C, but is often used at much lower temperatures (4–15 °C) to prevent thermal denaturation.

3. **Gene transfer method:** Microorganisms can be manipulated by transferring specific genes from one microorganism to other. Genes can be transferred by various genetic recombination methods mainly by transformation, conjugation, and transduction. The techniques of transformation and transduction represent the simplest methods available for getting recombinant DNA. In the context of cloning in *E. coli* cells, transformation refers to the uptake of plasmid DNA and

Table 11.1 Examples of gene transfer methods in microorganisms

Microorganism to modify	Gene transferred	Gene transfer method	References
P. putida mt-2 strain B13	TOL plasmid pWW0	Conjugation	
Alcaligenes sp. A7	Benzoate and phenol degrader genes	Conjugation	Schwien and Schmidt (1982)
P. putida strain B13	*xylXYZ* (toluate-1,2-dioxygenase) and *xylL*	Conjugation	Lehrbach et al. (1984)
P. putida strain B13	*nahG* (salicylate hydroxylase)	Conjugation	Lehrbach et al. (1984)
E. coli JM109	Plasmids *bphA1* (shuffled) and *bphA2A3A4BC* (KF707)	Electroporation	Kumamaru et al. (1998)
Sinorhizobium meliloti	plasmid pE43	Electro-transformation	Chen et al. (2005)
B. cepacia BU0072	pTOM-Bu61 plasmid from *B. cepacia* BU61	Conjugation	Taghavi et al. (2005)
Mycobacterium sp	Plasmid pNC950	Electroporation	Matsui et al. (2006)
P. putida KT2442, *P. stutzeri* 1317, and *A. hydrophila* 4AK4	Plasmid pKST11	Conjugation	Ouyang et al. (2007)
E. coli M15 (pREP4).	Functional *gcd*	Phage P1 mediated transduction	Tripura and Podile (2007)
E. coli S17-1	pASF101	Transformation	Menn et al. (2009)
P. protegens Pf-5	Cosmid X940	Transformation	Ayub et al. (2009)
A. vinelandii AvOP	pGDEGS1 and pGDEPS1	Transformation	Shashidhar and Rao (2009)
R. leguminosarum	*vktA* gene	Conjugation	Orikassa et al. (2010)
A. brasilense Sp245-Rif	Plasmids pFAJ0526, pFAJ0529 and pFAJ0535	Conjugation	Baudoin et al. (2010)
Pseudomonas A1501	Plasmid pUC4K (X06404)	Transformation	Setten et al. (2013)

transduction to the uptake of phage DNA. However, the most frequently used method is transformation. This process includes the microbial uptake of the exogenous genetic material through the cell membrane. This uptake can occur only at specific physiological stage of competence. Natural bacterial competence is inefficient to uptake DNA significantly, so, competence is induced in bacterial cells by chemical treatment (treating with calcium chloride). *Escherichia coli* is the most commonly used cloning host for generating competent cell by chemical treatments. Some of the examples to transfer the gene for modification in micro-organisms are shown in Table 11.1.

Transformation involves preparation of protoplasts using lysosome enzyme in the presence of polyethylene glycol to promote the uptake of DNA. Growth medium, growth phase, ionic composition of transformation buffers, and duration of treatment are the important factors that should be optimized before starting the process. However, the process of transduction involves packaging of phage with copies of desired genes which are in the form of concatemers linked through *cos* sites. Afterward, phage infects the host cell to release the DNA inside the bacteria through the injectisome apparatus. This process is used where the competent cells are inefficient to take up the DNA.

Conjugation is another method used to introduce plasmid DNA into microorganisms. In this process the donor strain contains both the gene of interest and the origin of replication on a plasmid which is transferred to recipient cell upon transient contact between them through the formation of conjugation bridge. After the transfer of genetic material, donor cells are eliminated with an antibiotic to which the recipient cells are resistant. There is horizontal gene transfer between the two strains which results in host cell with foreign gene. Moreover, it does not involve the tedious processes such as protoplast formation and regeneration as in the case of transformation. There are many other alternative methods to transfer the gene in organisms such as electroporation, liposome-mediated transformation, microinjection, etc., but electroporation is widely used for gene transfer method in the case of microorganism. The electroporation process involves the application of electric impulse for specific time interval to generate transient pores in the cell membrane, thereby allowing DNA transfer. Growth phase, cell density, electroporation parameters, and growth medium must be optimized to achieve desirable efficiency. Electroporation is often used when protoplast transformation is insufficient or ineffective. So, the methods available for getting recombinant DNA into cells depend on the type of host/vector system which ranges from very simple procedures to much more complicated ones.

4. **Selection and target gene expression:** Selection of recombinant cells from the large number of cells in which gene has been transferred is very critical process. Some cells get genetically modified, but majority of cells remain unmodified. The selection process is necessary as the number of recombinant cells is often significantly less than the number of nonrecombinant cells. To select the recombinant cells, various methods are used such as selectable marker, gene disruption method, positive selection method, colony PCR, DNA sequencing, etc. The selectable marker genes are important part of cloning vectors and are required for identification of transformed cells. Selectable marker gene usually codes for the antibiotic resistance or toxin resistance which gives the selective advantage to recombinant cells over the others. Usually, high-level expression of a selectable marker gene is required for complete elimination of nonrecombinant cells. Antibiotic resistance genes are routinely used as selectable markers, but these genes are not generally acceptable for the construction of recombinant microorganisms for environmental application (Akada et al. 2002). Gene disruption method is another method which involves insertion of target gene in the middle of previously present gene, thus disrupting the expression of the gene. A classic

example of gene disruption is the insertion of gene between the *lacZα* encoding sequence in plasmid DNA. The gene insertion disrupts the production of β-glucosidase enzyme (encoded by *lacZα*); as a result, recombinant microorganism is unable to utilize X-gal (substrate analog) in the presence of inducer isopropyl β-D-1-thiogalactopyranoside (IPTG) in growth media thus appears white on the medium, whereas nonrecombinants appear blue (commonly known as blue white colony selection). Another similar method is positive selection of recombinant vectors which is an effective and simple method for screening the presence of target gene. In this type of selection, the vector contains a lethal gene, and the target gene was inserted in multiple cloning sites present inside that lethal gene. The expression of the lethal gene is disrupted by ligation of a target DNA; as a result, only cells with recombinant plasmids can survive. This approach can save time and cost of selection since it typically yields more than 99% recombinant clones.

Genetically modified microorganisms should completely express the target gene in environmental condition which is necessary for the improvement in functional characteristics of microorganism. Expression of gene depends upon the presence of other gene sequence such as promoter sequence. This sequence is about 100–1000 base pairs long, present on genome near the transcription start sites of genes where the RNA polymerase will bind and initiate transcription. Thus, promoter sequence regulates the expression of the gene. There are two types of promoters—constitutive and inducible promoters. Constitutive promoters are continuously active and known as housekeeping genes, whereas inducible promoters become activated only under certain conditions such as the presence of an inducer. Promoter selection is very important to optimize the target gene's expression. The constitutive promoters are used for the continuous expression of a target gene. However, inducible promoters are used for expression of gene under specific set of environmental and biochemical conditions.

11.6 Applications of Genetically Modified Microbes in Agriculture

The potential of microbes is analyzed from their ecological impacts such as increased availability of nutrients in soil, improved crop productivity, plant growth promotion, and disease suppression. Their ecological impacts can be intensified by addressing the mechanisms for mutual interaction between microbial partners and crop plant. Most of the microbial interactions are regulated by specific molecules/signals which are responsible for key environmental processes, such as the biogeochemical cycling of nutrients and the maintenance of plant health as well as soil quality (Barea et al. 2004). The microbial interaction can be of two types:

(a) **Dead plant material-based interaction:** The microbial interactions with plant debris which lead to the degradation of detritus material and recycling of nutrients in the ecosystem, thus, affect energy and nutrient flows.

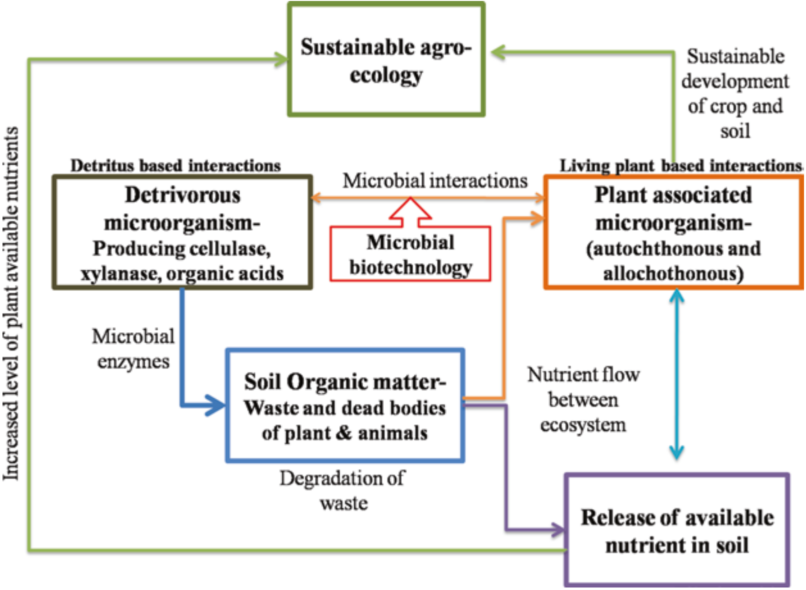


Fig. 11.5 Role of microbial interaction in maintaining sustainable agroecosystem

(b) **Living plant roots-based interactions:** The interaction between microorganisms and plant roots increases the production of beneficial secondary metabolites, availability of unavailable nutrients, and soil nutrient level and fertility, thus increasing the fitness of agroecosystem.

Both types of interactions are relevant to agronomy as well as ecology (Fig. 11.5). These interactions can be targeted by microbial biotechnology to produce genetically modified microbes which can improve the interaction between the microbes and crop plants leading to increased microbial ecological efficiencies.

These enhanced ecological efficiencies improve the communication between genetically modified microbes and their environment. As a result, genetically improved microbial population will have enhanced survival and better function and provide improved ecological services (Fig. 11.6).

11.6.1 Improvement in Phytostimulation Activity

Successful crop growth depends on the genetic makeup of the plant, adequate availability of nutrients, presence of beneficial microbes, and absence of phytopathogens. The presence of beneficial microorganisms increases availability of nutrients and growth-promoting metabolites in vicinity of plant roots. Plant growth-promoting rhizobacteria (PGPR) are such beneficial and obligate rhizoplane as well as rhizospheric bacteria which promote plant growth by a wide range of mechanisms such

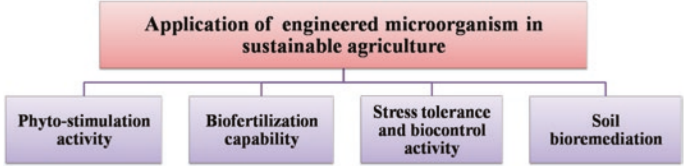


Fig. 11.6 Applications of genetic modified microorganism in sustainable agriculture

as release of beneficial metabolites and bioactive substances that have measurable impact on growth. Bioactive substances secreted by microbes include vitamins, phytohormones, and amino acids. These metabolites contribute to the host root respiration, root metabolism, root abundance, suppression of soilborne pathogens, etc. which result in improved mineral water uptake and disease suppression in inoculated plants.

The efficacy of PGPR depends upon microbial inoculant-crop compatibility, soil composition, moisture content, and most importantly mechanisms (direct or indirect) employed by PGPRs to facilitate plant growth. Various studies have shown that PGPR strains typically harbor more than one plant growth-promoting property. So, the genes encoding for these properties contribute to plant beneficial traits and can be selected by the interaction of these bacteria with plants. These genes include *nifHDK* (nitrogenase-encoding genes), *pqqBCDEFG* (pyrroloquinoline quinone-encoding genes), *ipdC/ppdC* (gene of the indole-3-pyruvate synthesis pathway), *nirK* (copper nitrite reductase gene), *acdS* (1-aminocyclopropane-1-carboxylate (ACC) deaminase gene), *hcnABC* (hydrogen cyanide genes), *phlACBD* (2,4-diacetylphloroglucinol), etc. for synthesis of various plant beneficiary metabolites as well as activities. Such information would bring fundamental insights into the associations of PGPR bacteria with plants which can be exploited for genetic manipulations to increased phytobeneficial potential (Bruto et al. 2009).

Indigenous plant growth-promoting bacteria associated with plants do not have uniform and expected effects on the growth and fitness of all host plants in different conditions (Long et al. 2008). It may be due to non-efficient colonization of microbes in rhizospheric area. The root colonization activity of plant growth-promoting rhizobacteria is a very important feature. Identification of genes associated in root colonization and other plant-microbe interactions are useful techniques for generating strains with enhanced competence for the rhizosphere. By this, the efficiency of plant-associated bacteria increased to such a level that can produce reliable results under the presence of variable environmental factors. There are enormous plant growth-promoting traits which can be targeted in genetic engineering to improve the performance of plant growth-promoting rhizobacteria in soil. Root-associated rhizobacteria can produce the plant growth hormone indole-3-acetic acid (IAA) which triggers the growth of the meristematic tissues of the root and shoot. The insertion of multiple copies of *ipdC* (coding an indole-3-pyruvate decarboxylase/phenylpyruvate decarboxylase) to rhizobacteria can improve the plant growth-promoting activity of recipient microorganism. The presence of *ipdC* construct in microorganism causes

increased shoot biomass which may be due to increased early nutrient acquisition and improved functioning of the root system in terms of nutrient acquisition (Bertrand et al. 2000). The potential to exploit soil microorganisms as plant growth promoters may be achieved by the development of more effective microbial inoculants through the genetic manipulation of microorganisms. However, genetic manipulation appears to offer the reliable and reproducible results in the field.

11.6.2 Enhanced Biofertilization Capability

Plant production confides on biogeochemical cycling, which is primarily driven by microbes and causes release of essential nutrients in soil ecosystem. Nutrients are important for the growth and development of plants as well as microorganisms. The rhizosphere harbors abundant and diverse species of bacteria and fungi that interplay between plant and soil nutrient (C, N, & P) dynamics. Functional activities of the beneficial rhizospheric microbes are related to plant nutrition, organic matter decomposition, nitrogen fixation, nutrient solubilization, mobilization, and transport (Philippot et al. 2013). Modification of characteristics of microorganisms by genetic manipulation can increase the availability of major nutrients in the soil.

Enhanced Nitrogen Fixation Nitrogen is a major component in building up of amino acids and proteins and usually is the most limiting nutrient. Nitrogen (N_2) fixation is the ability to fix atmospheric nitrogen and supply it in a usable form to the host plant. The process of conversion of atmospheric nitrogen into available forms that can be assimilated by plants is a unique process driven by prokaryotes. Some nitrogen-fixing organisms such as *Azospirillum* (associative N_2 fixer) and *Azotobacter* (free-living N_2 fixer), commonly associated with cereals in temperate zones, are able to improve cereal crop yields and are used as a biofertilizer for various crops. The nitrogen-fixing capability of microorganisms depends on the presence of *nif* genes which codes for nitrogenase enzyme. Nitrogenase enzyme is the key enzyme in nitrogen fixation which provides the site for reduction of dinitrogen to ammonia (available form). Genetic modification of microorganism by transferring the whole set of genes is required for the expression and functioning of nitrogenase enzyme which lead to release of significant amount of ammonia into medium, and hence, these modified microorganisms act as biofertilizers. Transformation of *Pseudomonas protegens* Pf-5 with genes encoding the nitrogenase of *Pseudomonas stutzeri* A1501 via the X940 cosmid is an example of increase in the biofertilizing capacity. The transformed strain showed high nitrogenase activity and released significant quantities of ammonium in medium. Similar to *Pseudomonas protegens* Pf-5, *Pseudomonas putida*, *Pseudomonas veronii*, and *Pseudomonas taetrolens* can be transformed with same cosmid which can cause the constitutive expression of nitrogenase activity and high ammonium production. Inoculation of *Arabidopsis*, alfalfa, tall fescue, and maize with Pf-5 X940 enhanced the soil ammonium concentration and plant productivity under nitrogen-deficient conditions (Setten et al. 2013).

Root-nodulating bacteria should have high competitiveness which is very critical for the successful use of rhizobial inoculants. Therefore, it is desirable that the inoculant strain be genetically modified to ensure that it will occupy a sufficient number of root nodules in the plant host. Nodulation competitiveness of several strains can be enhanced by genetic manipulation. This genetic manipulation consists of the modified expression of the regulatory *nifA* gene. The expression of *nif* genes, *nod* genes, and *ntr* operon in the microorganism which regulates nitrogen fixation, nitrogen uptake, and root nodulation, respectively, is regulated by *Nif A* gene (Germaine et al. 2013).

Enhanced Phosphorus Solubilization Phosphorus is the second major nutrient for crop plants which is present in soil in relatively larger amounts but in unavailable form. The release of microbial enzymes (phosphatases and phytases) and acids (formic acid, gluconic acid, succinic acid, etc.) causes breakdown of metal-bound insoluble phosphorus into plant available form. Molecular techniques are advantageous approach for obtaining and characterizing improved PSB strains (Igual et al. 2001). The direct oxidation of glucose to gluconic acid in the periplasmic space of gram-negative bacteria is catalyzed by quinoprotein glucose dehydrogenase (GDH). The genes for glucose dehydrogenase (*gcd*) along with glutamine synthetase (*gln*A) and phosphate transport system (*pts*) gene promoters have been mobilized into indigenous microorganism. The expression of these genes in transformed strains improved the biofertilizing potential in terms of mineral phosphate solubilization and plant growth-promoting activity with a small decrease in nitrogen fixation ability (Sashidhar and Rao 2009).

The metabolic pathways present in microorganisms have direct and indirect relations with other metabolic pathways. The little variation in one pathway can improve or diminish the production of other metabolites. The phosphorus-solubilizing activity and the production of the auxin indole-3-acetic acid (IAA) were co-expressed in some of the microorganisms although a direct correlation linking IAA production to P solubilization is not established. It has been reported that overproduction of IAA (with insertion of *ipcC/ipcD* gene) triggered the TCA cycle enzymes and caused increase in the excretion of malic, succinic, and fumaric acids (TCA cycle intermediates) which resulted in positive effects in both P-sufficient and P-limiting conditions (Bianco and Defez 2010). The presence of soil microorganisms causes enhanced utilization of inositol phosphate by plants (Richardson et al. 2001). It was observed that transformation of pyrroloquinoline quinone (PQQ) synthesizing genes into bacterial strains can increase the efficiency to solubilize more amount of insoluble phosphate as compared to parental strains (Rodriguez 2000). Hence, targeting the specific enzyme or biochemical pathway to genetically improve the microbes results in increased functional ability and biofertilization capacity of microorganism.

11.6.3 Improvement in Stress Tolerance Activity

The intense agricultural practices cause detrimental effects on soil ecology, soil irrigation needs, and human health, as well as posing threat to the environment which results in altered environmental conditions such as drought, temperature extremes, and soil salinity. These environmental conditions induce stress in plants. In addition to these environmental stresses, the presence of pathogenic microorganism/plant pests also induces stress condition in plants. The efficiency of plant growth and productivity decreases in stress conditions as most of the plant metabolism is focused to de-stress. Therefore, environmentally amiable approaches have to be employed to maintain proper growth in such adverse conditions. Microorganisms can provide alternative approach to cope the situation by reducing damage caused by environmental or pathogenic stress. So, basically stress can be of two types:

1. Abiotic stress
2. Biotic stress

11.6.3.1 Abiotic Stress Tolerance

Abiotic stress is induced in plants under the harsh and unfavorable environmental conditions. This stress affects plants throughout their life span, and plants have to develop mechanisms for increased survival in the presence of these stresses. The environmental changes such as extreme temperature, flooding, drought, freezing, salinity, strong light, change in pH, exposure to radiation, and heavy metals significantly affect the agriculture. Such adverse environmental conditions exert negative impact on crop productivity and pose a major problem for food security especially in tropical regions. So, to cope with the problem, there is an utmost need to develop the environmental friendly and cost-effective techniques. It involves the utilization of multifunctional microorganisms with an established role in stress management (provides induced systemic resistance to the plant by microbial growth). The stress tolerance response can be alleviated by inoculating crop seeds and seedlings with plant growth-promoting bacteria (PGPB). The microorganisms have to continually develop a complex stress tolerance system to survive with the changes in their external environment. Genetic engineering techniques help in improving stress tolerance potential of microbes in accordance to changing environment. It is believed that stress-induced responses are directly related with action of auxins (growth hormone). Variation in auxin metabolism (transport and catabolism) is induced by abiotic stresses which may be due to alteration in the expression of auxin-encoding genes (Carmen and Roberto 2012). The expression of PIN genes gets altered during drought or salinity which affects the auxin transport (Potters et al. 2009). This leads to increases in free auxin, which in turn inhibits root elongation and provides protection to plant against stresses. Targeting PIN gene by genetic engineering techniques can increase the survival of the plant in stressed conditions. Another enzyme, catalase (encoded by *Kat* gene), plays a very important role in degrading reactive oxygen species such as peroxides (H_2O_2) as well as significantly affects nodulation and nitrogen-fixing activities of root-nodulating bacteria. The root-nodulating bacteria had lower catalase

activity than the other genera of aerobic or facultative anaerobic bacteria; as a result, they have higher vulnerability to H_2O_2. The gene encoding for the catalase activity can improve the survival and nitrogen-fixing capability of root-nodulating bacteria in stress condition due to high oxygen concentration. So, the increase of catalase activity in rhizobial cells could be a valuable way to improve the nodulation, nitrogen-fixing ability, and biofertilizing ability in high oxygen conditions (Orikasa et al. 2010). During the stress period, 1-aminocyclopropane-1-carboxylate (ACC) is released by plants which is a precursor for ethylene hormone production. However, ethylene accumulation in the roots intensifies the plant stress (Babalola et al. 2007). Microbial degradation of ACC seems to be particularly important under stress such as cold, drought, saline soils, or heavy metals in contaminated flooded soils. The cloning of ACC deaminase genes and inserting them into plant-associated bacteria resulted in increased plant growth (Davoud et al. 2010).

11.6.3.2 Biotic Stress Tolerance

Pathogenic microorganisms are a major threat to plant growth, development, and productivity. Traditional methods such as crop rotation, breeding for resistant plant cultivars, and application of chemical pesticides seem to be insufficient to control plant diseases of important crops. The presence of pathogens/plant pests induces stress in crop plants. This leads to increased use of pesticides to control the diseases which become threat to environment. In the future, the greater reliance will be laid on biotechnological applications including the use of microorganisms as antagonists to control the pathogenic microorganisms. Therefore, interest in biological control has been increased in the past few years due to environmental concern over the use of chemicals. Biological control is generally defined as "any practice which can decrease the survival as well as activity of a pathogen through any other living organism (except man) and thus reduces the stress induced by the pathogen." After colonizing the rhizosphere, biocontrol agents must be able to defend the nutrient-rich sites under conditions of intense competition with indigenous/pathogenic microorganisms. Application of biocontrol agents to suppress diseases is often unsuccessful because of inconsistent performance under field conditions. This may be due to variable plant root colonization by the biocontrol agent, nutrient starvation due to competition, instability or degradation of the antibiotic, insufficient concentration of the released antibiotic, and genetic diversity of the pathogen. One approach to overcome some of these problems is to genetically modify biocontrol strains for enhanced and/or constitutive expression of biocontrol properties.

Pseudomonas fluorescens strain genetically modified for *Cry1Ac* and *Cry*1C genes encoding the δ-endotoxin proteins from *Bacillus thuringiensis* (Panetta 1993). This endotoxin persists longer in the environment when expressed inside *Pseudomonas* as compared to original host *B. thuringiensis*. This occurred due to the fact that after death of *Pseudomonas*, cell wall remained intact, whereas the *Bacillus* cell wall disintegrates (Soares and Quick 1990). The production of phenazine-1-carboxylic acid (PCA) or DAPG is an important trait in biological control agents and can lead to suppression of plant root diseases. It was reported that genetic modification of root-colonizing bacterium *Pseudomonas putida* WCS358r

by insertion of *phz* gene encoding for PCA enhances the ecological fitness of the plant and environment (Viebahn et al. 2003). The cloning of *phl*ACBDE operon, encoding 2,4 DAPG production, into a number of *Pseudomonas* strains, can result into enhanced biocontrol activity against plant microbial pathogens such as *Gaeumannomyces graminis* and *Ralstonia solanacearum* (Zhou et al. 2005).

Agrobacterium radiobacter produces an antibiotic agrocin 84 which is normally toxic to agrobacteria carrying a nopaline/agrocinopine-type Ti plasmid, but it can spread the plasmid by horizontal transfer method to other species. So, a mutant designated *A. radiobacter* strain K1026 was constructed which cannot transfer the modified agrocin plasmid to pathogenic agrobacteria, thereby retaining its capability to act as a biocontrol agent (Jones et al. 1988). This strain is particularly effective against *Agrobacterium tumefaciens*, the causative agent of crown gall disease of stone fruit trees and almonds. Another mechanism to control the growth of pathogen is to produce lytic enzymes such as chitinases, glucanases, and xylanase. *Pseudomonas putida* strain 101-9 was genetically engineered to chitinase production. It harbors the chitinase-expression vector pKAC9-p07 (degrade chitin, a major component of fungal cell walls) which resulted in overproduction of enzyme chitinase and suppressed the fungal pathogens (Ohno et al. 2011).

Genetic enhancement of biocontrol strains has mainly emphasized on the introduction of new biocontrol traits into plant-associated strains. However, to be a successful biocontrol agent, the microbe must have the ability to survive, compete, and maintain a high population in the target crop. Future research should also focus not only those traits involving colonization and effectiveness but also traits involved in stress responses such as desiccation and nutrient starvation. Also the genetic manipulation of root-associated traits to enhance establishment and proliferation will help biocontrol agents in the plant biosphere (Singh et al. 2011).

11.6.4 Genetic Engineering to Improve Bioremediation Potential of Microbes

Unprecedented growth of population, anthropogenic activities, and urbanization has increased pollutants in soil to critical levels. In day-to-day life, we use thousands of chemicals in the form of fuels, consumer products, industrial solvents, drugs, pesticides, fertilizers, and food additives. Traditional methods routinely used for the remediation of contaminated environmental soil include excavation, transport to specialized landfills, incineration, stabilization, and vitrification. Much interest is growing to use microorganism in bioremediation technologies to degrade toxic contaminants in environmental soil into less-toxic and/or nontoxic substances. The microorganisms are particularly effective for degradation of pollutants. Metabolic potential of microorganisms provide an effective mechanism for eliminating environmental pollutants. Using biotechnological techniques, plant-associated bacteria (rhizospheric and/or endophytic) can be engineered to produce specific enzymes which are capable of degrading toxic organic pollutants present in the environment. Genetic engineering of endophytic and rhizospheric bacteria is considered one of the

Table 11.2 Genetically modified microorganisms for degradation of organic compounds

Microorganism	Target gene for modification	Contaminant	References
Pseudomonas sp.	*dmpN*	Phenol	Selvaratnam et al. (1997)
Escherichia coli AtzA	Atrazine chlorohydrolase	Atrazine	Strong et al. (2000)
Pseudomonas fluorescens HK44	luxCDABE	Naphthalene	Sayler and Ripp (2000)
Cycloclasticus sp.	*phnA1, phnA2, phnA3, and phnA4*	PAH	Kasai et al. (2002)
Pseudomonas sp., *Bordetella* sp.	*nccA*	Nickel, cobalt, cadmium	Abou-shanab et al. (2003)
Burkholderia cepacia L.S.2.4	pTOD plasmid	Toluene	Barac et al. (2004)
Pseudomonas fluorescens F113rifpcbrrnBPI::GFP-MUT3	Operon bph, gfp	Chlorinated biphenyls	Boldt et al. (2004)
Pseudomonas putida KT2442	pNF142 plasmid, gfp	Naphthalene	Filnov et al. (2005)
Burkholderia cepacia VM1468	VM1468 pTOM-Bu61 plasmid	Toluene	Taghavi et al. (2005)
Rhodococcus sp. RHA1	*fcbABC* operon	2(4)-chlorobenzoate 2(4)-chlorobiphenyl	Rodrigues et al. (2006)
Staphylococcus aureus	*chrB*	Chromate	Aguilar-barajas et al. (2008)
Escherichia coli JM109	*Escherichia coli* JM109	Decolorize azo dyes, C.i. direct blue 71	Jin et al. (2009)
Pseudomonas putida PaW340(pDH5)	pDH5 plasmid	4-chlorobenzoic acid	
Bacillus subtilis, Bacillus cereus	*czcD*	Cobalt, zinc, cadmium	
Pseudomonas aeruginosa	*merA, merB*	Organic and inorganic mercury	Dash and Das (2012)

most promising new technologies for remediation of contaminated environmental sites (Dzantor 2007). In order to increase the bioremediation potential and/or metabolic activity of any microorganism, the insertion of certain functional genes into their genome is necessary. This phenomenon can be achieved by the insertion of new genes into the genomic complexion, insertion of new plasmid, alteration of metabolic pathways, and most importantly adaption of features toward the environmental conditions. Due to significant developments in the field of microbial biotechnology, there are numerous developments in genetically engineered microbes for bioremediation of toxic substances. The examples of selected GMMs degrading toxic organic compounds are listed in Table 11.2.

Improvement in existing catabolic pathways or extension of these pathways can be targeted to degrade some compounds which are not possible to degrade by using wild strain. The whole catabolic pathway may be encoded by a single microorganism or by a consortium of microorganisms, each performing one or more of the stages of bioremediation of xenobiotics. In this way, genetically modified microorganisms with improved degradation capabilities can be constructed. Alteration of gene sequences during the process of construction further improves the efficiency of the catabolic pathways. Genetic modification of rhizospheric bacteria with the *bph* operon could enable the bacteria to degrade polychlorinated biphenyls (PCBs) more efficiently than the wild-type rhizospheric bacteria. The genetic engineered *Pseudomonas fluorescens* HK44 was the first strain used for bioremediation because of special characteristics such as the absence of antibiotic resistance gene in vector, stable *bph* element, and nontransferable *bph* element making it more suitable for environment. Similarly, the insertion of naphthalene gene into microorganism is responsible for naphthalene degradation pathway (Sayler and Ripp 2000).

The enzymes mediate various metabolic pathways which are produced by the transcription and translation of specific genes. Microorganisms can be developed by hybrid gene clusters which alter the activity and substrate specificities of enzyme. *Deinococcus radiodurans*, the most radio-resistant organism, has been modified genetically to consume and digest toluene and the ionic form of mercury from nuclear wastes. A recombinant *Rhizobium tropici* strain expressing 1,9-α-dioxygenase enzyme was used for the degradation of dioxine-like compounds. Similarly various other degradative enzymes such as orthomonooxygenase are cloned for degradation of toluene in rhizospheric region of soil.

Genetic engineering offers a great scope for the use of natural ability of bacteria in construction of GMMs. Unfortunately, they are applicable mainly in laboratory conditions. The new approach to use plant-associated endophytic bacteria seems to be a very promising solution in remediation of contaminated areas. However, this field of study requires still much work in laboratory scale.

11.7 Achievements

Some examples of currently use genetically engineered microbes are listed below:

1. The first commercially available genetically modified biocontrol agent was a modified strain of *Agrobacterium radiobacter* strain K84 which has been marketed in Australia since 1989. This strain is used as a means of controlling *Agrobacterium tumefaciens*, the causative agent of crown gall disease which affects stone fruit trees and almonds.
2. *Agrobacterium radiobacter* K1026 (Jones and Kerr 1989) is a Tra$^-$ (transfer negative) derivative of *A. radiobacter* K84 which is a naturally occurring bacterium effective against crown gall. This mutant designated *A. radiobacter* strain K1026 was constructed so that it can no longer transfer the modified agrocin plasmid to pathogenic agrobacteria, thereby retaining its effectiveness as a biocontrol agent

(Jones et al. 1988). This strain is commercially available under the trade name "NoGall" and is also a registered biocontrol agent in the United States.

3. Biocontrol microbe that has been genetically modified and is commercially available is a *Pseudomonas fluorescens* strain. The *Cry1*Ac and *Cry*1C genes encoding the δ-endotoxin proteins from *Bacillus thuringiensis* were transferred into this *Pseudomonas* strain (Panetta 1993). This product is sold under the trade name "MVP bioinsecticide" and used to reduce crop damage from the diamondback moth (*Plutella xylostella*).
4. There was a transient commercialization of *Sinorhizobium* (*Rhizobium*) *meliloti* RMSPC-2 (EPA 1998) as seed inoculants for alfalfa. The strain had genes to enhance nitrogen fixation and nutrient utilization, as well as an antibiotic resistant marker gene.
5. The commercial transfer of the *Bt* delta endotoxin gene to the endophyte *Clavibacter xyli* for control of the corn earworm (Tomasino et al. 1995) lost to competition with the development of *Bt* genes transformed as integral parts of the plant cell.
6. *P. fluorescens* HK44 contained pUTK21 plasmid, the genes responsible for naphthalene degradation pathway. *Pseudomonas fluorescens* HK44 was the first strain used in the field experiment because of special characteristics such as the absence of antibiotic resistance gene in vector, stable *bph* element, and nontransferable *bph* element making it more suitable for environment.

11.8 Future

Genetic engineering is used to modify microorganisms capable to carry out unique processes. This becomes popular way of increasing the efficiency of microorganism in laboratory studies. Techniques which are used for improvement of microbial strains include engineering with single genes, alteration of the sequences of existing genes (both coding and controlling sequences), and pathway construction. But, the researchers should emphasize the ethical responsibilities before releasing a genetically modified microorganism into the environment.

The ethical problem raised by genetically modified microorganisms (GMMs) is mainly related to the impact they have on the Earth's biosphere. As microorganisms can be found everywhere, including in the ecological niches that are the most unsuited to life forms, the repercussions can be substantial and irreversible. The danger posed by these genetically modified organisms is therefore related both to their dispersal into the environment and to their potential for adaptation to a new environment. So, genetically modified microorganisms can alter the animal and plant microbial ecological balance hence disturb the environment to a greater or lesser extent. However, they may potentially transfer their modified genetic material to other organisms. This will lead to the appearance of new variants which may have the capability to significantly disrupt the environment. The studies carried out so far have shown that following appropriate regulations, genetically modified microorganisms can be applied safely in agriculture. Nowadays, the application and release

of genetically engineered bacteria directly in the environment are not accepted by the public and government. One reason behind this rejection is the presence of antibiotic resistant genes in transgenic strains which have been introduced in the bacteria during the allelic replacement process. Another reason may be the impact of recombinant microorganism on the indigenous soil microbial communities. Therefore, it is very difficult to predict the influence on environment which may be generated by the dispersal of genetically modified microorganisms. Several studies related to gene transfer will be necessary over the coming years in order to give an accurate assessment of the risks and consequently to develop GMMs which do not pose a risk to humans and their environment.

However, in view of the real technical difficulties relating to the detection of these microorganisms, their unlawful use, in particular by industrialists, is a cause for concern. As regards fundamental research on microorganisms which gives rise to new modified strains, the question of the real need for such research must be considered in the same way as with all research in all disciplines.

11.9 Conclusion

Microbes have immense potential to contribute to sustainable agriculture. Microbial inoculants are widely used for plant growth promotion, biocontrol, or bioremediation, but the results are inconsistent with low levels of success in varying field conditions. Genetic engineering paves a novel way to increase the reliability and performance of microbes to accomplish the particular target, but this will require further discovery of novel bacterial strains, genetic improvement of microbes, and improved inoculum delivery systems. In this context, genomic analysis and genetic engineering are helpful for obtaining improved bioformulations of promising beneficial microbes. This strategy will enable us to save considerable amounts of agrochemicals, especially chemical fertilizers and chemical pesticides, and hence our environment.

References

Abou-Shanab RA, Delorme TA, Angle JS, Chaney RL, Ghanem K, Moawad H, Ghozlan HA (2003) Phenotypic characterization of microbes in the rhizosphere of *Alyssum murale*. Int J Phytorem 5:367–379

Aguilar-Barajas PE, Cervantes C, Rensing C (2008) Expression of chromate resistance genes from *Shewanella* sp. strain ANA-3 in *Escherichia coli* Esther. FEMS Microbiol Lett 285:97–100

Akada R, Shimizu Y, Matsushita Y, Kawahata M, Hoshida H, Nishizawa Y (2002) Use of a *YAP1* overexpression cassette conferring specific resistance to cerulenin and cycloheximide as an efficient selectable marker in the yeast *Saccharomyces cerevisiae*. Yeast 19:17–28

Andrews M, Hodge S, Raven JA (2010) Positive plant microbial interactions. Annals Appl Biol 157:317–320

Ayub ND, Tribelli PM, López NI (2009) Polyhydroxyalkanoates are essential for maintenance of redox state in the Antarctic bacterium *Pseudomonas* sp. 14-3 during low temperature adaptation. Extremophiles 13:59–66

Babalola OO, Sanni AI, Odhiambo GD, Torto B (2007) Plant growth-promoting rhizobacteria do not pose any deleterious effect on cowpea and detectable amounts of ethylene are produced. World J Microbiol Biotechnol 23(6):747–752

Barac T, Taghavi S, Borremans B, Provoost A, Oeyen L, Colpaert JV, Vangronsveld J, van der Lelie D (2004) Engineered endophytic bacteria improve phytoremediation of water soluble, volatile, organic pollutants. Nat Biotechnol 22(5):583–588

Barea JM, Azcón R, Azcón-Aguilar C (2004) Mycorrhizal fungi and plant growth promoting rhizobacteria. In: Varma A, Abbott L, Werner D, Hampp R (eds) Plant surface microbiology. Springer-Verlag, Heidelberg, pp 351–371

Baudoin E, Lerner A, Mirza MS, Zemrany HE, Combaret CP, Jurkevich E, Spaepen S, Vanderleyden J, Nazaret S, Okon Y, Loccoz YM (2010) Effects of *Azospirillum brasilense* with genetically modified auxin biosynthesis gene *ipdC* upon the diversity of the indigenous microbiota of the wheat rhizosphere. Res Microbiol 161:219–226

Bertrand H, Plassard C, Pinochet X, Touraine B, Normand P, Cleyet- Marel JC (2000) Stimulation of the ionic transport system in *Brassica napus* by a plant growth-promoting rhizobacterium (*Achromobacter* sp.). Can J Microbiol 46:229–236

Bianco C, Defez R (2010) Improvement of phosphate solubilization and *medicago* plant yield by an indole-3-acetic acid-overproducing strain of *Sinorhizobium meliloti*. Appl Environ Microbiol 76(14):4626–4632

Boldt TS, Sørensen J, Karlson U, Molin S, Ramos C (2004) Combined use of different Gfp reporters for monitoring single-cell activities of a genetically modified PCB degrader in the rhizosphere of alfalfa. FEMS Microbiol Ecol 48(2):139–148

Bruto M, Combaret CP, Muller D, Loccoz YM (2009) Analysis of genes contributing to plant-beneficial functions in plant growth-promoting rhizobacteria and related Proteobacteria. Scientific Rep 4:6261

Carmen B, Roberto D (2012) Soil bacteria support and protect plants against abiotic stresses. Abiotic Stress Plants—Mech Adapt 7:144–170

Chen H, Gao K, Kondorosi E, Kondorosi A, Rolfe BG (2005) Functional genomic analysis of global regulator NolR in Sinorhizobium meliloti. Am Phytopathol Soc 18(12):1340–1352

Dale J W, Park SF (2007) *Molecular genetics of bacteria*. University of Surrey, pp. 137–244

Dash HR, Das S (2012) Bioremediation of mercury and importance of bacterial mer genes. Int Biodeterior Biodegra 75:207–213

Davoud F, Naser A, Nemat SB, Bagher Y (2010) Cloning and characterization of a plasmid encoded ACC deaminase from an indigenous *Pseudomonas fluorescens* FY32. Curr Microbiol 61:37–43

Dzantor EK (2007) Phytoremediation: the state of rhizosphere 'engineering' for accelerated rhizodegradation of xenobiotic contaminants. J Chem Technol Biotechnol 82:228–232

EPA (1998) Final rule: *Sinorhizobium meliloti* strain RMBPC-2: significant new use rule. Fed Regist 63:29646–29648

Filonov AE, Akhmetov LI, Puntus IF, ESikova TZ, Gafarov AB, Izmalkova TY, Sokolov SL, Kosheleva IA, Boronin AM (2005) The construction and monitoring of genetically tagged, plasmid-containing, naphthalene- degrading strains in soil. Microbiol 74(4):526–532

Germaine KJ, McGuinness M, Dowling DN (2013) Engineering of microbes for plant and soil systems. In: Gupta VK, Schmoll M, Mazutti MA, Maki M, Tuohy MG (eds) Applications of microbial engineering. CRC Press, Boca Raton, p 494

Hedin LO, Brookshire ENJ, Menge DNL, Barron AR (2009) The nitrogen paradox in tropical forest ecosystems. Annual Rev Ecol Evol System 40:613–635

Igual JM, Valverde A, Cervantes E, Velázquez E (2001) Phosphate-solubilizing bacteria as inoculants for agriculture: use of updated molecular techniques in their study. Agronomie 21:561–568

Jin R, Yang H, Zhang A, Wang J, Liu G (2009) Bioaugmentation on decolorization of C.I. Direct Blue 71 using genetically engineered strain *Escherichia coli* JM109 (pGEX-AZR). J Hazard Mater 163(2–3):1123–1128

Jones DA, Kerr A (1989) *Agrobacterium radiobacter* strain K1026, a genetically engineered derivative strain of strain K84, for biological control of crown gall. Pl Dis 73:15–18

Jones DA, Ryder MH, Clare BG, Farrand SK, Kerr A (1988) Construction of a Tra- deletion mutant of pAgK84 to safeguard the biological control of crown gall. Mol Gen Genet 212:207–214
Kasai Y, Kishira H, Harayama S (2002) Bacteria belonging to the genus *Cycloclasticus* play a primary role in the degradation of aromatic hydrocarbons released in a marine environment. Appl Environ Microbiol 68:5625–5633
Kumamaru T, Suenaga H, Mitsuoka H, Watannabe T, Furukawa H (1998) Enhanced degradation of polychlorinated biphenyls by direct evolution of biphenyl dioxygenase. Nat Biotechnol 16:663–666
Lehrbach PR, Zeyer J, Reineke W, Knackmuss HJ, Timmis KN (1984) Enzyme recruitment in vitro: use of cloned gene to extend the range of haloaromatics degraded by *Pseudomonas sp.* strain B13. J Bacteriol 158:1025–1032
Long HH, Schmidt DD, Baldwin IT (2008) Native bacterial endophytes promote host growth in a species-specific manner, phytohormone manipulations do not result in common growth responses. PLoS One 3(7):e2702. https://doi.org/10.1371/journal.pone.0002702
Matsui T, Saeki H, Shinzato N, Matsuda H (2006) Characterization of *Rhodococcus– E. coli* shuttle vector pNC9501 constructed from the cryptic plasmid of a propene-degrading bacterium. Curr Microbiol 52(6):445–448
Menn FM, Easter JP, Sayler GS (2009) Genetically engineered microorganism and bioremediation. In: Rehn HJ, Reed B (eds) Biotechnology set. Wiley, Hoboken, NJ, pp 441–463
Noble AD, Ruaysoongnern S (2010) The nature of sustainable agriculture. In: Dixon R, Tilston E (eds) Soil microbiology and sustainable crop production. Springer Science and Business Media B.V, Berlin, Heidelberg, pp 1–25
Ohno M, Kataoka S, Yamamoto-Tamura K, Akutsu TK, Hasebe A (2011) Biological control of *Rhizoctonia* damping-off of cucumber by a transformed *Pseudomonas putida* strain expressing a chitinase from a marine bacterium. JARC 45:91–98
Orikasa Y, Nodasaka Y, Ohyama T, Okuyama H, Ichise N, Yumoto I, Morita N, Wei M, Ohwada T (2010) Enhancement of the nitrogen fixation efficiency of genetically- engineered *Rhizobium* with high catalase activity. J Biosci Bioeng 110:397–402
Ouyang SP, Sun SY, Liu Q, Chen J, Chen GQ (2007) Microbial transformation of benzene to cis-3,5-cyclohexadien-1,2-diols by recombinant bacteria harboring toluene dioxygenase gene tod. Appl Microbiol Biotechnol 74(1):43–49
Panetta JD (1993) Engineered Microbes, the Cellcap system. In: Kim L (ed) Advanced engineered pesticides. Marcel Dekker, New York
Philippot L, Raaijmakers JM, Lemanceau P, van der Putten WH (2013) Going back to the roots: the microbial ecology of the rhizosphere. Nat Rev Microbiol 11:789–799
Potters G, Pasternak TP, Guisez Y, Jansen MAK (2009) Different stresses, similar morphogenic responses: integrating a plethora of pathways. Pl Cell Environ 32:158–169
Richardson AE, Hadobas PA, Hayes JE, O_Hara CP, Simpson RJ (2001) Utilization of phosphorus by pasture plants supplied with myo-inositol hexaphosphate is enhanced by the presence of soil micro-organisms. Pl Soil 229:47–56
Rodrigues JLM, Kachel A, Aiello MR, Quensen JF, Maltseva OV, Tsio TV, Tiedje (2006) Degradation of Aroclor 1242 dechlorination products in sediments by *Burkholderia xenovorans* LB400 (ohb) and *Rhodococcus sp. strain RHA1 (fcb)*. Appl Environ Microbiol 72(4):2476–2482
Rodriguez 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
Sashidhar B, Rao PA (2009) Transgenic expression of glucose dehydrogenase in *Azotobacter vinelandii* enhances mineral phosphate solubilization and growth of sorghum seedlings. Microbial Biotechnol 2(4):521–529
Sayler GS, Ripp S (2000) Field applications of genetically engineered microorganisms for bioremediation processes. Curr Opin Biotechnol 11(3):286–289
Schwin U, Schmidt E (1982) Improved degradation of mono chlorophenol by a constructed strain. Appl Environ Microbiol 44(1):33–39

Selvaratnam S, Schoedel BA, McFarland BL, Kulpa CF (1997) Application of the polymerase chain reaction (PCR) and reverse transcriptase/PCR for determining the fate of phenoldegrading *Pseudomonas putida ATCC* 11172 in bioaugmented sequencing batch reactor. Appl Microbiol Biotechnol 47:236–240

Setten L, Soto G, Mozzicafreddo M, Fox AR, Lisi C et al (2013) Engineering Pseudomonas protegens Pf-5 for nitrogen fixation and its application to improve plant growth under nitrogen-deficient conditions. PLoS One 8(5):e63666. https://doi.org/10.1371/journal.pone.0063666

Singh JS, Pandey VC, Singh DP (2011) Efficient soil microorganisms: a new dimension for sustainable agriculture and environmental development. Agric Ecosys Environ. 140:339–353

Soares G G, Quick T C (1990) MVP, A novel bioinsecticide for control of the Diamondback Moth. Chapter 15: In: Proceedings of the second international workshop, 10–14 Dec., Taiwan

Sosio M, Guisino F, Cappellano C, Bossi E, Puglia AM, Donadio S (2000) Artificial chromosomes for antibiotic-producing actinomycetes. Nat Biotechnol 18:343–345

Strong LC, McTavish H, Sadowsky MJ, Wackett LP (2000) Field-scale remediation of atrazine-contaminated soil using recombinant *Escherichia coli* expressing atrazine chlorohydrolase. Environ Microbiol 1:91–98

Taghavi S, Barac T, Greenberg B, Borremans B, Vangronsveld J, van der Lelie D (2005) Horizontal gene transfer to endogenous endophytic bacteria from poplar improves phytoremediation of toluene. Appl Environ Microbiol 71(12):8500–8505

Tomasino SF, Liester RT, Dimock MB, Breach RM, Kelly JL (1995) Field performance of *Clavibacter xyli* subsp. *cynodontis* expressing the insecticidal protein gene *crylA* (c) of *Bacillus thuringiensis* against European corn borer in field corn. Biol Control 5:442–448

Tripura CB, Podile AR (2007) Properties of chimeric glucose dehydrogenase improved by site-directed mutagenesis. J Biotechnol 131:197–204

UN Convention on Biological Diversity, Art. 2. Retrieved on January 3, 2017.

Viebahn M, Glandorf DCM, Ouwens TWM, Smit E, Leeflang P, Wernars K, Thomashow LS, van Loon LC, Bakker AHM (2003) Repeated introduction of genetically modified *Pseudomonas putida* WCS358r without intensified effects on the indigenous microflora of field-grown wheat. Appl Environ Microbiol 69(6):3110–3118

Zhou H, Wei H, Liu X, Wang Y, Zhang L, Tang W (2005) Improving biocontrol activity of *Pseudomonas fluorescens* through chromosomal integration of 2,4-diacetylphloroglucinol biosynthesis genes. Chinese Sci Bull 50:777–781

12 Stress Signalling in the Phytomicrobiome: Breadth and Potential

Sahana Basu and Gautam Kumar

Abstract

Higher plants continually compete with microbes to conserve their predominance within the particular niche. Plants have evolved constant relationships with a suite of microbes, known as the phytomicrobiome. The associates of phytomicrobiome exhibit symbiotic relationship. Plant-microbe and microbe-microbe interactions within the phytomicrobiome are carried out through the release of signalling compounds. Bacterial community within the phytomicrobiome communicates among themselves through quorum-sensing mechanism. Diversity, stability and resilience of microbial community in phytomicrobiome are the major determinants of plant health and productivity. Therefore, phytomicrobiome is under intensive investigation to improve our understanding of its strong effects on plant development, health and resistance to parasites. Exploration of the plant-microbe interactions within the phytomicrobiome is a promising avenue to improve crop productivity and agricultural sustainability. Understanding the regulation and relatedness of plant and microbial community may aid in engineering plants with improved pathogen resistance and novel symbiotic interactions. Comprehensive study of phytomicrobiome, especially the molecular signalling pathway, may provide new insights into the mechanism of plant disease management, successively inspiring new plant breeding strategies.

Keywords

Crop · Molecular signal · Phytomicrobiome · Symbiosis · Quorum sensing

S. Basu
Department of Biotechnology, Assam University, Silchar, Assam, India

G. Kumar (✉)
Department of Life Science, Central University of South Bihar, Gaya, Bihar, India
e-mail: gautam@cub.ac.in

© Springer Nature Singapore Pte Ltd. 2020
M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_12

12.1 Introduction: Phytomicrobiome

Phytomicrobiome is defined as the intimate, complex and subtle association between higher plants and the community of microbial organisms (such as bacteria, fungi and viruses), which can adhere to the external surfaces of plants or may colonize internal plant tissues (Smith and Zhou 2014). This symbiotic association leads to the formation of holobiont (Hartmann et al. 2014). Plant is a meta-organism having a persistent and regulated relationship with its phytomicrobiome (Berg et al. 2013). Phytomicrobiome includes three components—rhizomicrobiome, the community of microbes associated with plant root (Lundberg et al. 2012); phyllomicrobiome, above ground associated (Kembel et al. 2014); and endosphere, colonizing internal plant tissues (Berg et al. 2014). The lower group of plants have also been reported to form complex phytomicrobiomes, including highly specific associations (Bragina et al. 2013).

Phytomicrobiome has been estimated to be evolved about half a billion years ago, since plants colonized the terrestrial ecosystem (Knack et al. 2015). First terrestrial plants supposed to have poorly developed roots, making these plants requiring microbial support (Smith et al. 2015). As plants adapted to and dispersed through diverse terrestrial environments, evolving to grow under a range of conditions, it is probable that their associations with microbes also evolved. Higher plants and their associated phytomicrobiome affect each other in several ways (Berendsen et al. 2012). Microbial cohort of the phytomicrobiome employs inter-organismal signal compounds to alter the behaviour of the plants they associate with and the behaviour of the phytomicrobiome is regulated by the signal compounds from the plants. Thus, one organism within the phytomicrobiome alters the behaviour of another for its own benefit but often to the benefit of the other organism as well, leading to mutualistic symbiosis. Occasionally, the symbioses lead to the development of root nodules (Riely et al. 2006). Several symbioses require specific signalling pathways for intracellular accommodation of microbes (endosymbioses). Analysis of plant-microbe interactions within the phytomicrobiome can be exploited to (1) develop new approaches to crop growth promotion and (2) develop novel and more consistent biocontrol mechanisms for field crops (East 2013).

12.2 Different Types of Plant-Microbe Interactions

As plants evolved to acclimatize themselves to diverse terrestrial environments, the microbial community associated with the plants also evolved.

12.2.1 Mycorrhizal Symbiosis

Mycorrhiza (Greek ‘mykos’, fungus and ‘rhiza’, root) is a symbiotic association between roots of vascular plant and fungus (Smith and Read 2008). The term mycorrhiza refers to the intracellular (arbuscular mycorrhizal fungi) or extracellular (ectomycorrhizal fungi) fungal colonization in the plants’ rhizosphere (root system).

Arbuscular mycorrhizae form symbioses with more than 80% of the angiospermic plant species (Newman and Reddell 1987). Mycorrhizal fungi have existed more than 460 million years ago, since the first terrestrial plants appeared on this planet (Redeker et al. 2000). Fossil endomycorrhizal association occurred in the early Devonian period, demonstrating the association of plant roots with fungal elements of the rhizomicrobiome (Bonfante and Genre 2008).

Mycorrhizal fungi form an obligate symbiotic association with the host plants and assist the plants with the acquisition of essential mineral nutrients, particularly phosphorus (Smith et al. 2003). They form a network of fine filaments associated with plant roots to draw water and nutrients from the soil that the root system would not be able to access. Mycorrhizae play important roles in soil aggregation through hyphae networking (Rillig et al. 2003). The plant provides carbohydrates and other nutrients to the fungi. The carbohydrates are being utilized by the fungi for their growth and synthesis of glomalin (glycoprotein) molecules. These molecules result in better soil structure and increase soil organic matter content (Rillig et al. 2003). Arbuscular mycorrhizae also protect the host plants from herbivores and different environmental stresses including drought and salinity (Brem and Leuchtmann 2002; Miransari 2009; Liu et al. 2017).

There are two major groups of mycorrhizal fungi:

1. Endomycorrhizal fungi
2. Ectomycorrhizal fungi

12.2.1.1 Endomycorrhizal Fungi

Members of this group of fungi penetrate the plant cells where direct metabolic exchanges can occur. These fungi colonize trees, shrubs and herbaceous plants but do not form visible structures. Arbuscular mycorrhizal (AM) fungi are the most predominant endomycorrhizal fungi. They form finely branched structures called 'arbuscules' within the plant root cell and serve as a major metabolic exchange site between the plant and the fungus (Harrison 2005). Sac-like vesicular structures are also found in some species of AM fungi, emerging from hyphae, which serve as storage organs for lipids.

12.2.1.2 Ectomycorrhizal Fungi

These fungi are essentially found on trees and develop visible structures exclusively on the exterior of root cells without penetrating them. Their hyphae grow externally between root cells, forming dense growth known as a fungal mantle. These fungi form symbiotic relationships with most pines, spruces and some hardwood trees including beech, birch, oak and willow. They also form visible reproductive structures (mushrooms) at the feet of trees they colonize.

12.2.2 Nitrogen-Fixing Microorganisms

Leguminous plants develop a symbiotic relationship with nitrogen-fixing bacteria called rhizobia, allowing the bacteria to infect them within special structures known as nodules that are located along their roots. The nitrogen-fixing rhizobia include the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* which enter into symbiotic associations with their legume host plants and fix atmospheric dinitrogen inside root nodules differentiated for this purpose (Mabood et al. 2014). Plants utilize the nitrogen fixed inside the nodule and provide photosynthetically fixed carbon to the rhizobia. There has been an increase in rhizobia-based commercial inoculants as biofertilizers (Vessey 2003), and given the large quantities of fossil fuels used to produce nitrogen fertilizers and the steep rise in fossil fuel prices over recent years, a significant expansion in the use of biofertilizers is most likely to continue.

Besides rhizobia, there are other rhizobacteria that live and fix nitrogen outside of formal symbioses, and these are referred to as free-living or associative nitrogen-fixing bacteria; among them are *Azospirillum*, *Acetobacter*, *Herbaspirillum*, *Azoarcus* and *Azotobacter* (Steenhoudt and Vanderleyden 2000). These rhizobacteria have been getting a great deal of attention as biofertilizers as they are able to fix nitrogen in association with nonlegume plants and can be used in marginal lands as a low-input way to provide nutrients to crop plants (Boddey et al. 1991).

12.2.3 Cycad-Cyanobacterial Symbiosis

In all genera of cycads, in addition to normal geotropic roots, dichotomously branched apogeotropic roots are formed. These modified perennial lateral roots are referred to as coralloid because of their irregular, beady appearance (Chamberlain 1935). The coralloid roots are infected with endophytic cyanobacteria (blue-green algae). These endophytic algae associated with root tissues have been described as different species of *Anabaena* or *Nostoc* (Costa et al. 1999). The filamentous heterocystous are located in the intercellular spaces of a specialised, hollow, cylindrical zone within the cortex (Lindblad 2009). These symbiotic algae fix atmospheric nitrogen and supply it to the host plant in the form of nitrates. The blue-green algae in association with root tissues produce beneficial amino acids like asparagine and citrulline from the fixed nitrogen.

12.3 Molecular Signalling in Phytomicrobiome

The composition of phytomicrobiome is influenced by the complex matrix of plant-microbe and microbe-microbe communications, which are carried out through the release of signalling compounds. The social network of plants with major root endosymbionts—rhizobia and AM fungi—is decided by the genetic composition of host plants. The major beneficial plant-microbe associations—the rhizobium-legume

symbiosis (RLS) and arbuscular mycorrhiza (AM)—are regulated by a common set of signalling components that act downstream of both fungal and rhizobial signal perception and upstream of the activation of the appropriate response to either symbiont (Oldroyd, 2013). Secondary messengers, reactive oxygen and nitrogen species have also been found to be associated with RLS and AM signalling, but their actual role in relation to the common symbiotic pathway is unclear (Calcagno et al. 2012; Zhang et al. 2013).

12.3.1 Signalling in the Legume-Rhizobia and Mycorrhizal Symbioses: The Common Symbiotic Pathway

The gene regulation of common symbiotic pathway (CSP) has been extensively studied in *Medicago truncatula* and *Lotus japonicus* (Kevei et al. 2007; Shimoda et al. 2012). Early development of rhizobial symbiosis occurs through a chemical communication between the plant and rhizobium, present in the rhizosphere. Host plant roots release flavonoids as a signal to rhizobia. Rhizobia produce nodulation factors (Nod factors) that are recognized by the host plant to activate a CSP required for mycorrhizal and rhizobial symbioses (Fig. 12.1).

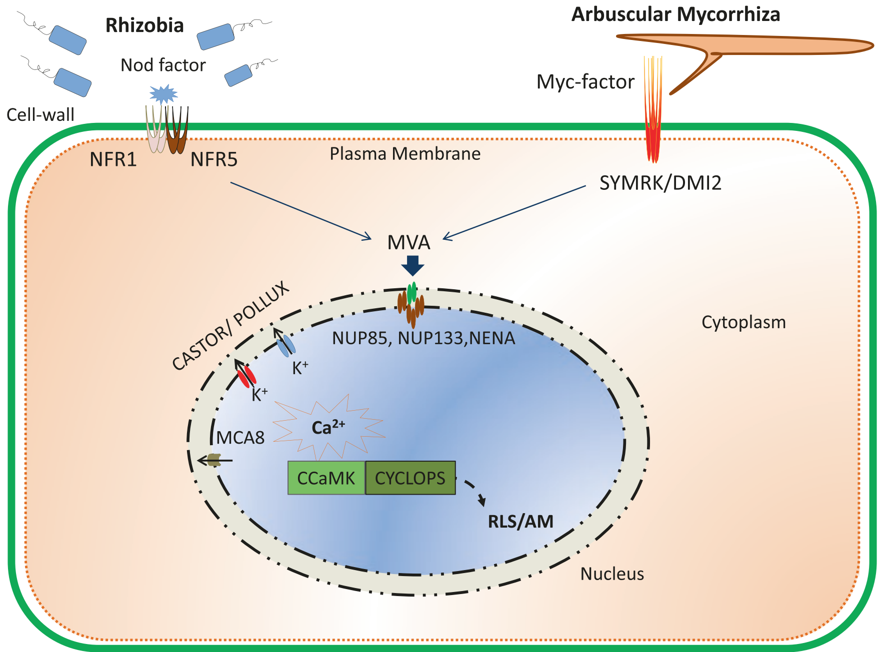


Fig. 12.1 Common signalling pathway in root-nodule symbiosis and arbuscular mycorrhiza (AM)

The first set of CSP proteins includes membrane-bound two LysM (lysin motives) receptor kinases NFR1/LYK3 (*nod factor receptor* 1/ *lysin motif receptor-like kinase* 3), NFR5/NFP (*nod factor receptor5/ nod factor perception*), a leucine-rich receptor kinase DMI2/SYMRK (*does not make infections2/symbiosis receptor kinase*) and the enzyme HMGR1 (HMGR1, 3-hydroxy-3-methylglutaryl-coenzyme A reductase1) (Madsen et al. 2003; Kevei et al. 2007; Lefebvre et al. 2010). NFR1/LYK3, a new common symbiotic gene 13, has also been found to be involved in mycorrhizal symbiosis. Signal transduction pathways are triggered by Nod and Myc factors in legume root cells. Nod- and contact-dependent Myc factors are recognized at the cell surface by receptor-like kinases (NFR1/LYK3, NFR5/NFP and SYMRK/DMI2). As a consequence of HMGR1 activation, mevalonate production can also be localized in the vicinity of the cytoplasmic face of the plasma membrane (Venkateshwaran et al. 2015).

A second cluster of CSP proteins is located on the nuclear membrane, comprising two cation channels (Castor and Pollux) and three nucleoporins (NENA, NUP85 and NUP133) at the core of the nuclear pore (Saito et al. 2007; Charpentier et al. 2008; Groth et al. 2010). These components are required for Nod factor-induced calcium oscillations (Oldroyd 2013). ATP-powered Ca^{2+} pump MCA8 is also bound to the nuclear envelope (Capoen et al. 2011). Intense oscillations in nuclear Ca^{2+} concentration (spiking) are observed during both AM and RLS establishment (Chabaud et al. 2016; Sieberer et al. 2012). Protein phosphorylation leads to Ca^{2+} influxes into the cytoplasm and Ca^{2+} spiking in the perinuclear region, mediated by Castor/Pollux and NUP gene products. Some unidentified channels have been assumed to release Ca^{2+} from the nuclear envelope lumen, which is sustained by the opposite flow of potassium ions (K^+) through Castor/Pollux (Venkateshwaran et al. 2012). MCA8 activity contributes to the restoration of basic nuclear Ca^{2+} concentration. Cytoplasmic Ca^{2+} oscillation has also been found to activate mycorrhiza-specific calcium-dependent protein kinases (MSCaPKs), which activate specific transcription factors (TF2) involved in the regulation of gene expression in AM (Lambais 2006).

The last group of CSP proteins are located in the nucleoplasm. Calcium oscillations are perceived through a nuclear-localized calcium and calmodulin-dependent protein kinase (CCaMK, known as DMI3 in *M. truncatula*) (Shimoda et al. 2012; Poovaiah et al. 2013). Activation of CCaMK induces symbiotic processes and initiates nodule organogenesis (Tirichine et al. 2006; Takeda et al. 2012). The enzyme, CCaMK, associates with and phosphorylates CYCLOPS (known as IPD3 in *M. truncatula*), indispensible for rhizobial and mycorrhizal colonization (Yano et al. 2008). Phosphorylated CYCLOPS regulates gene expression either directly, like in the NIN promoter (Nodule Inception) and induce nodulation in the absence of rhizobia (Singh et al. 2014), or through other transcription factors like NSP1 (*nodulation signalling pathway* 1), NSP2 (*nodulation signalling pathway* 2) and RAM1 (*required for arbuscular mycorrhization* 1) (Oldroyd, 2013).

DELLA proteins have been reported to promote nodule development and infection thread formation during root nodule symbiosis (Jin et al. 2016). These proteins can promote CCaMK–IPD3/CYCLOPS complex formation and increase the

phosphorylation of IPD3/CYCLOPS. DELLAs can form a protein complex with NSP2–NSP1 and are able to bridge a protein complex containing IPD3/CYCLOPS and NSP2. These particular proteins represent a missing link in the common symbiotic signalling pathway required for both rhizobial and mycorrhizal symbioses.

12.3.2 Communication Among Bacterial Community

Bacterial community within the phytomicrobiome communicates among themselves through a molecular signalling pathway, known as quorum sensing (QS). This mechanism is essential for within-species communication as well as for the crosstalk between species which defines the synergistic or antagonistic relationship of bacteria with their host plants (Straight and Kolter 2009). The QS signals in the phytomicrobiome can trigger immune responses and change the hormone profiles in plants, leading to growth responses and disease resistance (Hartmann and Schikora 2012; Hartmann et al. 2014). The QS mechanism regulates distinct microbial activities, including mobility, biofilm formation, virulence, symbiosis, antibiotic production and conjugation (Hartmann et al. 2009). Biofilm formation allows bacteria to adhere to host tissues, improves plant growth and root proliferation (*Azospirillum* in wheat) and acts as a biocontrol agent (*Bacillus subtilis*) (Farrar et al. 2014).

Plant-associated bacteria produce and utilize diffusible QS molecules (e.g. N-acyl homoserine lactones, AHLs) to signal to each other and to regulate their gene expression (Berendsen et al. 2012). Bacterial AHLs have been shown to affect the root development of *Arabidopsis* (Ortiz-Castro et al. 2008) and have been suggested to elicit induced systemic resistance, which allows the plants to resist pathogen attacks. Quorum-sensing circuits of plant-associated bacteria contain homologues of two *Vibrio fischeri* regulatory proteins—LuxI and LuxR. The LuxI-like proteins are responsible for the biosynthesis of AHLs that act as autoinducer and increase with the increasing cell-population density. At high concentrations, AHL binds to cognate cytoplasmic LuxR-like proteins and activates target gene transcription (Zhu and Winans 2001). Plant can also exploit this microbial communication system to manipulate gene expression in their associated microbial communities. LuxR-like proteins in plant-associated bacteria are stimulated by plant signals (Ferluga and Venturi, 2009). Some of the bacteria have the capacity to quench signals by degrading various plant and microbe-produced compounds in the rhizosphere that can negatively affect plants (Quiza et al. 2015).

12.4 Phytomicrobiome Signalling and Plant Growth

Microbial community has been found as one of the significant contributors for plant growth (Schmidt et al. 2014). The commonly recognized mechanisms of plant growth promotion are as follows:

1. Biofertilization
2. Phytohormone production
3. Biocontrol for disease suppression
4. Volatile signal compound production for disease control
5. Induction of plant disease resistance to phytopathogens

12.4.1 Biofertilization

Biofertilizer is the substance containing living microorganisms that promotes growth of host plants by increasing the availability of primary nutrients (Vessey 2003). When biofertilizer is applied to plant surfaces or soil, the microorganisms colonize the rhizosphere of the plant. Plant growth-promoting rhizobacteria (PGPR) act as biofertilizers and protect them from various forms of abiotic stress and soil-borne diseases (Yang et al. 2009). PGPR play a major role in improving plant health by (1) increasing nutrient availability for plants, (2) biological nitrogen fixation and (3) enhancing symbioses (Mabood et al. 2014). Nitrogen-fixing symbiotic rhizobia and nitrogen-fixing bacteria (free-living or associative) are the most commonly used bacterial biofertilizers. PGPR also stimulate plant growth by solubilizing plant growth-promoting nutrients, making it substantially more available to plants. For instance, phosphate-solubilizing bacteria mobilize phosphorus that is one of the essential nutrients in plant growth (Kim et al. 1998; Rodriguez and Fraga 1999). Some rhizobacteria produce siderophores that enhance iron availability to plants (Bloemberg and Lugtenberg 2001). Several other bacteria play a significant role in micronutrient availability to plants (Fasim et al. 2002).

12.4.2 Phytohormone Production

Phytohormones play a significant role in regulating the physiology and growth of crop plants. Therefore, the production of phytohormones by PGPR has extreme agricultural importance. Production of phytohormone—indole-3-acetic acid (IAA) by rhizobacteria—causes plant growth promotion. Selected strains of *Pseudomonas* spp. have been found to promote plant growth and drive developmental plasticity in plant roots by producing IAA (Zamioudis et al. 2013). PGPR also produce cytokinins, important in the control of cell division, chloroplast development and bud formation (Serdyuk et al. 1995). A range of bacterial species including *Proteus mirabilis*, *P. vulgaris*, *Klebsiella pneumoniae*, *Bacillus megaterium*, *B. cereus* and *Azospirillum* spp. have been reported to produce IAA, gibberellin, cytokinin (zeatin) and abscisic acid (Karadeniz et al. 2006). *Rhizobium leguminosarum* strains promote early plant growth and development of canola and lettuce by producing IAA and cytokinins (Noel et al. 1996). Rhizobacteria can also regulate ethylene biosynthesis in plants. Some rhizobacteria produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase that lowers ethylene level in plants by cleaving and hydrolysing plant-produced ACC. Thus, the plants

become more resistant to a wide variety of environmental stresses (Glick 2005). Moreover, PGPR produce salicylates, which are able to induce systemic acquired resistance in colonized plants (Ryals et al. 1996).

12.4.3 Biocontrol for Disease Suppression

Plant growth and development are also enhanced by PGPR. These rhizobacteria control harmful microorganisms through a range of complex mechanisms.

1. Production of broad-spectrum antibiotics
2. Production of narrow-spectrum bacteriocins
3. Production of extracellular lytic enzymes

12.4.3.1 Production of Broad-Spectrum Antibiotics

Antibiotics are compounds recognized by the host plant that activate an immune response via high-affinity cell surface pattern-recognition receptors (Dang et al. 2013). The role of antibiosis in rhizobacteria-mediated control of plant pathogens has been an area of extensive research during the past several decades (Whipps 2001). Pseudomonads produce a wide range of antibiotics including 2,4-diacetylphloroglucinol (DAPG), amphisin, oomycin A, phenazine, hydrogen cyanide, pyoluteorin, pyrrolnitrin and cyclic lipopeptides (Raaijmakers et al. 2002; de Souza et al. 2003; Nielsen and Sorensen 2003). Besides pseudomonads, *Streptomyces*, *Bacillus* and *Stenotrophomonas* spp. have also been reported to produce antibiotic substances, such as oligomycin A, zwittermicin A, kanosamine and xanthobaccin (Milner et al. 1995, 1996; Kim et al. 1999; Nakayama et al. 1999). Antibiotics synthesis by bacteria is affected by several factors, like carbon source, pH of the growth medium, growth temperature and availability of trace elements (Ownley et al. 1992; Duffy and Defago 1997). The presence of particular nutrient types and the age of the host plants also affect the biosynthesis of antibiotics by selected bacterial strain (Picard et al. 2000). The addition of glucose, as a carbon source to *P. fluorescens*, stimulates the biosynthesis of DAPG, while it inhibits the biosynthesis of pyoluteorin, a dominant antimicrobial compound (Duffy and Defago 1999).

12.4.3.2 Production of Narrow-Spectrum Bacteriocins

Bacteriocins are proteinaceous antimicrobial peptides, produced by some rhizobacteria, which show bactericidal or bacteriostatic effects against bacteria closely related to the producer strain (Jack et al. 1995). Recently, it has been shown that *Bacillus thuringiensis* produces some bacteriocins, like nisin, thuricin 17 (T17) and bacthuricin F4 (BF4) that show antimicrobial activity against a broad range of closely related bacterial species (Jung et al. 2011).

12.4.3.3 Production of Extracellular Lytic Enzymes

Biocontrol of plant pathogens is also employed through the production of extracellular lytic enzymes. These cell wall-degrading enzymes exert antifungal activities, contributing to the biocontol activity of the rhizobacteria. The enzymes include chitinases, glucanases, cellulases and proteases that cause lysis and degradation of the fungal cell wall. Some actinomycete isolates have been reported to cause lysis of *Phytophthora* cell walls by hydrolysing cell wall glucans. Cell wall glucan is hydrolysed by producing ß-1,3-glucanase, ß-1,4-glucanase and ß-1,6-glucanase. Thus, causing *Phytophthora fragariae* var. rubi, a pathogen causing raspberry root rot is being controlled (Valois et al. 1996). Chitinase-producing bacteria like *Enterobacter agglomerans* (Chernin et al. 1995), *Bacillus cereus* (Pleban et al. 1997) and *Paenibacillus illinoisensis* (Jung et al. 2003) have decreased the incidence of disease symptoms caused by *Rhizoctonia solani* in cotton and cucumber. *Paenibacillus illinoisensis* also controls the blight disease in pepper (*Capsicum annuum* L.) caused by *Phytophthora capsici* (Jung et al. 2005). Extracellular proteases are also important biocontrol agents. Proteases produced by *Stenotrophomonas maltophilia* W81 are involved in the biocontrol of *Pythium ultimum* in sugar beet (Dunne et al. 1997). *Micromonospora carbonacea* produces cellulase and shows biocontrol activity against *Phytophthora cinnamomi*, the causal organism of root rot in *Banksia grandis* (El-Tarabily et al. 1996).

Lytic enzymes produced by rhizobacteria are of great importance in the biological control of fungal plant pathogens. *Paenibacillus* sp. 300 and *Streptomyces* sp. 385 produce chitinase and ß-1,3-glucanase, which play a crucial role in the biological control of *Fusarium oxysporum* f. sp. *cucumerinum* causing *Fusarium* wilt of cucumber (*Cucumis sativus*) (Singh et al. 1999). *Pseudomonas cepacia* also produces ß-1,3 glucanase that lyses fungal cell walls and decreases the incidence of diseases caused by *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium ultimum* (Fridlender et al. 1993).

12.4.4 Volatile Signal Compound Production for Disease Control

Volatile signal molecules such as methyl salicylate, methyl jasmonate (MeJA) and ethylene play a crucial role in plant communication. These compounds are released at very low concentrations by plants and act as signals to neighbouring plants (Baldwin et al. 2006). Some of the PGPR strains have also been found to communicate with plants by producing volatile compounds that are responsible for plant growth and systemic resistance (Ryu et al. 2003, 2004). The PGPR strains *Bacillus subtilis* GB03 and *Bacillus amyloliquefaciens* IN937a release two volatile compounds, 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol (2,3-B), which promote growth of *Arabidopsis* plants (Ping and Boland 2004).

12.4.5 Induction of Plant Disease Resistance to Phytopathogens

Growth and yield of plants are affected by several biotic stresses, including fungal, bacterial or viral infections. Plants activate diverse defensive pathways to encounter the deleterious effect of stress.

12.4.5.1 Systemic Acquired Resistance

Systemic acquired resistance (SAR) refers to a distinct signal transduction pathway that plays an important role in the ability of plants to defend themselves against pathogens. Activation of SAR pathway is associated with numerous cellular defence responses, such as synthesis of PR (pathogenesis-related) proteins, phytoalexins, accumulation of active oxygen species and rapid alterations in cell wall, which result in the development of a broad-spectrum, systemic resistance (Ryals et al. 1996). Salicylic acid (SA) plays a central role in SAR and its induction of pathogenesis-related proteins in plants (Gaffney et al. 1993). Several rhizobacteria have been described to synthesize SA under iron-limiting conditions, triggering the SAR pathway and inducing systemic resistance in plants (Press et al. 1997). SA produced by *Pseudomonas aeruginosa* 7NSK2 has been reported to activate SAR pathway in bean plants (De Meyer et al. 1999). The SAR pathway acts as a modulator of disease resistance mechanisms by converting a compatible plant-pathogen interaction into an incompatible one.

12.4.5.2 Induced Systemic Resistance

Induced systemic resistance (ISR) is the resistance mechanism activated by biotic or abiotic factors. ISR allows the plants to endure pathogen attack that could be lethal, without the presence of these bacterial factors. Selected strains of PGPRs suppress diseases by antagonism between the bacteria and soil-borne pathogens by inducing a systemic resistance in plants (Choudhary et al. 2007). ISR protects plants that are able to resist subsequent pathogen infections (van Loon 1997). ISR does not provoke a visible hypersensitive response, as it is induced by PGPR (Wei et al. 1991). Rhizobacteria-mediated stimulation of ISR is considered different from SAR, as ISR is not SA dependent and involves jasmonic acid and ethylene in a signal transduction cascade leading to improved disease resistance (Pieterse et al. 1998).

12.5 Plant-Microbe Interaction for Improving the Efficiency of Ecosystem

Abiotic stresses, including drought, extreme temperatures, soil salinity, acidity, alkalinity and heavy metals, cause severe loss in crop yield. Response of leguminous plants to various stresses depends on the host plant reaction, which can be influenced by the rhizobia and the symbiotic process (Yang et al. 2009). Tolerance and nodulating capacity of *Rhizobium* and *Bradyrhizobium* to different abiotic stresses have been elaborately studied (Grover et al. 2010). Mycorrhiza also plays a significant role in the alleviation of soil stresses (Miransari 2010). AM fungi lead to enhanced plant growth and production under different stress conditions (Daei et al. 2009).

12.5.1 Drought

Drought is one of the major abiotic stresses limiting plant growth and productivity under arid and semi-arid conditions (Feng et al. 2002). Plants have acquired various strategies for drought tolerance (Turner et al. 2000). Plants usually overexpress zeatin for delayed leaf senescence as a drought tolerance mechanism (Xu et al. 2012). Production of extracellular polymeric substances or exo-polysaccharides (EPS) by the soil microbes has been reported as one of the drought tolerance mechanisms (Vanderlinde et al. 2010). Microbial intraspecies difference was observed under different water conditions. *Bradyrhizobium* ORS 3257 was found to grow well under favourable water conditions, while *Bradyrhizobium* ORS 3260 grows well under water-stressed conditions (Krasova-Wade et al. 2006).

Mycorrhizal symbiosis also plays a significant role in alleviating drought stress. Mycorrhizal plants can grow much better under drought conditions (Subramanian et al. 2006). AM influence plant growth under water stress by stabilizing soil structure through the binding of soil particles with glomalin that alters soil structure and moisture retention capability (Auge 2001). Moreover, higher uptake of nutrient, enhancement of root surface area and dense root growth improve the drought tolerance of mycorrhizal plants (Subramanian et al. 2006). Under drought stress, mycorrhiza contributes in plant water movement, influencing hydration and physiological processes of plants (Auge 2001). Mycorrhizal plants under stress conditions can absorb several forms of nitrogen that are unavailable to plants, causing higher plant growth (Subramanian et al. 2006). Under drought conditions, mycorrhizal plants increase biomass through the higher accumulation of organic products, like proline, glycine-betaine, carbohydrates (sucrose, mannitol) and inorganic ions (K^+, Cl^-) (Ruiz-Lozano et al. 2006). AM symbiosis enhances drought tolerance of host plant through altering plant physiology and gene expression (Ruiz-Lozano et al. 2006). Mycorrhizal plants also produce antioxidant enzymes under drought stress that reduce the effect of stress and sustain plant growth (Ruiz-Lozano 2003).

12.5.2 Extreme Temperatures

Agricultural cultivation is often exposed to different abiotic stresses including extreme temperatures affecting crop productivity. High temperatures cause enhanced transpirational water loss leading to drought stress, which results in delay in nodulation, reduced nodule number, rhizobial growth, rate of colonization and infection events. The optimum temperature for rhizobial growth has been reported to be 28–31°C. Rhizobia grown under high temperature have been found to lose their infectiveness and symbiotic properties (Hartel and Alexander 1984). Some of the N_2 fixing *Rhizobium* strains have been reported to be heat-tolerant (Hungria and Franco 1993). Stress adaptation of microorganisms involves the lipopolysaccharide (LPS) and extracellular polymeric substances/exo-polysaccharides (EPS) (Nandal et al. 2005). Small heat-shock proteins have also been found to play significant roles in heat resistance of microbial associates of the phytomicrobiome

(Michiels et al. 1994; Munchbach et al. 1999). Higher induction of chaperone genes have also been observed in heat-tolerant isolates than in heat-sensitive isolates of the same species, which help the hydrophobic domains of the target protein to regain their native structure since they get denatured upon stress (Hartl and Hayer-Hartl 2009; Alexandre and Oliveira 2011).

Mycorrhizal fungi have been found to ameliorate temperature stress in thermophilic plants (Bunn et al. 2009). Elevated temperatures limit plants' available habitat by inhibiting their root growth. AM fungi can extend extra-radical hyphae into the soils that increase host plants' access to water and nutrients by extending the growth of extra-radical hyphae into the soils.

12.5.3 Salinity

Salinity is the most detrimental abiotic stress for sustainable agricultural production in the arid and semi-arid tropical ecosystems and accounts for about 40% of the world's land surface (Zhan et al. 1991). Salinity disturbs ion homeostasis of plants and interferes with internal solute balance (Kumar et al. 2009). Salinity affects bacterial infection process, nodule growth and biological nitrogen fixation (Zhang et al. 1991). *Rhizobial* species have been found to exhibit variation in their salt sensitivity, such as *R. meliloti* (Zhang et al. 1991) and *R. japonicum* (Yelton et al. 1983) which are salt tolerant, whereas *R. leguminosarum* is salt sensitive (Chein et al. 1992). Salinity tolerance in rhizobia is associated with the production of compatible solutes [trehalose, N-acetyl glutaminyl glutamine amide (NAGGN) and glutamate], osmoprotectants [betaine, glycine-betaine, proline-betaine, glucans, trehalose, sucrose, ectoine, 3-dimethyl sulfoniopropionate (3-dimethylpropiothetin or DMSP), 2-dimethyl sulfonioacetate (2-dimethyl thetin or DMSA)] and pipecolic acid and cations (Ca^{2+}, K^{+}) (Chen 2011; Streeter 2003; Sugawara et al. 2010). Salt tolerance also comprises a number of gene families, including the production of glycine-betaine (AraC) and proline-betaine (betS/prb) (Boscari et al. 2004), sucrose (zwf) and trehalose (zwf) (Barra et al. 2003) and cation efflux (phaA2/phaD2/phaF2/phaG2) (Jiang et al. 2004).

Arbuscular mycorrhizal fungi have significant contribution in alleviation of salt stress. Increased salinity increases the dependency of plants on AM symbiosis indicating the influence of AM to reduce the effect of salinity stress (Tian et al. 2004). AM biologically enhance plant growth and crop production under salinity stress (Daei et al. 2009). AM enhance salt tolerance in host plants by improving of nutrient (N and P) uptake, improved leaf respiration and transpiration that increases the gaseous (carbon dioxide and water vapour) exchange through stomata and eventually affect water use efficiency of host plants. AM also increase the concentration of osmolytes (carbohydrates and electrolytes) in plant roots that can alleviate salinity stress on plant (Daei et al. 2009). AM improve leaf chlorophyll content under salinity stress by minimizing the inhibitory effect of Na on magnesium (Mg) absorption photosynthesis and increase Mg uptake which is necessary for chlorophyll formation (Miransari 2009). AM also stimulate root development and enhance nutrient

uptake by increasing root hydraulic conductivity (Giri et al. 2003). AM also stabilize the K^+/Na^+ ratio under saline conditions and increase K^+ uptake resulting in sustainable plant growth (Giri et al. 2003; Daei et al. 2009).

12.5.4 Soil Acidity

Soil acidity (low pH) is another abiotic stress that leads to crop failures by limiting plant growth and productivity. Soil pH reduces the mobilization and availability of nutrients to plants (Haynes 1990; Marschner 1995). High concentration of protons in the acid soil results in the failure of rhizobia-legume symbiosis (Richardson et al. 1988). Some strains of *Rhizobium*, *Azorhizobium* and *Bradyrhizobium* are low pH tolerant due to the production of extracellular polysaccharide or polyamines (glutamate) concentration in the cell (Graham et al. 1994). Acid tolerant *R. leguminosarum* bv. *trifolii* has been reported to accumulate higher level of potassium and phosphorus than an acid-sensitive strain (Watkin et al. 2003).

Mycorrhizal fungi modify the pH of their root environment. For instance, *Scutellospora calospora* formed detectable extra-radical mycelium at lower pH (van Aarle et al. 2002). Hayman and Tavares (1985) have reported AM fungi *Glomus clarum* to stimulate plant growth at considerably low pH (pH 4). The AM-colonized plants grown on acidic soils have been observed to accumulate increased K, Ca and Mg as compared to neutral or alkaline soils (Harrier and Watson 2003). Low soil pH reduces phosphorus (P) availability for plants which is a major element for plant growth and development. AM fungi increase host plants' access to limiting nutrients P in acid soils (Seguel et al. 2013).

12.5.5 Heavy Metals

Industrial revolution has increased the environmental pollution by the toxic metals due to the dumping of solid wastes and use of industrial waste waters for irrigation. Heavy metals damage plant growth and productivity. Heavy metals are non-degradable in nature and thus often enter the food chain causing serious illness to human beings. Bioremediation is one of the popular practices to control the heavy metal accumulation in the soil (Gianfreda and Rao 2004). Microorganisms including different species of *Bacillus*, *Pseudomonas*, *Azotobacter*, *Enterobacter* and *Rhizobium* have been found to accelerate the process of phytoremediation (Ma et al. 2011). Increasing concentrations of heavy metals have been found to alter the expression of symbiotic nod genes in *Rhizobium* sp. (Stan et al. 2011). Involvement of EPS and LPS was also found in *Rhizobia* for influencing heavy metal resistance by forming complexes with metal ions through electrostatic interactions (Liu et al. 2001).

Mycorrhiza plays a crucial role in reducing the heavy metal concentrations of the soil. The AM hyphae allow the fungus to absorb high levels of heavy metals from the rhizosphere and consequently decrease its translocation from plant roots to shoots (phytostabilization) (Leyval et al. 2002). Symbiotic association of

metallophytes (plants that can grow under heavy metals stress) with AM reduces heavy metal uptake by the plants and improves their ability to grow under heavy metal stress (Berreck and Haselwandter 2001). AM keep heavy metals out of plants or reduce concentrations in plants through immobilization of heavy metals by binding with insoluble glycoprotein (glomalin) produced by AM hyphae (Hildebrandt et al. 2007). The AM colonization with root of metallophytes leads to the expression of metallothionein proteins producing genes that increase the heavy metal tolerance of plants (Rivera-Becerril et al. 2005). Different species of AM, including *Glomus intraradices*, are able to enhance tolerance of plants such as tomato, corn and *Medicago truncatula* to heavy metal stress (Wulf et al. 2003; Hildebrandt et al. 2007). The AM symbiosis can regulate the transcription of a number of genes involved in heavy metal tolerance, including metal transporter genes (Hildebrandt et al. 2007). However, AM also produce antioxidant enzymes, including glutathione S-transferase, superoxide dismutase, cytochrome P450 and thioredoxin that alleviate the stress of reactive oxygen species (ROS), thus decreasing the oxidative stress of heavy metals on plants (Hildebrandt et al. 2007). Thus, the combined effects of bacteria and AM can enhance plant tolerance to heavy metals by promoting plant growth (through phytohormone IAA production) and increasing AM activity in heavy metal containing soils (Vivas et al. 2003).

12.5.6 Pesticides

Frequent application of pesticide and their slow degradation rate lead to their accumulation in the soil that affects plant growth by altering morpho-physiological features of plants. Use of pesticide beyond the recommended level reduces microbial density on soil and also affects the growth of nitrogen-fixing bacteria (Martinez-Toledo et al. 1996; Mathur 1999). Several microorganisms have been reported to have the ability to degrade the pesticides due to the presence of degradative genes in their plasmids/transposons/chromosomes (Kumar et al. 1996).

The chemical residues of several pesticides used worldwide have exceeded the food safety standards as they create public health risk. AM fungi play a significant role in organic uptake and translocation by plants, dissipation and degradation of organics in soils including atrazine, PAHs (polycyclic aromatic hydrocarbon), DDT and weathered p,p-DDE in soils (Joner et al. 2001; White et al. 2006; Wu et al. 2008; Huang et al. 2009). The extra-radical hyphae of *G. intraradices* have been reported to hydrolyse 5-bromo-4-chloro-3-indolyl phosphate and phenolphthalein diphosphate (Koide and Kabir 2000). AM colonization of *Cynara cardunculus* has been found to be unaffected by phoxim (Marin et al. 2002). Thus, AM fungi contribute to enhanced biodegradation of organophosphorus pesticides in soils (Wang et al. 2011). Furthermore, AM fungi are widely used as natural fertilizers, as they provide mineral nutrients and water to their host plants (Smith and Read 2008). AM fungal hyphae colonize the host plant's root cortex and form highly branched structures that mediate the nutrient exchange (Balestrini et al. 2015). AM fungi also improve the soil structure and aggregation (Rillig et al. 2015)

12.6 Conclusion

Phytomicrobiome is a complex, structured and dynamic community that can improve the overall efficiency of an ecosystem by influencing plant growth, health, productivity and functions. Therefore, symbiotic plant-microbe associations can be successfully applied for improving the agricultural strategies to attain food security. Exploration of novel 'omics tools will certainly contribute in more sustainable agriculture.

References

Alexandre A, Oliveira S (2011) Most heat-tolerant rhizobia show high induction of major chaperone genes upon stress. FEMS Microbiol Ecol 75:28–36

Auge RM (2001) Water relations, drought and vesicular arbuscular mycorrhizal symbiosis. Mycorrhiza 11:3–42

Baldwin IT, Halitschke R, Paschold A, von Dahl CC, Presto CA (2006) Volatile signaling in plant-plant interactions: "Talking Trees" in the genomics era. Science 311:812–815

Balestrini R, Lumini E, Borriello R, Bianciotto V (2015) Plant-soil biota interactions. In: Paul EA (ed) Soil microbiology, ecology and biochemistry. Academic Press; Elsevier, London, pp 311–338

Barra L, Pica N, Gouffi K, Walker GC, Blanco C, Trautwetter A (2003) Glucose 6-phosphate dehydrogenase is required for sucrose and trehalose to be efficient osmoprotectants in *Sinorhizobium meliloti*. FEMS Microbiol Lett 229:183–188

Berendsen RL, Pieterse CMJ, Bakker PAHM (2012) The rhizosphere microbiome and plant health. Trends Plant Sci 8:478–486

Berg G, Zachow C, Müller H, Philipps J, Tilcher R (2013) Next-generation bio-products sowing the seeds of success for sustainable agriculture. Agronomy 3:648–656

Berg G, Grube M, Schloter M, Smalla K (2014) Unraveling the plant microbe: looking back and future perspectives. Front Microbiol 5:148

Berreck M, Haselwandter K (2001) Effect of the arbuscular mycorrhizal symbiosis upon uptake of cesium and other cations by plants. Mycorrhiza 10:275–280

Bloemberg GV, Lugtenberg BJJ (2001) Molecular basis of plant growth promotion and biocontrol by rhizobacteria. Curr Opin Plant Biol 4:343–350

Boddey RM, Urquiaga S, Reis V, Dobereiner J (1991) Biological nitrogen fixation associated with sugar cane. Plant Soil 137:111–117

Bonfante P, Genre A (2008) Plants and arbuscular mycorrhizal fungi: an evolutionary developmental perspective. Trends Plant Sci 9:402–498

Boscari A, Mandon K, Poggi MC, Le Rudulier D (2004) Functional expression of Sinorhizobium meliloti BetS, a high-affinity betaine transporter, in Bradyrhizobium japonicum USDA110. Appl Environ Microbiol 70:5916–5922

Bragina A, Berg C, Müller H, Moser D, Berg G (2013) Insights into functional bacterial diversity and its effects on Alpine bog ecosystem functioning. Sci Rep 3:1955

Brem D, Leuchtmann A (2002) Intraspecific competition of endophyte infected vs uninfected plants of two woodland grass species. Oikos 96:281–290

Bunn R, Lekberg Y, Zabinski C (2009) Arbuscular mycorrhizal fungi ameliorate temperature stress in thermophilic plants. Ecology 90(5):1378–1388

Calcagno C, Novero M, Genre A, Bonfante P, Lanfranco L (2012) The exudate from an arbuscular mycorrhizal fungus induces nitricoxide accumulation in *Medicago truncatula* roots. Mycorrhiza 22:259–269

Capoen W, Sun J, Wysham D, Otegui MS, Venkateshwaran M, Hirsch S, Miwa H, Downie JA, Morris RJ, Ane JM, Oldroyd GE (2011) Nuclear membranes control symbiotic calcium signaling of legumes. Proc Natl Acad Sci U S A 108:14348–14353

Chabaud M, Gherbi H, Pirolles E, Vaissayre V, Fournier J, Moukouanga D, Franche C, Bogusz D, Tisa LS, Barker DG, Svistoonoff S (2016) Chitinase-resistant hydrophilic symbiotic factors secreted by *Frankia* activate both Ca^{2+} spiking and NIN gene expression in the actinorhizal plant *Casuarina glauca*. New Phytol 209:86–93

Chamberlain CJ (1935) Gymnosperms, structure and function. University of Chicago Press, Chicago

Charpentier M, Myriam Charpentier A, Bredemeier R, Wanner G, Takeda N, Schleiff E, Parniske M (2008) *Lotus japonicus* CASTOR and POLLUX are ion channels essential for perinuclear calcium spiking in legume root endosymbiosis. Plant Cell 20:3467–3479

Chein CT, Maundu J, Cavaness J, Daudurand LM, Orser CS (1992) Characterization of salt-tolerant and salt-sensitive mutants of *Rhizobium leguminosarum* biovar *viciae* strain C1204b. FEMS Microbiol Lett 90:135–140

Chen P (2011) Symbiotic effectiveness, competitiveness and salt tolerance of lucerne rhizobia. RMIT University, Australia

Chernin L, Ismailov Z, Haran S, Chet I (1995) Chitinolytic Enterobacter agglomerans antagonistic to fungal plant pathogens. App Env Microbiol 61:1720–1726

Choudhary DK, Prakash A, Johri BN (2007) Induced systemic resistance (ISR) in plants: mechanism of action. Indian J Microbiol 47:289–297

Costa JL, Paulsrud P, Lindblad P (1999) Cyanobiont diversity within coralloid roots of selected cycad species. FEMS Microbiol Ecol 28:85–91

Daei G, Ardekani M, Rejali F, Teimuri S, Miransari M (2009) Alleviation of salinity stress on wheat yield, yield components, and nutrient uptake using arbuscular mycorrhizal fungi under field conditions. J Plant Physiol. 166:217–225

Dang JL, Horvath DM, Staskawicz BJ (2013) Pivoting the plant immune system from dissection to deployment. Science 341:746–751

De Meyer G, Capieau K, Audenaert K, Buchala A, Metraux JP, Hofte M (1999) Nanogram amounts of salicylic acid produced by the rhizobacterium *Pseudomonas aeruginosa* 7NSK2 activate the systemic acquired resistance pathway in Bean. Mol Plant-Microbe Interact 12:450–458

de Souza JT, de Boer M, de Waard P, van Beek TA, Raaijmakers JM (2003) Biochemical, genetic, and zoosporicidal properties of cyclic lipopeptide surfactants produced by *Pseudomonas fluorescens*. Appl Environ Microbiol. 69:7161–7172

Duffy BK, Defago G (1997) Zinc improves biocontrol of *Fusarium crown* and root rot of tomato by *Pseudomonas fluorescens* and represses the production of pathogen metabolites inhibitory to bacterial antibiotic biosynthesis. Phytopathology 87:1250–1257

Duffy BK, Defago G (1999) Environmental factors modulating antibiotic and siderophore biosynthesis by *Pseudomonas fluorescens* biocontrol strains. Appl Environ Microbiol 65:2429–2438

Dunne C, Crowley JJ, Moenne-Loccoz Y, Dowling DN, de Bruijn FJ, O'Gara F (1997) Biological control of *Pythium ultimum* by *Stenotrophomonas maltophilia* W81 is mediated by an extracellular proteolytic activity. Microbiology 143:3921–3931

East R (2013) Soil science comes to life: Plants may be getting a little help with their tolerance of drought and heat. Nature 501:18–19

El-Tarabily KA, Sykes ML, Kurtboke ID, Hardy GE, St J, Barbosa AM, Dekker RFH (1996) Synergistic effects of a cellulase-producing *Micromonospora carbonacea* and an antibiotic-producing *Streptomyces violascens* on the suppression of Phytophthora cinnamomi root rot of *Banksia grandis*. Can J Bot 74:618–624

Farrar K, Bryant D, Cope-Selby N (2014) Understanding and engineering beneficial plant-microbe interactions: plant growth promotion in energy crops. Plant Biotechnol J 12:1193–1206

Fasim F, Ahmed N, Parsons R, Gadd GM (2002) Solubilization of zinc salts by a bacterium isolated from the air environment of a tannery. FEMS Microbiol Lett 213:1–6

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

Ferluga S, Venturi V (2009) OryR is a LuxR-Family protein involved in interkingdom signalling between pathogenic *Xanthomonas oryzae* pv. *oryzae* and Rice. J Bacteriol 191:890–897

Fridlender M, Inbar J, Chet I (1993) Biological control of soilborne plant pathogens by a b-1,3-glucanase-producing *Pseudomonas cepacia*. Soil Biol Biochem 25:1211–1221

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261:754–756

Gianfreda L, Rao MA (2004) Potential of extra cellular enzymes in remediation of polluted soils: a review. Enzyme Microb Technol 35:339–354

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 Soils 38:170–175

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

Graham PH, Draeger KJ, Ferrey ML, Conroy MJ, Hammer BE, Martínez E, Aarons SR, Quinto C (1994) Acid pH tolerance in strains of *Rhizobium* and *Bradyrhizobium*, and initial studies on the basis for acid tolerance of *Rhizobium tropici* UMR1899. Can J Microbiol 40:198–207

Groth M, Takeda N, Perry J, Uchida H, Draxl S, Brachmann A, Sato S, Tabata S, Kawaguchi M, Wang TL, Parniske M (2010) NENA, a *Lotus japonicus* homolog of Sec13, is required for rhizodermal infection by arbuscular mycorrhiza fungi and rhizobia but dispensable for cortical endosymbiotic development. Plant Cell 22:2509–2526

Grover M, Ali SZ, Sandhya V, Rasul A, Venkateswarlu B (2010) Role of microorganisms in adaptation of agriculture crops to abiotic stresses. World J Microbiol Biotechnol 27:1231–1240

Harrier LA, Watson CA (2003) The role of arbuscular mycorrhizal fungi in sustainable cropping systems. Adv Agron 79:185–225

Harrison MJ (2005) Signaling in the arbuscular mycorrhizal symbiosis. Annu Rev Microbiol 59:19–42

Hartel PG, Alexander M (1984) Temperature and desiccation tolerance of cowpea rhizobia. Can J Microbiol 30:820–823

Hartl FU, Hayer-Hartl M (2009) Converging concepts of protein folding in vitro and in vivo. Nat Struct Mol Biol 16:574–581

Hartmann A, Schikora A (2012) Quorum sensing of bacteria and trans-kingdom interactions of N-acylhomoserine lactones with eukaryotes. J Chem Ecol 38:70–713

Hartmann A, Schmid M, Tuinen D, Berg G (2009) Plant-driven selection of microbes. Plant Soil 321:235–257

Hartmann A, Rothballer M, Hense BA, Schroder P (2014) Bacterial quorum sensing compounds are important modulators of microbe-plant interactions. Front Plant Sci 5:131

Hayman DS, Tavares M (1985) Plant growth responses to vesicular arbuscular mycorrhiza. New Phytol 100:367–377

Haynes RJ (1990) Active ion uptake and maintenance of cation-anion balance: a critical examination of their role in regulating rhizosphere pH. Plant Soil 126:247–264

Hildebrandt U, Regvar M, Bothe H (2007) Arbuscular mycorrhiza and heavy metal tolerance. Phytochemistry 68:139–146

Huang HL, Zhang SZ, Wu NY, Luo L, Christie P (2009) Influence of *Glomus etunicatum*/*Zea mays* mycorrhiza on atrazine degradation, soil phosphatase and dehydrogenase activities, and soil microbial community structure. Soil Biol Biochem 41:726–734

Hungria M, Franco AA (1993) Effects of high temperature on nodulation and nitrogen fixation by Phaseolus vulgaris L. Plant Soil 149:95–102

Jack WE, Tagg JR, Ray B (1995) Bacteriocins of Gram-positive bacteria. Microbiol Rev 59:171–200

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

Jin Y, Liu H, Luo D, Yu N, Dong W, Wang C, Zhang X, Dai H, Yang J, Wang E (2016) DELLA proteins are common components of symbiotic rhizobial and mycorrhizal signalling pathways. Nat Commun 7:12433–12446

Joner EJ, Johansen A, Loibner AP, de la Cruz MA, Szolar OH, Portal JM, Leyval C (2001) Rhizosphere effects on microbial community structure and dissipation and toxicity of polycyclic aromatic hydrocarbons (PAHs) in spiked soil. Environ Sci Technol 35(13):2773–2777

Jung WJ, An KN, Jin YL, Park RD, Lim KT, Kim KY, Kim TH (2003) Biological control of damping-off caused by *Rhizoctonia solani* using chitinase producing *Paenibacillus illinoisensis* KJA-424. Soil Biol Biochem 35:1261–1264

Jung WJ, Jin YL, Kim KY, Park RD, Kim TH (2005) Changes in pathogenesis-related proteins in pepper plants with regard to biological control of phytopthora blight with Paenibacillus illinoisensis. Biocontrol 50:165–178

Jung WJ, Mabood F, Souleimanov A, Smith DL (2011) Induction of defense-related enzymes in soybean leaves by class IId bacteriocins (thuricin 17and bacthuricin F4) purified from *Bacillus* strains. Microbiol Res 167:14–19

Karadeniz A, Topeuoglu SF, Inan S (2006) Auxins, gibberellin, cytokinin and abscisic acid production in some bacteria. World J Microbiol Biotechnol 22:1061–1064

Kembel SW, O'Connor TK, Arnold HK, Hubbell SP, Wright SJ, Green JL (2014) Relationships between phyllosphere bacterial communities and plant functional traits in a neotropical forest. Proc Natl Acad Sci U S A 38:13715–13720

Kevei Z, Lougnon G, Mergaert P, Horvath GV, Kereszt A, Jayaraman D, Zaman N, Marcel F, Regulski K, Kiss GB, Kondorosi A, Endre G, Kondorosi E, Ane JM (2007) 3-hydroxy-3-methylglutaryl coenzyme A reductase1 interacts with NORK and is crucial for nodulation in *Medicago truncatula*. Plant Cell 19:3974–3989

Kim KY, Jordan D, McDonald GA (1998) Effect of phosphate solubilizing bacteria and vesicular-arbuscular-mycorrhizae on tomato growth and soil microbial activity. Biol Fertil Soil 26:79–87

Kim BS, Moon SS, Hwang BK (1999) Isolation, identification and antifungal activity of a macrolide antibiotic, oligomycin A, produced by *Streptomyces libani*. Can J Bot 77:850–858

Knack JJ, Wilcox LW, Delaux PM, Ane JM, Piotrowski MJ, Cook ME, Graham JM, Graham LE (2015) Microbiomes of streptophyte algae and bryophytes suggest that a functional suite of microbiota fostered plant colonization of land. Int J Plant Sci 176:405–420

Koide RT, Kabir Z (2000) Extraradical hyphae of the mycorrhizal fungus Glomus intraradices can hydrolyse organic phosphate. New Phytol. 148:511–517

Krasova-Wade T, Diouf O, Ndoye I, Sall CE, Braconnier S, Neyra M (2006) Water condition effects on rhizobia competition for cowpea nodule occupancy. African J Biotech 5:1457–1463

Kumar S, Mukerji KG, Lai R (1996) Molecular aspects of pesticide degradation by microorganisms. Crit Rev Microbiol 22:1–26

Kumar G, Purty RS, Sharma MP, Singla-Pareek SL, Pareek A (2009) Physiological responses among *Brassica* species under salinity stress show strong correlation with transcript abundance for SOS pathway-related genes. J Plant Physiol 166:507–520

Lambais MR (2006) Unraveling the signalling and signal transduction mechanisms controlling arbuscular mycorrhiza development. Sci Agric 63(4):405–413

Lefebvre B, Timmers T, Mbengue M, Moreau S, Herve C, Toth K, Bittencourt-Silvestre J, Klaus D, Deslandes L, Godiard L, Murray JD, Udvardi MK, Raffaele S, Mongrand S, Cullimore J, Gamas P, Niebel A, Ott T (2010) A remorin protein interacts with symbiotic receptors and regulates bacterial infection. Proc Natl Acad Sci U S A 107:2343–2348

Leyval C, Joner EJ, del Val C, Haselwandter K (2002) Potential of arbuscular mycorrhizal fungi for bioremediation. In: Gianinazzi S, Schuepp JM, Barea JM, Haselwandter K (eds) Mycorrhizal technology in agriculture, from genes to bioproducts. Birkhauser Verlag, Basel, Switzerland, pp 175–186

Lindblad P (2009) Cyanobacteria in symbiosis with Cycads in prokaryotic symbionts in plants. Microbiol Monogr 8:225–233. Pawlowski K

Liu Y, Lam MC, HHP F (2001) Adsorption of heavy metals by EPS of activated sludge. Water Sci Technol 43:59–66

Liu H, Chen W, Wu M, Wu R, Zhou Y, Gao Y, Ren A (2017) Arbuscular mycorrhizal fungus inoculation reduces the drought-resistance advantage of endophyte-infected versus endophyte-free *Leymus chinensis*. Mycorrhiza 27(8):791–799

Lundberg DS, Lebeis SL, Paredes SH, Yourstone S, Gehring J, Malfatti S, Tremblay J, Engelbrektson A, Kunin V, del Rio TG, Edgar RC, Eickhorst T, Ley RE, Hugenholtz P, Tringe SG, Dangl JL (2012) Defining the core *Arabidopsis thaliana* root. Nature 488:86–90

Ma Y, Prasad MNV, Rajkumar M, Freitas H (2011) Plant growth promoting rhizobacteria and endophytes accelerate phytoremediation of metalliferous soils. Biotechnology Adv 29(2):248–258

Mabood F, Zhou X, Smith DL (2014) Microbial signaling and plant growth promotion. Can J Plant Sci 94:1051–1063

Madsen EB, Madsen LH, Radutoiu S, Olbryt M, Rakwalska M, Szczyglowski K, Sato S, Kaneko T, Tabata S, Sandal N, Stougaard J (2003) A receptor kinase gene of the LysM type is involved in legume perception of rhizobial signals. Nature 425:637–640

Marin M, Ybarra M, Fe A, Garcia-Ferriz L (2002) Effect of arbuscular mycorrhizal fungi and pesticides on Cynara cardunculus growth. Agric Food Sci Finland. 11:245–251

Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic Press Ltd, London

Martinez-Toledo MV, Salmeron V, Rodelas B, Pozo C, Gonzalez-Lopez J (1996) Studies on the effects of the herbicide simazine on microflora of four agricultural soils. Environ Toxicol Chem 15:1115–1118

Mathur SC (1999) Future of Indian pesticides industry in next millennium. Pestic Inf 24:9–23

Michiels J, Verreth C, Vanderleyden J (1994) Effects of temperature stress on bean nodulating *Rhizobium* strains. Appl Environ Microbiol 60:1206–1212

Milner JL, Raffel SJ, Lethbridge BJ, Handelsman J (1995) Culture conditions that influence accumulation of zwittermicin A by *Bacillus cereus* UW85. Appl Microbiol Biotechnol 43:685–691

Milner JL, Silo-Suh L, Lee JC, He H, Clardy J, Handelsman J (1996) Production of kanosamine by *Bacillus cereus* UW85. Appl Environ Microbiol 62:3061–3065

Miransari M (2009) Contribution of arbuscular mycorrhizal symbiosis to plant growth under different types of soil stress. Plant Biol 12:563–569

Miransari M (2010) Contribution of arbuscular mycorrhizal symbiosis to plant growth under different types of soil stress. Plant Biol 12:563–569

Munchbach M, Nocker A, Narberhaus F (1999) Multiple small heat shock proteins in *Rhizobia*. J Bacteriol 181:83–90

Nakayama T, Homma Y, Hashidoko Y, Mizutani J, Tahara S (1999) Possible role of xanthobaccins produced by *Stenotrophomonas* sp. strain SB-K88 in suppression of sugar beet damping-off disease. Appl Environ Microbiol 65:4334–4339

Nandal K, Sehrawat AR, Yadav AS, Vashishat RK, Boora KS (2005) High temperature-induced changes in exo-polysaccharides, lipopolysaccharides and protein profile of heat-resistant mutants of *Rhizobium* sp. (*Cajanus*). Microbiol Res 160:367–373

Newman EI, Reddell P (1987) The distribution of mycorrhizas among families of vascular plants. New Phytol 106:745–751

Nielsen TH, Sorensen J (2003) Production of cyclic lipopeptides by *Pseudomonas fluorescens* strains in bulk soil and in the sugar beet rhizosphere. Appl Environ Microbiol 69:861–868

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

Oldroyd GED (2013) Speak, friend, and enter: signalling systems that promote beneficial symbiotic associations in plants. Nat Rev Microbiol 11:252–263

Ortiz-Castro R, Martinez-Trujillo M, Lopez-Bucio J (2008) N-acyl-L-homoserine lactones: a class of bacterial quorum-sensing signals alter post-embryonic root development in *Arabidopsis thaliana*. Plant Cell Environ 31:1497–1509

Ownley BH, Weller DM, Thomashow LS (1992) Influence of in situ and in vitro pH on suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens*. 2–79. Phytopathology 82:178–184

Picard C, Di Cello F, Ventura M, Fani R, Guckert A (2000) Frequency and biodiversity of 2,4-diacetylphloroglucinol-producing bacteria isolated from the maize rhizosphere at different stages of plant growth. Appl Environ Microbiol 66:948–955

Pieterse CMJ, Van Wees SCM, Van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ, van Loon LC (1998) A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. Plant Cell 10:1571–1580

Ping L, Boland W (2004) Signals from the underground: bacterial volatiles promote growth in *Arabidopsis*. Trends Plant Sci 9:263–266

Pleban S, Chernin L, Chet I (1997) Chitinolytic activity of an endophytic strain of *Bacillus cereus*. Lett Appl Microbiol 25:284–288

Poovaiah BW, Du L, Wang H, Yang T (2013) Recent advances in calcium/calmodulin-mediated signaling with an emphasis on plant: microbe interactions. Plant Physiol 163:531–542

Press CM, Wilson M, Tuzun S, Kloepper JW (1997) Salicylic acid produced by *Serratia marcescens* 90-166 is not the primary determinant of induced systemic resistance in cucumber or tobacco. Mol Plant-Microbe Interact 10:761–768

Quiza L, St-Arnaud M, Yergeau E (2015) Harnessing phytomicrobiome signalling for rhizosphere microbiome engineering. Front Plant Sci 6:507

Raaijmakers JM, Vlami M, de Souza JT (2002) Antibiotic production by bacterial biocontrol agents. A Van Leeuw J Microb 81:537–547

Redeker D, Kodner R, Graham L (2000) Glomalean fungi from the Ordovician. Science 289:1920–1921

Richardson AE, Henderson AP, James GS, Simpson RJ (1988) Consequences of soil acidity and the effect of lime on the nodulation of *Trifolium subterraneum* L. growing in an acid soil. Soil Biol Biochem 20:439–445

Riely BK, Mun JH, Ane JM (2006) Unravelling the molecular basis for symbiotic signal transduction in legumes. Mol Plant Pathol 7:197–207

Rillig MC, Maestre FT, Lamit LJ (2003) Microsite differences in fungal hyphal length, glomalin, and soil aggregate stability in semiarid Mediterranean steppes. Soil Biol Biochem 35:1257–1260

Rillig MC, Aguilar-Trigueros CA, Bergmann J, Verbruggen E, Veresoglou SD, Lehmann A (2015) Plant root and mycorrhizal fungal traits for understanding soil aggregation. New Phytol 205:1385–1388

Rivera-Becerril F, van Tuinen D, Martin-Laurent F, Metwally A, Dietz KJ, Gianinazzi S, Gianinazzi-Pearson V (2005) Molecular changes in *Pisum sativum* L. roots during arbuscular mycorrhiza buffering of cadmium stress. Mycorrhiza 16:51–60

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

Ruiz-Lozano JM (2003) Arbuscular mycorrhizal symbiosis and alleviation of osmotic stress. New perspectives for molecular studies. Mycorrhiza 13:309–317

Ruiz-Lozano JM, Porcel R, Aroca R (2006) Does the enhanced tolerance of arbuscular mycorrhizal plants to water deficit involve modulation of drought-induced plant genes? New Phytol 171:693–698

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner HY, Hunt MD (1996) Systemic acquired resistance. Plant Cell 8:1809–1819

Ryu CM, Farag MA, Hu CH, Reddy MS, Wei HX, Pare PW, Kloepper JW (2003) Bacterial volatiles promote growth in Arabidopsis. Proc Natl Acad Sci USA 100:4927–4932

Ryu CM, Farag MA, Hu CH, Reddy MS, Kloepper JW, Pare PW (2004) Bacterial volatiles induce systemic resistance in *Arabidopsis*. Plant Physiol 134:1017–1026

Saito K, Yoshikawa M, Yano K, Miwa H, Uchida H, Asamizu E, Sato S, Tabata S, Imaizumi-Anraku H, Umehara Y, Kouchi H, Murooka Y, Szczyglowski K, Downie JA, Parniske M, Hayashi M, Kawaguchi M (2007) NUCLEOPORIN85 is required for calcium spiking, fungal and bacterial symbioses, and seed production in *Lotus japonicus*. Plant Cell 19:610–624

Schmidt R, Koberl M, Mostafa A, Ramadan EM, Monschein M, Jensen KB, Bauer R, Berg G (2014) Effects of bacterial inoculants on the indigenous microbiome and secondary metabolites of chamomile plants. Front Microbiol 64:111

Seguel A, Cumming JR, Klugh-Stewart K, Cornejo P, Borie F (2013) The role of arbuscular mycorrhizas in decreasing aluminium phytotoxicity in acidic soils: a review. Mycorrhiza 23(3):167–183

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

Shimoda Y, Han L, Yamazaki T, Suzuki R, Hayashi M, Imaizumi-Anraku H (2012) Rhizobial and fungal symbioses show different requirements for calmodulin binding to calcium calmodulin-dependent proteinkinase in *Lotus japonicus*. Plant Cell 24:304–321

Sieberer BJ, Chabaud M, Fournier J, Timmers AC, Barker DG (2012) A switch in Ca^{2+} spiking signature is concomitant with endosymbiotic microbe entry into cortical root cells of *Medicago truncatula*. Plant J 69:822–830

Singh PP, Shin YC, Park CS, Chung YR (1999) Biological control of Fusarium wilt of cucumber by chitinolytic bacteria. Phytopathology 89:92–99

Singh S, Katzer K, Lambert J, Cerri M, Parniske M (2014) CYCLOPS, a DNA-binding transcriptional activator, orchestrates symbiotic root nodule development. Cell Host Microbe 15:139–152

Smith SE, Read DJ (2008) Mycorrhizal symbiosis, 3rd edn. Academic Press, Cambridge

Smith DL, Zhou X (2014) An effective integrated research approach to study climate change in Canada. Can J Plant Sci 94:995–1008

Smith SE, Smith FA, Jakobsen I (2003) Mycorrhizal fungi can dominate phosphate supply to plants irrespective of growth responses. Plant Physiol 133:16–20

Smith DL, Subramanian S, Lamont JR, Bywater-Ekegärd M (2015) Signaling in the phytomicrobiome: breadth and potential. Front. PlantSci 6:709–717

Stan V, Gament E, Cornea CP, Voaides C, Dusa M, Plopeanu G (2011) Effects of heavy metal from polluted soils on the *Rhizobium* diversity. Not Bot Hort Agrobot Cluj 39:88–95

Steenhoudt O, Vanderleyden J (2000) *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol Rev 24:487–506

Straight PD, Kolter R (2009) Inter species chemical communication in bacterial development. Annu Rev Microbiol 63:99–118

Streeter JG (2003) Effect of trehalose on survival of *Bradyrhizobium japonicum* during desiccation. J Appl Microbiol 95:484–491

Subramanian K, Santhanakrishnan P, Balasubramanian P (2006) Responses of field grown tomato plants to arbuscular mycorrhizal fungal colonization under varying intensities of drought stress. Sci Horticult 107:245–253

Sugawara M, Cytryn EJ, Sadowsky MJ (2010) Functional role of *Bradyrhizobium japonicum* trehalose biosynthesis and metabolism genes during physiological stress and nodulation. Appl Environ Microbiol 76:1071–1081

Takeda N, Maekawa T, Hayashi M (2012) Nuclear-localized and deregulated calcium- and calmodulin-dependent protein kinase activates rhizobial and mycorrhizal responses in Lotus japonicus. Plant Cell 24:810–822

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

Tirichine L, Imaizumi-Anraku H, Yoshida S, Murakami Y, Madsen LH, Miwa H, Nakagawa T, Sandal N, Albrektsen AS, Kawaguchi M, Downie A, Sato S, Tabata S, Kouchi H, Parniske M,

Kawasaki S, Stougaard J (2006) Deregulation of a Ca^{2+}/calmodulin-dependent kinase leads to spontaneous nodule development. Nature 441:1153–1156

Turner NC, Wright GC, Siddique KHM (2000) Adaptation of grain legumes (pulses) to water-limited environments. Adv Agron 71:193–231

Valois D, Fayad K, Barbasubiye T, Garon M, Dery C, Brzezinski R, Beaulieu C (1996) Glucanolytic actinomycetes antagonistic to *Phytophthora fragariae* var. *rubi*, the causal agent of raspberry root rot. Appl Environ Microbiol 62:1630–1635

van Aarle IM, Olsson PA, Soderstrom B (2002) Arbuscular mycorrhizal fungi respond to the substrate pH of their extraradical mycelium by altered growth and root colonization. New Phytol 155:173–182

van Loon LC (1997) Induced resistance in plants and the role of pathogenesis-related proteins. Eur J Plant Pathol 103:753–765

Vanderlinde EM, Harrison JJ, Muszynski A, Carlson RW, Turner RJ, Yost CK (2010) Identification of a novel ABC transporter required for desicattion tolerance, and biofilm formation in *Rhizobium leguminosarum* bv. *viciae* 3841. FEMS Microbiol Ecol 71:327–340

Venkateshwaran M, Cosme A, Han L, Banba M, Satyshur KA, Schleiff E, Parniske M, Imaizumi-Anraku H, An JM (2012) The recent evolution of a symbiotic ion channel in the legume family altered ion conductance and improved functionality in calcium signaling. Plant Cell 24:2528–2545

Venkateshwaran M, Jayaraman D, Chabaud M, Genre A, Balloon AJ, Maeda J, Forshey K, den Os D, Kwiecien NW, Coon JJ, Barker DG, An JM (2015) A role for the mevalonate pathway in early plant symbiotic signaling. Proc Natl Acad Sci U S A 112:9781–9786

Vessey JK (2003) Plant gowth promoting rhizobacteria as biofertilizers. Plant Soil 255:571–586

Vivas A, Azcon R, Biro B, Barea JM, Ruiz-Lozano JM (2003) Influence of bacterial strains isolated from lead-polluted soil and their interactions with arbuscular mycorrhizae on the growth of *Trifolium pratense* L. under lead toxicity. Can J Microbiol 49:577–588

Wang FY, Tong RJ, Shi ZY, Xu XF, He XH (2011) Inoculations with arbuscular mycorrhizal fungi increase vegetable yields and decrease phoxim concentrations in carrot and green onion and their soils. PLoS One 6(2):e16949

Watkin ELJ, O'Hara GW, Glenn AR (2003) Physiological responses to acid stress of an acid-soil tolerant and an acid-soil sensitive strain of *Rhizobium leguminosarum* biovar *trifolii*. Soil Biol Biochem 35:621–624

Wei G, Kloepper JW, Tuzun S (1991) Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth promoting rhizobacteria. Phytopathology 81:1508–1512

Whipps JM (2001) Microbial interactions and biocontrol in the rhizosphere. J Exp Bot 52:487–511

White JC, Ross DW, Gent MPN, Eitzer BD, Mattina MJI (2006) Effect of mycorrhizal fungi on the phytoextraction of weathered p,p-DDE by Cucurbita pepo. J Hazard Mater 137:1750–1757

Wu N, Zhang S, Huang H, Shan X, Christie P, Wang Y (2008) DDT uptake by arbuscular mycorrhizal alfalfa and depletion in soil as influenced by soil application of a non-ionic surfactant. Environ Pollut 151(3):569–575

Wulf A, Manthey K, Doll J, Perlick A, Franken P, Linke B, Meyer F, Kuster H, Krajinski F (2003) Transcriptional changes in response to arbuscular mycorrhiza development in the model plant *Medicago truncatula*. Mol Plant Microbe 16:306–314

Xu J, Xiao-Lin L, Luo L (2012) Effects of engineered *Sinorhizobium meliloti* on cytokinin synthesis and tolerance of alfalfa to extreme drought stress. Appl Environ Microbiol 78:8056

Yang J, Kloepper JW, Ryu CM (2009) Rhizosphere bacteria help plants tolerate abiotic stress. Trends Plant Sci 14(1):1–4

Yano K, Yoshida S, Muller J, Singh S, Banba M, Vickers K, Markmann K, White C, Schuller B, Sato S, Asamizu E, Tabatae S, Murooka Y, Perry J, Wang TL, Kawaguchi M, Imaizumi-Anraku H, Hayashi M, Parniske M (2008) CYCLOPS, a mediator of symbiotic intracellular accommodation. Proc Natl Acad Sci U S A 105:20540–20545

Yelton MM, Yang SS, Edie SA, Lim ST (1983) Characterization of an effective salt-tolerant fast-growing strain of *Rhizobium japonicum*. J Gen Microbiol 129:1537–1547

Zamioudis C, Mastranesti P, Dhonukshe P, Blilou I, Pieterse CMJ (2013) Unraveling root developmental programs initiated by beneficial *Pseudomonas* spp. Bacteria. Plant Physiol 162:304–318
Zhan HJ, Lee CC, Leigh JA (1991) Induction of the second exopolysaccharide (EPS) in Rhizobium meliloti SU47 by low phosphate concentrations. J Bacteriol 173:7391–7394
Zhang XP, Karsisto M, Harper R, LindstroEm K (1991) Diversity of Rhizobium bacteria isolated from the root nodules of leguminous trees. Int J Syst Evol Bacteriol 41:104–113
Zhang RQ, Zhu HH, Zhao HQ, Yao Q (2013) Arbuscular mycorrhizal fungal inoculation increases phenolic synthesis in clover roots via hydrogen peroxide, salicylic acid and nitric oxide signaling pathways. J Plant Physiol 170:74–79
Zhu J, Winans SC (2001) The quorum-sensing transcriptional regulator TraR requires its cognate signaling ligand for protein folding, protease resistance, and dimerization. Proc Natl Acad Sci U S A 98:1507–1512

A Simple Procedure for Isolation, Culture of Protoplast, and Plant Regeneration

13

Indu Kumari

Abstract

This chapter presents different steps involved in isolation and culture of protoplast; discusses basic steps involved in the developmental sequence from protoplast to plant; and overviews a simple procedure for protoplast isolation, culture, and plant regeneration. Protoplasts are naked plant cells without the cell wall, but they possess plasma membrane and other components of cell. Protoplasts are potentially totipotent individuals at the single-cell level. They have the potential to regenerate a cell wall, dedifferentiate, divide mitotically and form unlimited growing cell clones, and differentiate shoot and root meristems or embryos which regenerate whole plant.

Keywords

Isolation · Culture · Embryos · Cell clones · Protoplast

13.1 Introduction

The protoplasts are isolated, single, and naked plant cells. Protoplast is the content which is enclosed by plasma membrane. The term protoplast means spherical plasmolysed content of plant cell covered by plasma membranes or naked cell without cell wall. Protoplasts are naked plant cells without the cell wall, but they possess plasma membrane and all other cellular components. Protoplast represents the functional plant cells but for the lack of the barrier cell wall. Protoplasts of different species can be fused to generate a hybrid and this process is known as protoplast fusion or somatic hybridization.

I. Kumari (✉)
Department of Botany, Nirmala College, Doranda, Ranchi, India

© Springer Nature Singapore Pte Ltd. 2020
M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_13

The term protoplast was introduced by Hanstein in 1880. The first isolation of protoplasts was achieved by Klercker employing a mechanical method. A real research in protoplast was started by Cocking (1960). He used an enzymatic method for the removal of cell wall. Protoplasts can be isolated directly from the different parts of whole plant which contains parenchymatous tissue or indirectly from the in vitro-grown plant tissue or callus tissue.

Takebe et al. (1971) regenerated complete plants from leaf protoplasts of tobacco that increased the potential of protoplast culture methods. Several species of plants have been regenerated by using protoplasts of different plants (Bajaj 1974; Davey and Short 1973; Frearson et al. 1973; Grun and Chu 1978; Hess et al. 1973; Sink and Power 1977; Wilson et al. 1980; Wallin and Eriksson 1972).

Protoplast cultures have been used in genetic engineering for the transfer of DNA and extrachromosomal bodies like chloroplast, mitochondria, plasmids, and *nif* (nitrogen fixing) genes.

13.2 Sources of Explant for Protoplast Isolation

The protoplasts can be isolated from a variety of tissues including leaves, roots, in vitro shoot cultures, callus, cell suspension, and pollen. Among these, the mesophyll tissue of fully expanded leaves of young plants or new shoots is used most frequently. In addition, callus and suspension cultures also serve as good sources for protoplast isolation. Young cell suspensions are ideal for isolation of protoplasts in large quantities. Cell suspension cultures may provide a very good source of protoplast.

13.3 Techniques of Isolation, Culture and Regeneration of Protoplast

The basic techniques of isolation, culture, and regeneration of protoplast are following:

1. **Sterilization of Samples**: Samples like mature leaves are collected from healthy plants which are washed in tap water to remove adhering soil particles and sterilized with sodium hypochlorite solution.
2. **Rinsing in Suitable Osmoticum**: After 10 min, sample is properly washed with sterile distilled water or MS medium adjusted to a suitable pH and buffer to maintain osmotic pressure. Washing should be done for about six times to remove the traces of sodium hypochlorite.
3. **Plasmolysis of Cells**: The lower epidermis covered by thin wax cuticle is removed with a forceps. Stripping should be done from midrib to margin of lamina. The stripped surface of leaf is kept in mannitol solution (13% W/V) for 3 h to allow plasmolysis of cells.
4. **Peeling of Lower Epidermis**: Thereafter, leaves of plant are peeled off and used for isolation of protoplasts.

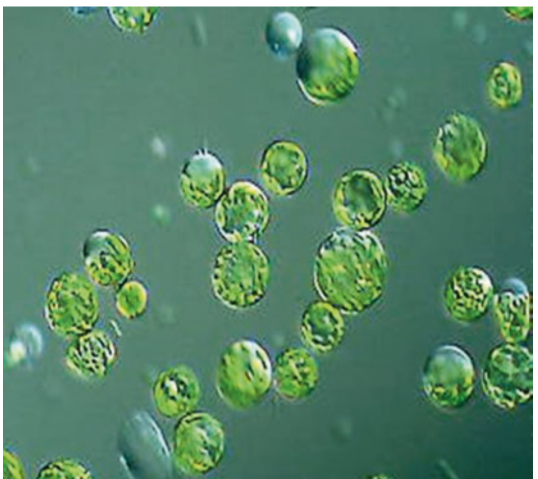


Fig. 13.1 Isolated protoplasts (source: Bhojwani and Rajdan)

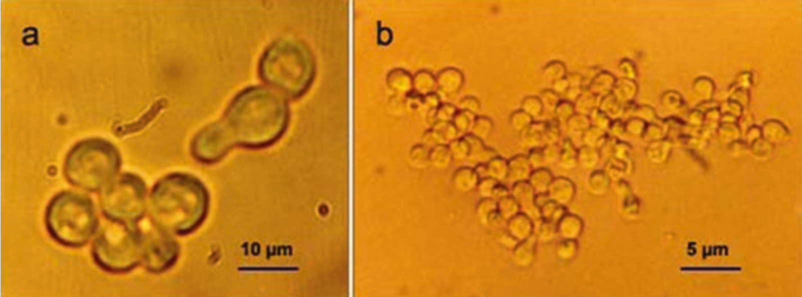


Fig. 13.2 Protoplasts obtained from *Trichoderma harzianum* by means of enzymatic action (source: Bhojwani and Rajdan)

5. **Isolation of Protoplast:** Each plant cell is enclosed by cellulose wall which is attached with each other by middle lamella ,made up of pectin. Protoplast is the content which is enclosed by plasma membrane. It is essential to get isolated protoplast, to remove the pectin material to obtain the single cells and then to remove cell wall (Figs. 13.1 and 13.2). Protoplasts may be isolated by any one of the two following ways: (a) mechanical method and (b) enzymatic method.

13.3.1 Mechanical Method

In mechanical method, cells are kept in a suitable plasmolyticum (in plasmolysed cells, protoplasts shrink away from cell wall) and cut with a fine knife, so that protoplasts are released from cells cut through the cell wall, when the tissue is again deplasmolysed. Mechanical method is suitable for isolation of protoplasts from

vacuolated cells such as onion bulbs, scales, and radish roots. The mechanical method was used as early as 1892. This technique involves the following stages:

Step 1. A small piece of epidermis from a plant is selected.
Step 2. The cells are subjected to plasmolysis. This causes protoplasts to shrink away from the cell walls.
Step 3. The tissue is dissected to release the protoplasts.

13.3.1.1 Limitation of Mechanical Method

Mechanical method for isolation of protoplasts is restricted to certain tissues with vacuolated cells. This method is not suitable for isolating protoplast from meristematic and less vacuolated cells. It gives poor yield of protoplasts and also the viability of protoplasts is low. It is laborious and tedious.

13.3.2 Enzymatic Method

This method involves the use of enzymes to dissolve the cell wall for releasing protoplasts. The enzymatic method is almost invariably used now for the isolation of protoplasts. Protoplasts can be isolated from plant tissues or cultured cells by enzymatic digestion to remove the cell walls. It gives large quantities of protoplasts, where cells are not broken and osmotic shrinkage is minimum. Sometimes mechanical and enzymatic methods are combined, where cells are first separated mechanically and later protoplast is isolated through enzymatic method. The credit of developing high-yield protoplast isolation through enzymatic method goes to Cocking (1960). He used crude cellulase from the fungus *Myrothecium verrucaria* to dissolve cell wall and release the protoplasts from tomato roots. Later this method with suitable modifications and using purified enzymes has been extensively used by other scientists.

13.3.2.1 Enzymes for Protoplast Isolation

The enzymes that can digest the cell walls are required for protoplast isolation. Chemically, the plant cell wall is mainly composed of cellulose, hemicellulose, and pectin which can be, respectively, degraded by the enzymes cellulase, hemicellulase, and pectinase. The enzymes are usually used at a pH 4.5–6.0 and temperature 25–30 °C with a wide variation in incubation period that may range from half an hour to 20 h. The success of protoplast isolation depends especially on the condition of the tissue and the combination of enzymes being used. After release of protoplast into the suspension, for removal of enzymes the protoplasts are collected in centrifuge tube as pellet and washed several times with the osmoticum.

The enzymatic method could be used as a one-step method (direct method), or as a two-step method (sequential method).

13.3.2.2 The One-Step Method

Protoplasts are isolated directly from the tissue by using two enzymes, cellulase and pectinase, simultaneously.

13.3.2.3 The Two-Step Method

Cells are first isolated from callus or tissue by using pectinase and to this cell suspension cellulase is added to digest the cell wall and release protoplasts.

An enzymatic solution used for protoplast isolation contains the enzymes and a sugar as an osmoticum to prevent the plasma membrane from rupturing. Some salts and nutrients are also used as osmoticum. Generally 50 mM CaCl2 is added to increase the stability of released protoplasts.

13.4 Advantages of Enzymatic Method

Enzymatic method is a very widely used technique for the isolation of protoplasts. It includes good yield of viable cells and minimal or no damage to the protoplasts.

13.5 Purification of Protoplasts

Leaf debris is removed with forceps, and enzyme solution containing protoplasts is filtered with a steel or a nylon mesh (45 mm). The cell clumps and undigested tissues can be removed by filtration. This is followed by centrifugation and washing of the protoplasts (Gregory and Cocking, 1965; Power and Cocking, 1969; Evans et al. 1980a, b; Schenk and Hilderbrandt 1969). Filtrate is centrifuged at 75 × g for 5 min and supernatant is decanted. Again a fresh MS medium plus 13% mannitol is added

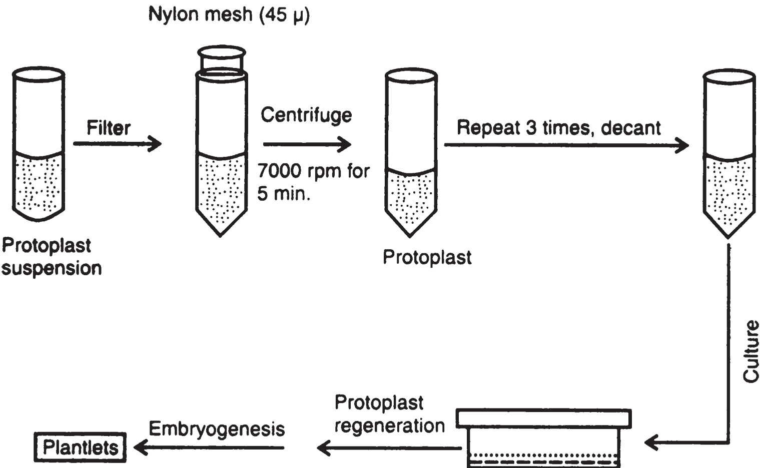


Fig. 13.3 Protoplast purification and regeneration (source: Bhojwani and Rajdan)

to centrifuge. The protoplasts settle to the bottom of the centrifuge tube while the supernatant is removed with the help of a pipette. Repeated washing with nutrient medium, centrifugation, and decantation are done for about three times. Traces of enzyme are removed by washing the protoplasts twice or thrice with the medium (Fig. 13.3).

13.6 Viability of Protoplasts

It is essential to ensure that the isolated protoplasts are healthy and viable so that they are capable of undergoing sustained cell divisions and regeneration.

13.6.1 There Are Several Methods to Assess the Protoplast Viability

- Fluorescein diacetate (FDA) staining method—Fluorescence microscopy detects the dye accumulates inside viable protoplasts.
- Phenosafranine stain is used which is specific for dead protoplasts that show red color, while the viable cells remain unstained.
- Exclusion of Evans blue dye by intact membranes.
- Cell wall formation can be detected by staining with 0.1% calcofluor white (CPW) fluorescent stain. Calcofluor white (CFW) stain binds to the newly formed cell walls which emit fluorescence.
- Oxygen uptake by protoplasts can be measured by oxygen electrode.
- The ability of protoplasts to undergo continuous mitotic divisions (direct measure).
- Photosynthetic activity of protoplasts.

13.7 Protoplast Culture and Regeneration

Protoplast regeneration which may also be regarded as protoplast development occurs in two stages: first formation of cell wall and second development of callus/whole plant.

13.7.1 Formation of Cell Wall

The process of cell wall formation in cultured protoplasts starts within a few hours after isolation. From the protoplast solution of known density (about 10^5 protoplast/mL) about 1 mL suspension is poured on sterile and cooled-down nutrient medium in Petri dishes. The plates are incubated at 25 °C in a dim white light. Protoplast develops cell wall within two to several days under suitable conditions. The process of cell wall development requires continuous supply of nutrients, particularly carbon source such as sucrose.

As the cell wall development occurs, the protoplasts lose their characteristic spherical shape. The newly developed cell wall by protoplasts can be detected by staining with 0.1% calcofluor white (CPW) fluorescent stain, while the presence of a proper wall is essential for a regular division. On the other hand, protoplasts with poorly regenerated cell wall show budding and fail to undergo normal mitosis.

13.7.2 Development of Callus/Whole Plant

Protoplasts are cultured either in semisolid agar or liquid medium. Sometimes, protoplasts are first allowed to develop cell wall in liquid medium, and then transferred to agar medium. Agarose is the most frequently used agar to solidify the culture media. In agar cultures, the protoplasts remain in a fixed position, divide, and form cell clones. The advantage with agar culture is that clumping of protoplasts is avoided. But liquid culture is easy to dilute and transfer. Density of the cells can be manipulated as desired. For some plant species, the cells cannot divide in agar medium, and therefore liquid medium is the only choice. Osmotic pressure of liquid medium can be altered as desired.

13.7.2.1 Culture Media

The culture media for protoplast culture contains nutritional components and osmoticum.

13.7.2.2 Nutritional Components

The nutritional requirements of protoplasts are similar to callus and suspension cultures. Mostly, MS and B5 media with suitable modifications are used. The medium for protoplast culture should be devoid of ammonium, and the quantities of iron and zinc should be less. The concentration of calcium should be 2–4 times higher than used for cell cultures. This is needed for membrane stability. The vitamins used for protoplast cultures are the same as used in standard tissue culture media. Glucose is the preferred carbon source by protoplasts although a combination of sugars such as glucose and sucrose can be used. High auxin/kinetin ratio is suitable to induce cell divisions while high kinetin/auxin ratio is required for regeneration.

13.7.3 Osmoticum

Osmoticum broadly refers to the reagents or chemicals that are added to increase the osmotic pressure of a liquid. The isolation and culture of protoplasts require osmotic protection until they develop a strong cell wall. In fact, if the freshly isolated protoplasts are directly added to the normal culture medium, they will burst. Thus, addition of an osmoticum is essential for both isolation and culture media of protoplast to prevent their rupture. The osmotica are of two types—nonionic and ionic.

13.7.3.1 Nonionic Osmotica

The nonionic substances most commonly used are soluble carbohydrates such as mannitol, sorbitol, glucose, fructose, galactose, and sucrose. Mannitol, being metabolically inert, is most frequently used.

13.7.3.2 Ionic Osmotica

Potassium chloride, calcium chloride, and magnesium phosphate are the ionic substances in use to maintain osmotic pressure. When the protoplasts are transferred to a culture medium, the use of metabolically active osmotic stabilizers (e.g., glucose, sucrose) along with metabolically inert osmotic stabilizers (mannitol) is advantageous. As the growth of protoplasts and cell wall regeneration occurs, the metabolically active compounds are utilized, and this results in the reduced osmotic pressure so that proper osmolarity is maintained.

13.8 Culture and Regeneration of Protoplast

As the cell wall formation around protoplasts is complete, the cells increase in size. The protoplasts, which are capable of dividing, undergo first division within 2–7 days and form small cell colonies after 2–3 weeks. With suitable manipulations of nutritional and physiological conditions, the cell colonies may be grown continuously, and form visible colonies (macroscopic colonies). These colonies are then transferred to an osmotic-free (mannitol- or sorbitol-free) medium for further development to form callus. The callus can also be subcultured. With induction and appropriate manipulations, the callus can undergo organogenic or embryo genic differentiation to finally form the whole plant. A general view of the protoplast isolation, culture, and regeneration is represented in Fig. 13.4.

Plant regeneration can be done from the callus obtained either from protoplasts or from the culture of plant organs. There are however certain differences in these two calluses. The callus derived from plant organs carries preformed buds or organized structures, while the callus from protoplast culture does not have such structures.

The first success of regeneration of plants from protoplast cultures of *Nicotiana tabacum* was achieved by Takebe et al. (1971). Since then, several species of plants have been regenerated by using protoplasts (Binding et al., 1978; Cocking and Pojnar 1969; Cocking 1972; Dos Santos et al. 1980; Harada 1973; Vasil and Vasil 1974, 1980; Zieg and Outka 1980). Some examples of plant species regenerated from protoplasts are listed in Table 13.1.

13.9 Conclusions

The isolation, culture, and fusion of protoplasts are a fascinating field in plant research. Protoplasts have a wide range of applications. A whole plant can be regenerated by protoplast culture. It is easy to perform single-cell cloning with protoplasts.

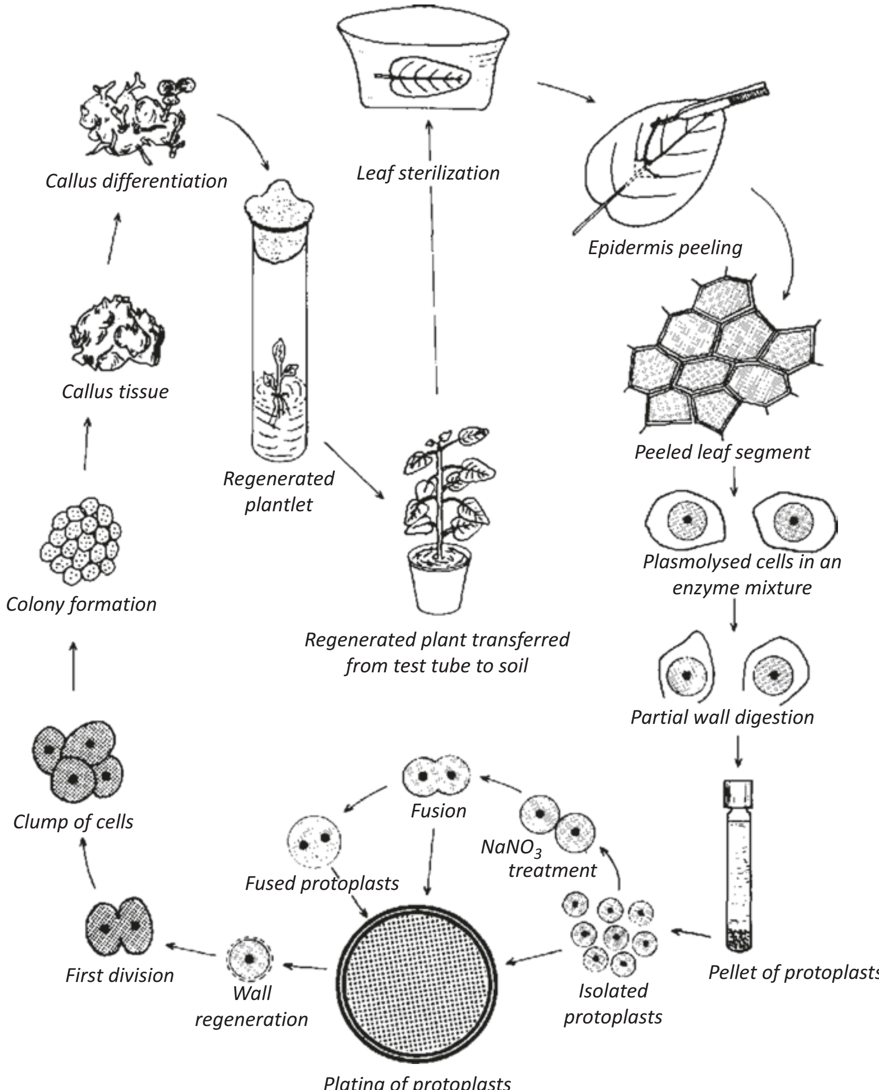


Fig. 13.4 Major steps of protoplast isolation, culture, and regeneration of plant

Protoplast fusion or somatic hybridization can produce hybrids. Protoplasts are excellent materials for studies of ultrastructure of cell. Protoplasts are useful for studies of membrane function like transport and uptake processes. Isolation of cell organelles and chromosomes is easy from protoplasts. Genetic transformations can be achieved through genetic engineering of protoplast DNA. It is easy to isolate mutants from protoplast cultures.

Table 13.1 Selected examples of plant species regenerated from protoplasts

Category	Plant species
Cereals	*Oryza sativa*, *Zea mays*
Vegetables	*Brassica oleracea*, *Capsicum annuum*
Ornamentals	Chrysanthemum sp., Rosa sp.
Woody trees	*Prunus avium*, *Larix eurolepsis*
Oil crops	*Brassica napus*, *Helianthus annuus*
Legumes	*Glycine max*

References

Bajaj YPS (1974) Potentials of protoplast culture work in agriculture. Euphytica 23:633–649

Binding H, Behls R, Schieder O, Sopory SK, Wenzel G (1978) Regeneration of mesophyll protoplasts isolated from dihaploid clones of *Solanum tuberosum*. Physiol Plant 43:52–54

Cocking EC (1960) A method for the isolation of plant protoplasts and vacuoles. Nature (London) 187:927–929

Cocking EC (1972) Plant cell protoplasts—isolation and development. A Rev Plant Physiol 23:29–50

Cocking EC, Pojnar E (1969) An electron microscopic study of the infection of isolated tomato fruit protoplasts by tobacco mosaic virus. J Gen Virol 4:305–312

Davey MR, Short KC (1973) The isolation, culture, and fusion of legume and corn flower leaf protoplasts. In: Protoplastes et fusion de cellules somatiques végétales. Colloq Int CNRS, Paris, pp 437–444

Dos Santos AVP, Outka DE, Cocking EC, Davey MR (1980) Organogenesis and somatic embryogenesis in tissues derived from leaf protoplasts and leaf explants of *Medicago sativa*. Z Pflanzenphysiol 99:261–270

Evans DA, Crocomo OJ, De Carvalho MTV (1980a) Protoplast isolation and subsequent callus regeneration in sugarcane. Z Pflanzenphysiol 98:355–358

Evans DA, Wetter LR, Gamborg OL (1980b) Somatic hybrid plants of *Nicotiana glauca* and *Nicotiana tabacum* obtained by protoplast fusion. Physiol Plant 48:225–230

Frearson EM, Power JB, Cocking EC (1973) The isolation, culture, and regeneration of *Petunia* leaf protoplasts. Dev Biol 33:130–137

Gregory DW, Cocking EC (1965) The large scale isolation of protoplasts from immature tomato fruit. J Cell Biol 24:143–146

Grun P, Chu LJ (1978) Development of plants from protoplasts of *Solanum* (Solanaceae). Am J Bot 65:538–543

Harada H (1973) A new method for obtaining protoplasts from mesophyll cells. Z Pflanzenphysiol 69:77–80

Hess D, Potrykus I, Doon G, Durand T, Hoffmann F (1973) Transformation experiments in higher plants: prerequisites for the use of isolated protoplasts (isolation from mesophyll and callus cultures, uptake of proteins and DNA, and regeneration of whole plants). In: Protoplastes et fusion de cellules somatiques végétales. Colloq Int CNRS, Paris, pp 343–351

Power JB, Cocking EC (1969) A simple method for the isolation of very large numbers of leaf protoplasts. Biochem J 111:33

Schenk RU, Hilderbrandt AC (1969) Production of protoplasts from plant cells in liquid culture using purified commercial cellulases. Crop Sci 9:629–631

Sink KC, Power JB (1977) The isolation, culture, and regeneration of leaf protoplasts of *Petunia parviflora* Juss. Plant Sci Lett 10:335–340

Takebe I, Labib G, Melchers G (1971) Regeneration of whole plants from isolated mesophyll protoplasts of tobacco. Naturwissenschaften 58:318–320

Vasil V, Vasil IK (1974) Regeneration of tobacco and *Petunia* plants from protoplasts and culture of corn protoplasts. In Vitro 10:83–96

Vasil V, Vasil IK (1980) Isolation and culture of cereal protoplasts II. Embryogenesis and plantlet formation from protoplasts of *Pennisetum americanum*. Theor Appl Genet 56:97–99

Wallin A, Eriksson T (1972) Plating of protoplasts from cell suspension cultures of *Daucus carota*. In: Protoplastes et fusion de cellules somatiques végétales. Colloq Int. CNRS, Paris, pp 301–307

Wilson HM, Styer DJ, Conard PL, Durbin RD, Helgeson JP (1980) Isolation of sterile protoplasts from unsterilized leaves. Plant Sci Lett 18:151–154

Zieg RG, Outka DE (1980) The isolation, culture and callus formation of soybean pod protoplasts. Plant Sci Lett 18:105–114

Plant Antimicrobial Peptides: Next-Generation Bioactive Molecules for Plant Protection

14

Paomipem Phazang, Neelam Prabha Negi, Meenakshi Raina, and Deepak Kumar

Abstract

Plants are under constant attack by diverse groups of pathogenic microorganisms but are able to survive and thrive harmoniously with these organisms. To accomplish this, they produce bioactive molecules called antimicrobial peptides (AMPs) constitutively or after receiving chemical cues and downstream processing of the signals. The AMPs are low-molecular-weight molecules with broad-spectrum activity and less cytotoxicity affecting not only pathogenic microbes but also neoplastic cells. They play an important role in the innate immunity of plants. Studies have shown that heterologous expression of AMPs in plants conferred disease resistance. In this chapter we have discussed two major families of plant AMPs, elaborating their mode of action and their use in plant protection. We have also highlighted the plant-based expression systems for AMPs in brief and addressed its application in agriculture and therapeutic purposes.

Keywords

Antimicrobial peptides · Plant defense · Pathogenesis-related proteins

P. Phazang
School of Life Sciences, Jawaharlal Nehru University, New Delhi, India

N. P. Negi
University Institute of Biotechnology, Chandigarh University, Mohali, Punjab, India

M. Raina · D. Kumar (✉)
Department of Botany, Central University of Jammu, Jammu, India
e-mail: deepakkumar@cujammu.ac.in

© Springer Nature Singapore Pte Ltd. 2020
M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_14

14.1 Introduction

Antimicrobial peptides (AMPs) are short sequence peptides, with usually fewer than 50 amino acid residues reported in living systems. AMPs are an important part of the innate immune system and these small molecules possess antifungal, antiparasitic, antibacterial, and antiviral activity (Sels et al. 2008). Hence, antimicrobial peptides are considered a first line of defense in plants and animals against pathogen attack (Egorov et al. 2005). AMPs are ubiquitous and are found from microbes to plants to animals. These peptides can be circular, linear, or polycyclic. Circular peptides include bacteriocins from bacteria, cyclotides from plants, and theta-defensins from animals. Lantibiotics from bacteria are polycyclic peptides (Egorov et al. 2005; Tam et al. 2015). Plants produce antimicrobial peptides as host defense molecules. In general, plants fight infections using their two-branched innate immune system. The first branch consists of the transmembrane pattern recognition receptors (PRRs) recognizing the pathogen-associated molecular patterns (PAMPs) common to many types of microbes. This branch is known as PAMP-triggered immunity (PTI). The second branch is the effector-triggered immunity (ETI). ETI gets activated upon the release of virulence factors by successful pathogens that have "outdone" the PTI. A characteristic feature of ETI is the induction of systemic acquired resistance (SAR), a hallmark of "plant memory." Downstream signaling of both pathways leads to ROS production, hormone biosynthesis, and signaling and generation of pathogenesis-related proteins (PR) (Jones and Dangl 2006; Egorov et al. 2005).

PR proteins were first discovered in tobacco leaves upon infection by tobacco mosaic virus. They were later defined as induced proteins upon pathogenic challenge. They play an important role in the systemic acquired resistance. Until recently, 17 PR families have been identified (Table 14.1). They have antimicrobial or insecticidal properties. This chapter discusses about the PR proteins that have antimicrobial activities. They are also known as antimicrobial peptides (AMPs). Major plant AMP families are PR-13/thionins, PR-12/defensins, PR-14/lipid transfer proteins, hevein-like peptides, knottins, and snakins (Van Loon and Van Strien 1999; Epand and Vogel 1999).

Plant AMPs are Cys rich that enable them to form disulfide bonds, thereby contributing to their compact structures. In general they share many common characteristics with microbes, animals, and insects and they include molecular forms, amphipathic nature, and positive charge (Tam et al. 2015; Hammami et al. 2009).

Plant AMPs are expressed constitutively or upon microbial challenge and are often tissue specific also. They are similar to the vertebrate immunoglobulin as they also have hypervariable sequences encased and exhibit molecular diversity. The defining features of plant AMPs include moderate size with molecular weight ranging from 2 kDa to 6 kDa, being cationic, and presence of 2–6 intramolecular disulfide bonds; "peptide promiscuity" as a single peptide displays multiple functions in addition to being antimicrobial. They are all ribosomally derived and bioprocessed from precursors containing three domains, viz. N-terminal, C-terminal, and AMP domain. The presence of the disulfide bonds not only allows them to have compact

Table 14.1 List of pathogenesis-related (PR) proteins with their properties

Family	Representative member(s)	Size (kDa)	Properties
PR-1	Tobacco PR-1a	15	Antifungal
PR-2	Tobacco PR-2	30	β-1,3-Glucanase
PR-3	Tobacco P, Q	25–30	Chitinase I, II, IV, V, VI
PR-4	Tobacco R	15–20	Chitinase I, II
PR-5	Tobacco S	25	Thaumatin-like
PR-6	Tomato/potato inhibitor I	8	Proteinase inhibitor
PR-7	Tomato P_{69}	75	Endoproteinase
PR-8	Cucumber chitinase	28	Chitinase III
PR-9	Tobacco lignin-forming peroxidase	35	Peroxidase
PR-10	Parsley PR-1	17	Ribonuclease-like
PR-11	Tobacco class V chitinase	40	Chitinase I
PR-12	Radish Rs-AFP3	5	Defensin
PR-13	Arabidopsis THI2.1	5	Thionin
PR-14	Barley LTP4	9	Lipid transfer protein
PR-15	Barley OxOa	20	Oxalate oxidase
PR-16	Barley OxOLP	20	Oxalo oxidase-like
PR-17	Tobacco PRp27	27	Unknown

(Source: Van Loon and Van Strien 1999; Epand and Vogel 1999)

secondary and tertiary structures but also offers high chemical, thermal, and enzymatic stability (Tam et al. 2015; Harris et al. 2009). This chapter gives an insight into the major classes of plant AMPs and their applications in agriculture and for diverse therapeutic purposes.

14.2 Antimicrobial Microbial Peptides (AMPs) from Plants

Plants made their innate immune system besides microbial attack through numerous lines of defense, with native and general creation of secondary metabolites, proteins, and ROS with antimicrobial activity. Plants prevent dispersion of pathogens by hypersensitive response. There are six major families of plant AMPs, namely thionins, defensins, hevein-like peptides, knottin-type peptides (linear and cyclic), lipid transfer proteins, and snakins. These families are classified based on their Cys motifs and their tertiary structures as a result of the distinctive disulfide bonds. The major families of plant AMPs discussed in the chapter are represented in Table 14.2.

14.2.1 Thionins

Thionins are peptides with molecular size ranging from 45 to 48 amino acids. Eight-cysteine-type (8-C) and six-cysteine-type (6-C) thionins are the two main subgroups of this peptide family. The 8-C and 6-C thionins are characterized by the presence of 3- and 4-disulfide bonds, respectively. All thionins have common 3D structures:

Table 14.2 List of major families of plant AMPs

Peptide	Disulfide bonds (No.)	Disulfide motif	Structural motif	Some representative
6C Thionin	3	2-C-0-C-11-C-8-C-5-C-7-C-6	Gamma (Γ) fold β1-α1-α2-β2-coil motif	Crambin, viscotoxin
8C Thionin	4	2-C-0-C-7-C-3-C-8-C-3-C-1-C-7-C-6		αl-Purothionin, β-purothionin
8C Defensin	4	2-C-10-C-5-C-3-C-9-C-6-C-1-C-3-C	CSαβ motif β1-coil-α-β2-β3	NaD1
10C Defensin	5	2-C-3-C-6-C-5-C-2-C-0-C-9-C-6-C-1-C-3-C		PhDs
LTP1	4	3-C-9-C-14-C-0-C-19-C-1-C-22-C-13-C-4	Hydrophobic cavity α1-α2-α3-α4-coil	Ace-AMP1 (onion)
LTP2	4	1-C-7-C-13-C-0-C-8-C-1-C-23-C-6-C		DIR1 (Arabidopsis)
Knottins	6	2-C-6-C-8-C-0-C-3-C-10-C-3	Cystine knot short β strand and coil	PAFP-S
6C hevein	3	3-C-4-C-4-C-0-C-5-C-6-C-1	Gly&Cys-rich central β-strands and (short helical) side coils	Ac-AMP1
8C hevein	4	2-C-8-C-4-C-0-C-5-C-6-C-5-C-3-C-		Hevein
10C hevein	5	2-C-3-C-3-C-4-C-0-C-5-C-6-C-5-C-1-C-1-C-2		EAFP1
Snakins	6	4-C-3-C-3-C-8-C-3-C-2-CC-2-C-1-C-11-C-1-C-12-C-1	α-Helices	Snakin-1

(Source: Tam et al. 2015)

they all exist as L-shape. Two antiparallel α-helices and two antiparallel β-strands form the long and short arms, respectively. The L-shape molecular structure has the hydrophobic residues clustered on the outer surface and the hydrophilic residues at the inner surface, thus imparting the amphipathic property to the peptides. Examples of 8-C thionins include αl-purothionin and β-purothionin. Crambin and viscotoxin are few examples of 6-C thionins (Rao 2015; Stec 2006; Clore et al. 1987).

Thionins have been isolated from both monocots and dicots. They are expressed in leaves, roots, and endosperm of seeds. It has been reported in barley, *Arabidopsis thaliana*, and certain dicots that different genes control the expression of thionins in different tissues and hence the tissue-specific expression is observed (Ponz et al. 1983; Steinmuller et al. 1986). It has also been shown that the plant hormone methyl jasmonate plays an important role in signal transduction for thionin expression (Gausing 1987; Epple et al. 1995).

Thionins are ribosomally derived and are synthesized as preproproteins. The preproproteins have a mature thionin domain between two domains, i.e., the signal sequence domain at the N-terminus and the prodomain at the C-terminus. Studies have shown that the proprotein domain at the C-terminus plays an important role as chaperones in the intravesicular trafficking of the proteins (Ponz et al. 1983; Pelegrini and Franco 2005).

14.2.2 Defensins

Plant defensins are the most abundant plant AMPs. They have molecular size ranging from 45 to 54 amino acids (Gao et al. 2000; Terras et al. 1992). Because of the size similarity they share with thionins they were mistaken as one of them and named them as γ-thionin subgroups. But the molecular structure analysis revealed that they are structurally unrelated. Defensins showed the presence of an α-helix and triple-stranded antiparallel β-sheets. Another characteristic feature defining defensins is the formation of two disulfide bonds between two cysteines of CXXXC segment of α-helix to the CXC segment of the β-strand. This structure is known as cysteine-stabilized α-helix motif and is found in insect defensins as well (Terras et al. 1995; Bruix et al. 1993).

Defensins are found in almost all plant species. Like thionins they also show tissue-specific expression. Localization studies showed that they are expressed in the peripheral cell layer of most tissues like leaves, seeds, pods, and flower organs (Bruix et al. 1993-Finkina et al. 2008). These peptides are derived from two precursors: (a) precursor containing an N-terminal signal peptide and mature defensin domain and (b) precursor with additional propeptide at the C-terminal and only few defensins like the flower specific are synthesized from this precursor (Kader 1996).

14.2.3 Lipid Transfer Proteins (LTP)

Lipid transfer proteins (LTP) were first identified in an *in vitro* bioassay for measuring the transfer of phospholipids from artificial liposomes to mitochondria. Thus, this class of peptides derived their name from their ability to transfer phospholipids from a donor to an acceptor (Bloj and Zilversmit 1977; Gomar et al. 1998). They are classified into two subgroups, LTP1 and LTP2, based on their molecular sizes and also their tertiary structures. LTP1 subfamily consists of peptides with molecular size ranging from 90 to 95aa. They have four α-helices stabilized by four disulfide bonds (between CysI and CysVI, CysII and CysIII, CysIV and CysVII, and CysV and CysVIII) and a flexible C-terminal coil. LTP2 subfamily is made of peptides with molecular size of 70aa. These peptides have three extended helices, two single-turn helices, and four disulfide bonds between CysI and CysV, CysII and CysIII, CysIV and CysVII, and CysVI and CysVIII. The helices in LTP1 and LTP2 form a hydrophobic cavity, which accommodates a variety of lipids. The presence of 8 cysteine residues forming four disulfide bridges and the hydrophobic and aromatic

residues at 12 invariable positions of all the peptides are the characteristic features of this class of peptides (Tassin et al. 1998; Pallaghy et al. 1994).

LTP expression has been observed in various plant species. Studies in *Arabidopsis,* barley, maize, and broccoli have shown the expression of LTPs in a variety of plant organs including embryos, cotyledons, leaves, stems, siliques, and various flower organs. The expression levels were highest in the epidermal layer of the organs and all around abscised regions (Bloj and Zilversmit 1977; Tassin et al. 1998). Synthesis of LTPs also occurs via precursors containing a N-terminal with signal peptide of 20–25aa and a mature domain with 8 cysteine residues at the C-terminal (Tassin et al. 1998; Pallaghy et al. 1994).

14.2.4 Knottin-Type Peptides

Knottins are the smallest but the most diverse in functions among the plant AMPs. They have a molecular size of 30 amino acids. The prototypic tertiary structure of knottins is defined by the presence of six cysteine residues forming a cysteine knot because of the disulfide bonds between CysI and CysIV, CysII and CysV, and CysIII and CysVI. The characteristic structural motif is a compact triple-stranded β-sheet and a long loop connecting the first to the second β-strand. They mostly exist in two molecular forms, i.e., linear or cyclic. Inhibitors of α-amylase, trypsin, and carboxypeptidase families and cyclotides are representatives of this class of AMPs (Tam et al. 2015; Franco 2011; Craik et al. 1999).

Their bioactive functions include hormone-like functions, and enzyme-inhibitory, cytotoxic, antimicrobial, insecticidal, and anti-HIV activities (Franco 2011). This diversity in function also known as "peptide promiscuity" is another characteristic of knottin-type peptides (Franco 2011; Heitz et al. 1999).

They are found in dicot plants of the *Rubiaceae, Violaceae, Cucurbitaceae, Fabaceae,* and *Solanaceae* families to a monocot plant of the *Poaceae* family (Gruber et al. 2008; DeBolle et al. 1993). Studies in transgenic tobacco overexpressing knottin-type peptide from *Mirabilis jalapa* showed extracellular expression in leaves (De Bolle et al. 1995; Nguyen et al. 2011). They are mostly synthesized from a precursor having a signal peptide, a prodomain, a mature protein domain, and a short C-terminal tail. However, variations in synthesis have been identified. Cliotides identified from *Clitoria ternatea* showed that they originate from chimeric precursors consisting of albumin-1 chain A and cyclotide domains (Ireland et al. 2006). Violacin A, a naturally occurring linear cyclotide from *Viola odorata*, lacks the essential bioprocessing signal, the C-terminal Asn residue required for cyclization due to the presence of a stop codon earlier in the C-terminal sequence (Archer 1960).

14.2.5 Hevein-Like Peptides

Hevein was first identified from the latex of the rubber tree, *Hevea brasiliensis*. It showed antifungal activity and is attributed to the presence of the chitin-binding domain (Van Parijs et al. 1991; Lipkin et al. 2005). Peptides identified to contain the hevein domain were later named as hevein-like peptides. They are basic peptides with molecular size of 25–45aa. They have 3–5 disulfide bonds based on the number of cysteine residues. Thus they are subclassified as 6C, 8C, and 10C hevein-like peptides. They are also characterized by the presence of cysteine knot but differ in structures from those of knottins. The defining structural motif is the coil-β1-β2-coil-β3 and a short arm that separates the β_1 and β_2 coils although variations are observed in the length of the arm. They are rich in Gly and aromatic residues and these residues are conserved across the peptides. Like hevein, hevein-like peptides showed toxicity to chitin-containing fungi and other plant fungal pathogens (Andreev et al. 2012; Segura et al. 1999).

Very little is known about the expression patterns of hevein-like peptides. The rubber seedlings showed expression of hevein in the leaves and stem but not in the roots. Wounding and application of abscisic acid also triggered the accumulation of hevein transcripts in the leaves and stem. Hevein and hevein-like peptides are processed from precursors containing three domains: a signal peptide of 25aa at the N-terminal, a mature peptide of 33aa, and a 34aa C-terminal region, which undergo cleavage during posttranslational modification (Segura et al. 1999).

14.2.6 Snakins

This class of peptides was first isolated from potato tubers and was named snakins by Sengura and coworkers (1999) because of the similarity in sequence to the hemotoxin of snakes (Berrocal-Lobo et al. 2002). Two subtypes of snakins have been identified from potato, *Solanum tuberosum: snakin-1 having 63aa and snakin-2 having 66aa residues.* They are cationic peptides and contain 12 cysteine residues and are predicted to have 2 α-helices with disulfide bonds between CysI and CysIX, CysII and CysVII, CysIII and CysIV, CysV and CysXI, CysVI and CysXII, and CysVIII and CysX. Snakins show both antibacterial and antifungal activities and are recognized components of constitutive and inducible plant defense systems (Berrocal-Lobo et al. 2002; Van Loon et al. 1994).

14.2.7 Mode of Action of Plant AMPs

The cationic charge and the amphipathic nature play an important role in the antimicrobial activities of the plant AMPs. In general, the cationic peptides bind to the negatively charged phospholipids and liposaccharides of Gram-negative bacteria and teichoic acid of Gram-positive bacteria and cause disruption of the lipid membrane bilayer. This in turn causes membrane collapse and cell lyses. Intrinsic factors

such as the peptides' ability to self-assemble and oligomerize and extrinsic factors like that of the phospholipid composition of the membrane, head group size, and membrane fluidity play a critical role in exerting their antimicrobial activities (Sitaram and Nagaraj 1999; Carmona et al. 1993).

The mode of action of plant AMPs can be broadly categorized into three types: (a) the barrel-stave mechanism, (b) the toroidal pore or wormhole mechanism, and (c) the carpet mechanism (Yeamn and Yount 2003; Carmona et al. 1993). In the barrel-stave model, the cationic peptides initially aggregate and interact with each other forming a barrel ring. After reaching the threshold concentration, the peptides orient perpendicularly to the membrane of the microbes. This orientation allows the interaction of the hydrophobic portions of the peptides with the head group of the phospholipids of the bilayer membrane of the bacteria, thus forming a pore in the membrane. The barrel-stave complex formed thus acts as a hydrophilic channel on the membrane allowing cell lyses to occur (Yeamn and Yount 2003). The toroidal pore or the wormhole model takes place in two stages. In the first step, when the peptide concentration is low also known as the inactive state, the peptides align parallel to the membrane and the polar regions interact with the lipid head groups of the membrane. The second step occurs when the peptide concentration reaches the threshold level. At this stage the peptides reorient themselves perpendicular to the membrane causing the formation of the hydrophilic transmembrane pore. The barrel-stave and the toroidal pore models are very similar except that the pore formation in the latter takes place in two stages depending on the peptide concentration (Yeamn and Yount 2003; Carmona et al. 1993). The carpet model mode of action of the cationic peptides aggregates by electrostatically binding themselves forming a carpet-like structure on top of the microbial membrane. The peptide aggregate then binds to the phospholipids of the bilayer membrane, causing disturbance on the membrane fluidity. The membrane damage of the pathogenic microbes occurs in a dispersion-like manner unlike the channel formation observed in the other two models (Carmona et al. 1993).

14.2.8 Plant AMPs as Plant Protection Agents: A Plausible Application in Agriculture

The primary objective of plant biotechnology is to enroot crop plants with superior ability to resist disease caused by bacterial and fungal pathogens, thereby increasing production. Genetic transformation of plants has helped in establishing the roles of various plant AMPs. Antimicrobial peptide genes taken from plants and overexpressing them in model as well as crop plants have shown the potential of the peptides for protection against phytopathogens (Oard and Enright 2006). Tobacco plants expressing a barley thionin gene under the CaMV 35S promoter showed reduced lesion symptoms when challenged with either *Pseudomonas syringae* pv. *Tabaci* or *Pseudomonas syringae* pv. *Syringae* (Oard and Enright 2006). β-Purothionin isolated from wheat endosperm conferred increased resistance to *Pseudomonas syringae* pv. Tomato and *F. oxysporum* in *Arabidopsis* (Oard and

Enright 2006). Another thionin from wheat, puroindolin genes *pinA and pinB*, was overexpressed in rice and was found to give increased tolerance to *Magnaporthe grisae* and *Rhizoctonia solani* (Krisnamurthy et al. 2001). Defensins isolated from various plant sources have also been investigated. Transgenic tobacco plants expressing the cDNA of radish defensin, Rs-AFP2, showed sevenfold reduction in lesion size on the leaves upon infection by *Alternaria longipes*, a foliar fungal pathogen. The alfalfa defensin, Alf-AFP expressed in potato protected against *Verticillium dahliae* (Terras et al. 1992), and pea defensin, DRR206 expressed in canola and tobacco, showed protection against *Leptosphaeria maculans* (Wang et al. 1999). BSD1 cabbage defensin expressed in tobacco conferred resistance to *Phytophthora parasitica* (Park et al. 2002a, b). WT1 wasabi defensin expressed in rice protected against *Magnaporthe grisea* (Kanzaki et al. 2002), eggplant expressing the Dm-AMP1 dahlia defensin was able to show resistance to pathogens like *Botrytis cinerea* and *Verticillium alboatrum* (Turrini et al. 2004), and Mj-AMP1 jalapa defensin expressed in tomato protected against *Alternaria solani* (Schaefer et al. 2005).

Overexpression of the PR-14 gene from barley coding for a lipid transfer protein in tobacco and *Arabidopsis thaliana* conferred enhanced resistance against *Pseudomonas syringae pv. Tabaci* (Chassot et al. 2007). It has also been reported of the resistance provided to *Arabidopsis thaliana* against *Botrytis cinerea* by overexpression of three endogenous LTP (PR-14)-like genes (Chassot et al. 2007).

In addition, it may also be mentioned that some synthetic antimicrobial peptides have gained similar status as plant-protecting agents. Overexpression of *MsrA*1, a synthetic AMP, a cecropin-melittin cationic chimera peptide in potato, showed protection against *Phytophthora cactotrum, Fusarium solani*, and *Erwinia carotovora* (Osusky et al. 2000). In *Brassica juncea* overexpression of *MsrA*1 has shown resistance to *Alternaria brassicae* and *Sclerotinia sclerotiorum*, two of the most devastating pathogens of *B. juncea* crops (Rustagi et al. 2014). Another synthetic peptide D4E1 also showed its antimicrobial property against *Colletotrichum destructivum* in transgenic tobacco (Cary et al. 2000). Poplar plants also expressing the D4E1 showed resistance against *Agrobacterium tumefaciens* and *Xanthomonas populi pv. populi* (Mentag et al. 2003).

These reports of transgenic model and crop plants expressing the plant AMPs showing enhanced resistance to pathogens are paving way for their large-scale application in agriculture. Today, the world is challenged with issues related to food security. Farmers across the globe are not able to produce enough food for the ever-increasing population. Mass destruction of crops by phytopathogens and abiotic stress factors is the main reason attributing to the shortage of food. Thus, one of the major goals of plant biotechnology is to develop crop plants that would show resistance to these stress factors and at the same time bring about sustainable agricultural developments. There is an urgent need to come up with new plant-protecting agents to replace the chemical pesticides that have been used extensively and have caused enormous damage on our agricultural lands. Plant AMPs thus seem to be one of the potential alternative biocontrol agents discovered so far. The various facets of the antimicrobial properties displayed by them not only highlight the inbuilt

mechanism and strategies adapted by plants but also give us a status on "plant health." If plant molecular biotechnology and plant breeding as well as other interdisciplinary science collaborate, we would be able to further tap the AMPs of plant sources for beneficial use in agriculture.

14.3 Conclusion

The diversity displayed by plant AMPs in terms of structure and function is spectacular. Their constitutive and inducible expression patterns reveal that they function not only to fight against pathogens but also as part of the basal plant machineries. Though many of them have been isolated/discovered and extensively investigated, we still need novel strategies to understand the structure-function relationships to be able to fully understand their mechanisms for beneficial exploitation. There is no denial that they are advantageous as they are of plant origin and are essential components of the plant innate immune system and have been used by them to fight back against the constant attack by microbes. In addition, they are also effective against plant-feeding insects. Thus, the antimicrobial property (antibacterial, antiviral, and antifungal) and insecticidal property confer plant AMPs as potential plant-protecting agents.

References

Andreev YA, Korostyleva TV, Slavokhotova AA, Rogozhin EA, Utkina LL, Vassilevski AA, Grishin EV, Egorov TA, Odintsova TI (2012) Genes encoding hevein-like defense peptides in wheat: Distribution, evolution, and role in stress response. Biochimie 94:1009–1016

Archer BL (1960) The proteins of *Hevea brasiliensis* latex. 4. Isolation and characterization of crystalline hevein. Biochem J 75:236–240

Berrocal-Lobo M, Segura A, Moreno M, Lopez G, Garcia-Olmedo F, Molina A (2002) Snakin-2, an antimicrobial peptide from potato whose gene is locally induced by wounding and responds to pathogen infection. Plant Physiol 128:951–961

Bloj B, Zilversmit DB (1977) Rat liver proteins capable of transferring phosphatidylethanolamine. Purification and transfer activity for other phospholipids and cholesterol. J Biol Chem 252(5):1613–1619

Bruix M, Jimenez MA, Santoro J, Gonzalez C, Colilla FJ, Mendez E, Rico M (1993) Solution structure of gamma 1-h and gamma 1-p thionins from barley and wheat endosperm determined by 1H-NMR: A structural motif common to toxic arthropod proteins. Biochemistry 32:715–724

Carmona MJ, Molina A, Fernandez JA, Lopez-Fando JJ, Garcia-Olmedo F (1993) Expression of the α-thionin gene from barley in tobacco confers enhanced resistance to bacterial pathogens. Plant J 3:457462

Cary JW, Rajasekaran K, Jaynes JM, Cleaveland TE (2000) Transgenic expression of a gene encoding a synthetic antimicrobial peptide results in inhibition of fungal growth *in vitro* in planta. Plant Sci 154:171–181

Chassot C, Nawrath C, Metraux JP (2007) Cuticular defects lead to full immunity to a major plant pathogen. Plant J 49:972–980

Clore GM, Sukumaran DK, Nilges M, Gronenborn AM (1987) Three-dimensional structure of phoratoxin in solution: combined use of nuclear magnetic resonance, distance geometry, and restrained molecular dynamics. Biochemistry 26:1732–1745

Craik DJ, Daly NL, Bond T, Waine C (1999) Plant cyclotides: a unique family of cyclic and knotted proteins that defines the cyclic cystine knot structural motif. J Mol Biol 294:1327–1336

De Bolle MFC, Eggermont K, Duncan RE, Osborn RW, Terras FRG, Broekaert WF (1995) Cloning and characterization of two cDNA clones encoding seed-specific anti-microbial peptides from *Mirabilis jalapa* L. Plant Mol Biol 28:713–721

DeBolle MFC, David KMM, Rees SB, Vanderleyden J, Cammue BPA, Broekaert WF (1993) Cloning and characterization of a cDNA encoding an antimicrobial chitin-binding protein from amaranth, *Amaranthus caudatus*. Plant Mol Biol 22(1187–1):190

Egorov TA, Odintsova TI, Pukhalsky VA, Grishin EV (2005) Diversity of wheat anti-microbial peptides. Peptides 26:2064–2073

Epand RM, Vogel HJ (1999) Diversity of antimicrobial peptides and their mechanisms of action. Biochim Biophys Acta 1462:11–28

Epple P, Apel K, Bohlmann H (1995) An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. Plant Physiol 109:813–820

Finkina EI, Shramova EI, Tagaev AA, Ovchinnikova TV (2008) A novel defensin from the lentil (*Lens culinaris*) seeds. Biochem Biophys Res Commun 371:860–865

Franco OL (2011) Peptide promiscuity: an evolutionary concept for plant defense. FEBS Lett 585:995–1000

Gao AG, Hakimi SM, Mittanck CA, Wu Y, Woerner BM, Stark DM, Shah DM, Liang J, Rommens CM (2000) Fungal pathogen protection in potato by expression of a plant defensin peptide. Nat Biotechnol 18:1307–1310

Gausing K (1987) Thionin genes specifically expressed in barley leaves. Planta 171:241–246

Gomar J, Sodano P, Sy D, Shin DH, Lee JY, Suh SW, Marion D, Vovelle F, Ptak M (1998) Comparison of solution and crystal structures of maize nonspecific lipid transfer protein: A model for a potential in vivo lipid carrier protein. Proteins 31:160–171

Gruber CW, Elliott AG, Ireland DC, Delprete PG, Dessein S, Goransson U, Trabi M, Wang CK, Kinghorn AB, Robbrecht E et al (2008) Distribution and evolution of circular miniproteins in flowering plants. Plant Cell 20:2471–2483

Hammami R, BenHamida J, Vergoten G, Fliss I (2009) Phytamp: a database dedicated to antimicrobial plant peptides. Nucleic Acids Res 37:D963–D968

Harris F, Dennison SR, Phoenix DA (2009) Anionic antimicrobial peptides from eukaryotic organisms. Curr Protein Pept Sci 10:585–606

Heitz A, Le-Nguyen D, Chiche L (1999) Min-21 and min-23, the smallest peptides that fold like a cystine-stabilized beta-sheet motif: design, solution structure, and thermal stability. Biochemistry 38:10615–10625

Ireland DC, Colgrave ML, Nguyencong P, Daly NL, Craik DJ (2006) Discovery and characterization of a linear cyclotide from *Viola odorata*: Implications for the processing of circular proteins. J Mol Biol 357:1522–1535

Jones JDG, Dangl JL (2006) The plant immune system. Nature 444:323–329

Kader JC (1996) Lipid-transfer proteins in plants. Annu Rev Plant Physiol Plant Mol Biol 47:627–654

Kanzaki H, Nirasawa S, Saitoh H (2002) Overexpression of the wasabi defensin gene confers enhanced resistance to blast fungus (*Magnaporthe grisea*) in transgenic rice. Theor Appl Genet 105:809–814

Krisnamurthy K, Balconi C, Sherwood JE, Giroux MJ (2001) Wheat puroindolines enhance fungal disease resistance in transgenic rice. Mol Plant-Microbe Interact 14:1255–1260

Lipkin A, Anisimova V, Nikonorova A, Babakov A, Krause E, Bienert M, Grishin E, Egorov T (2005) An antimicrobial peptide ar-amp from amaranth (*Amaranthus retroflexus L.*) seeds. Phytochemistry 66:2426–2431

Mentag R, Lukevich M, Morency MJ, Seguin A (2003) Bacterial disease resistant of transgenic hybrid poplar expressing the synthetic antimicrobial peptide D4E1. Tree Physiol 23:405–411

Nguyen GK, Zhang S, Nguyen NT, Nguyen PQ, Chiu MS, Hardjojo A, Tam JP (2011) Discovery and characterization of novel cyclotides originated from chimeric precursors consisting of albumin-1 chain a and cyclotide domains in the Fabaceae family. J Biol Chem 286:24275–24287

Oard SV, Enright FM (2006) Expression of antimicrobial peptides in plants to control phytopathogenic bacteria and fungi. Plant Cell Rep 25:561–572

Osusky M, Zhou G, Osuska L, Hancock RE, Kay WW, Misra S (2000) Transgenic plants expressing cationic peptide chimeras exhibit broad spectrum resistance to phytopathogens. Nat Biotechnol 18:1162–1166

Pallaghy PK, Nielsen KJ, Craik DJ, Norton RS (1994) A common structural motif incorporating a cystine knot and a triple-stranded beta-sheet in toxic and inhibitory polypeptides. Protein Sci 3:1833–1839

Park HC, Kang YH, Chun HJ, Koo JC, Cheong YH, Kim CY, Kim MC, Chung WS, Kim JC, Yoo JH et al (2002a) Characterization of a stamen-specific cDNA encoding a novel plant defensin in Chinese cabbage. Plant Mol Biol 50:59–69

Park CH, Kang YH, Chun HJ (2002b) Characterization of a stamen-specific cDNA encoding a novel plant defensin in Chinese cabbage. Plant Mol Biol 50:59–69

Pelegrini PB, Franco OL (2005) Plant gamma-thionins: novel insights on the mechanism of action of a multi-functional class of defense proteins. Int J Biochem Cell Biol 37:2239–2253

Ponz F, Paz-Ares J, Hernandez-Lucas C, Carbonero P, Garcia-Olmedo F (1983) Synthesis and processing of thionin precursors in developing endosperm from barley (*Hordeum vulgare l.*). EMBO J 2:1035–1040

Rao AG (2015) Antimicrobial peptides. Mol Plant-Microbe Interact 8:6–13

Rustagi A, Kumar D, Shekhar S, Yusuf MA, Misra S, Sarin NB (2014) Transgenic *Brassica juncea* plants expressing *MsrA*1, a synthetic cationic antimicrobial peptide, exhibit resistance to fungal phytopathogens. Mol Biotechnol 56:535–545

Schaefer SC, Gasic K, Cammue B, Broekaert W, Van Damme EJM, Peumans WJ, Korban SS (2005) Enhanced resistance to early blight in transgenic tomato lines expressing heterologous plant defense genes. Planta 222:858–866

Segura A, Moreno M, Madueno F, Molina A, Garcia-Olmedo F (1999) Snakin-1, a peptide from potato that is active against plant pathogens. Mol Plant-Microbe Interact 12:16–23

Sels J, Mathys J, De Coninck BM, Cammue BP, De Bolle MF (2008) Plant pathogenesis-related (pr) proteins: a focus on PR peptides. Plant Physiol Biochem 46:941–950

Sitaram N, Nagaraj R (1999) Interaction of antimicrobial peptides with biological and model membranes: structural and charge requirements for activity. Biochim Biophys Acta 1462:29–54

Stec B (2006) Plant thionins—the structural perspective. Cell Mol Life Sci 63:1370–1385

Steinmuller K, Batschauer A, Apel K (1986) Tissue-specific and light-dependent changes of chromatin organization in barley (*Hordeum vulgare*). Eur J Biochem 158:519–525

Tam JP, Wang S, Wong KH, Tan WL (2015) Antimicrobial peptides from plants. Pharmaceuticals 8:711–757

Tassin S, Broekaert WF, Marion D, Acland DP, Ptak M, Vovelle F, Sodano P (1998) Solution structure of Ace-AMP1, a potent antimicrobial protein extracted from onion seeds. Structural analogies with plant nonspecific lipid transfer proteins. Biochemistry 37:3623–3637

Terras FR, Schoofs HM, De Bolle MF, Van Leuven F, Rees SB, Vanderleyden J, Cammue BP, Broekaert WF (1992) Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus l.*) seeds. J Biol Chem 267:15301–15309

Terras FR, Eggermont K, Kovaleva V, Raikhel NV, Osborn RW, Kester A, Rees SB, Torrekens S, Van Leuven F, Vanderleyden J et al (1995) Small cysteine-rich antifungal proteins from radish: Their role in host defense. Plant Cell 7:573–588

Turrini A, Sbrana C, Pitto L (2004) The antifungal Dm-AMP1 protein from Dahlia merckii expressed in *Solanum melongena* is released in root exudates and differentially affects pathogenic fungi and mycorrhizal symbiosis. New Phytol 163:393–403

Van Loon LC, Van Strien EA (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol Mol Plant Pathol 55:85–97

Van Loon LC, Pierpont WS, Boller T, Conejero V (1994) Recommendations for naming plant pathogenesis-related proteins. Plant Mol Biol Rep 12:245–264

Van Parijs J, Broekaert WF, Goldstein IJ, Peumans WJ (1991) Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis)* latex. Planta 183:258–264

Wang Y, Nowak G, Culley D, Hadwiger LA, Fristensky B (1999) Constitutive expression of pea defense gene DRR206 confers resistance to blackleg (*Leptosphaeria maculans*) disease in transgenic canola (*Brassica napus*). Mol Plant-Microbe Interact 12:410–418

Yeamn MR, Yount NY (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacol Rev 55:27–55

15 Nitrogen Stress in Plants and the Role of Phytomicrobiome

Garima Malik, Navneet Singh, and Sunila Hooda

Abstract

Nitrogen (N) being an important macronutrient for plants is a major factor that determines its growth and yield. Nitrogen uptake, nutrition, and signaling have received a lot of attention in the last few decades. More recently, the focus of the research has shifted to regulatory networks within or outside the N metabolism. We know that N is not just the essential nutrient required to support the optimal plant growth and yield, but is also an important signal involved in a wide array of plant responses to a broad range of biotic and abiotic stresses including nutrient deficiency, light, salinity, and drought. The recent progress in the genome sequencing data has allowed us to draw a more comprehensive picture of the molecular and structural diversities of the genes and the encoded proteins involved in morphological and physiological responses to N. Most plants have the ability to enhance nutrient acquisition through symbiosis—close and long-term relationship of microbes with plants. The current review focuses on the most exciting developments in the field of microbes and its role in N stress.

Keywords

Nitrogen · Nitrate · Diazotrophs · Biological nitrogen fixation · Sustainable agriculture

G. Malik
R.G. (PG) College, C.C.S University, Meerut, Uttar Pradesh, India

N. Singh
John Curtin School of Medical Research, Australian National University, Canberra, ACT, Australia

S. Hooda (✉)
Ram Lal Anand College, University of Delhi, New Delhi, India

© Springer Nature Singapore Pte Ltd. 2020
M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_15

15.1 Introduction

Nitrogen (N) is an essential macronutrient required in large amount for proper plant development (Marschner 1995; Epstein and Bloom 2005; Miller et al. 2007). Its accessibility is a major factor that governs plant growth and vigor in both natural and agricultural ecosystems (Galloway and Cowling 2002). N plays an important role in diverse aspects of the plant's life cycle and affects all levels of plant function, from metabolism to resource allocation, growth, and development including plant morphology, nutrient availability, net photosynthesis, root and shoot biomass production, and synthesis of essential biomolecules such as nucleic acids, proteins, phospholipids, chlorophyll, growth hormones, many cofactors, and secondary metabolites (Crawford 1995, Marschner 1995, Tschoep et al. 2009). N is a fundamental constituent of nucleotides and amino acids, primary building block of nucleic acids and proteins, respectively, present in each and every cell of the plant body. It is also an essential part of the chlorophyll molecule, which imparts green color to the plant leaves and is involved in the synthesis of food material for the plant via photosynthesis. Apart from photosynthesis, it performs a crucial role in various other metabolic and physiological processes of plants—it promotes growth and differentiation of different vegetative and reproductive parts of plants. Furthermore, it stimulates rapid early growth in plant seedlings, enhances dry matter of leafy vegetables, improves quality and quantity of fruit set along with seed production, and increases the protein content of fodder and forage crops (Marschner 2011). It also facilitates the uptake and utilization of other essential nutrients such as potassium (K) and phosphorous (P) and thereby controls overall growth and survival of plants (Bloom 2015). Deficiency of N in plants leads to chlorosis (appearing first on older leaves, usually starting at the tips), stunted growth (due to reduction in cell division), restricted growth of lateral buds (from which leaves, stem, and branches develop), lowered protein content of vegetative parts and seeds, reduced flower set, and overall reduction in yield and quality (Uchida 2000). Likewise, excessive application of N has negative effects on plant and adversely affects plant growth, promotes extra dark-green color on the leaves, reduces the fruit quantity and quality, and accelerates senescence (Uchida 2000).

N is present in the biosphere in various chemical forms and the earth's atmosphere is composed of 80% of molecular nitrogen (N_2) (Sanhueza 1982). However, plants cannot directly use an unreactive molecular form of N. N_2 enters into the global N cycle in three predominant ways (Kraiser et al. 2011): (a) through biological nitrogen fixation (BNF), i.e., conversion of N_2 to ammonia by microorganisms/prokaryotes; (b) by atmospheric nitrogen fixation (ANF), i.e., conversion of N_2 to nitrate (NO^{3-}) by means of lightning and photochemical reactions; and (c) by the Haber–Bosch industrial N fixation, i.e., conversion of N_2 to ammonia (Marschner 1995). Without human interference, BNF and ANF would be the only sources of new N in the environment. Fritz Haber in 1908 discovered how ammonia (a chemically reactive and highly usable form of N) could be synthesized artificially by reacting atmospheric N_2 with hydrogen in the presence of iron at high pressures and

temperatures (Erisman et al. 2008). His discovery transformed the world and later in 1918 he was awarded the Nobel Prize in Chemistry.

Once N is fixed in the form of NO_3^- and ammonium, it can be used for the biosynthesis of N-containing metabolites (e.g., nucleotides, amino acids, small polypeptides, urea) by various life forms, and finally returned back to the atmosphere as elemental form (Marschner 1995, Miller et al. 2007). The ability of a plant to capture fixed N from the soil depends on plant species, physical and chemical properties of soil, and various environmental factors. The use of N by plants involves various steps inclusive of uptake, assimilation, translocation, recycling, and remobilization. Proper growth and development of plants require an optimum supply of N. The N supply is indispensable for plants and increases in N supply have been exploited to increase the yield of agricultural crops in order to provide food for the fast-growing global human population. However, N is one of the most expensive nutrients to supply and chemical fertilizers signify the major cost in crop production for farmers. It has been estimated that more than 100 million metric tonnes of nitrogenous fertilizers are added annually to the soil worldwide and almost half of the human population depends on N fertilizer for their food supply (Good et al. 2004, Erisman et al. 2008). However, the excessive use of these chemical fertilizers is also the main source of soil and water pollution. In order to overcome these problems, various plant scientists are now focusing on developing plant varieties with better inherent N use efficiency (NUE), thus reducing environmental degradation and paving a path for sustainable agriculture production (Hirel et al. 2007).

15.1.1 Biological Nitrogen Fixation (BNF)

Biologically available N or fixed N is essential to sustain life on earth. The lack of biologically available N is often the limiting factor in the productivity of crop plants and thus food production. In order for N to be assimilated by plants, it must be fixed or combined or reduced in the form of ammonium (NH_4^+) or NO_3^- ions (Mancinelli 1996). The global biogeochemical N cycle allows the interconversions of N compounds to keep a comparatively small amount of fixed N in a constant exchange with atmospheric N_2 (Cabello et al. 2004). BNF, a process where molecular N_2 is reduced in multiple electron transfer reactions resulting in the synthesis of ammonia and the release of hydrogen, is carried out by a group of prokaryotic microorganisms called nitrogen-fixing organisms (diazatrophs), distributed across the archaeal and eubacterial domains (Kim and Rees 1992; Raymond et al. 2004). Due to their prominent function in the global N cycle, diazotrophs are present in virtually all ecosystems ranging from rhizosphere which includes the ocean to specialized root nodules in legume plants. Diazotrophs are capable of N fixation due to the presence of a nitrogenase enzyme complex that carries out one of the most metabolically expensive processes of N_2 fixation in biology, requiring large amounts of both reducing power and high-energy phosphate (ATP) (Simpson and Burris 1984). Nitrogenase is an ATP-dependent enzyme complex that consists of two metalloproteins, a molybdenum-iron (MoFe) protein heterotetramer (dinitrogenase) and an

iron-protein (Fe-protein) homodimer (dinitrogenase reductase) encoded by the nitrogen fixation (*nif*) genes (Hageman and Burris 1978; Peters et al. 2011). Supplementary genes in the *nif* operon code for proteins are involved in the biosynthesis of nitrogenase cofactor, along with proteins required for electron transport to nitrogenase and regulation, and some proteins of unfamiliar functions (Dean and Jacobsen 1992; Rubio and Ludden 2008). On the basis of the composition of their metal centers, nitrogenase can be categorized into three broad categories: iron and molybdenum (Fe/Mo), iron and vanadium (Fe/V), or iron only (Fe), of which Fe/Mo type is the most common one (Mus et al. 2016). Nitrogenase catalyzes the conversion of N_2 to NH_4^+ as represented by the following equation (Halbleib and Ludden 2000):

$$N_2 + 10H^+ + 8e^- + 16MgATP \rightarrow 2NH_4^+ + H_2 + 16MgADP + 16Pi$$

The nitrogenase enzyme complex is sensitive to O_2, which irreversibly inactivates the enzyme. Thus N-fixing microorganisms have evolved various mechanisms which concomitantly permit the supply of O_2 required for obtaining chemical energy while protecting nitrogenase from its poisonous effect (Rubio and Ludden 2008; Peters et al. 2011). The requirement of high energy for N fixation and the oxygen sensitivity of nitrogenase impose considerable physiological constraints on N-fixing organisms (Argudo et al. 2005).

15.1.2 Phytomicrobiome and Its Role in N Fixation

A plant sustains an ecosystem. It would be ideal to consider it from a systems perspective. The term "*phytomicrobiome*" refers to the microbial community associated with the plant system. There is an intricate relationship of a plant with its relatively constant type of microorganisms. The plant-beneficial microbiome confers growth-promoting effects on plants by mobilizing nutrients in the soil, supporting under abiotic stress and biotic stress and bioremediating the soil polluted with heavy metals (Miransari 2011; Richardson et al. 2009; Ahemad and Malik 2011). The indispensable role of microorganisms in building and conserving soil fertility has been recognized ever since the commencement of agriculture.

Different plant compartments harbor different niches of bacteria. Generally, these niches are divided into three broad habitats based on the plant parts they inhabit—*phyllosphere* (habitat defined by aerial plant parts, particularly stem and leaves), *endosphere* (encompassing habitat inside plant organs both above- and belowground), and *rhizosphere* (microbial habitat around the roots, both inside and outside soil). Evident loss of N from aerial parts of the plants in the form of gaseous ammonia and unavailability of a suitable form of N in the soil for plant uptake are reasons for N stress in plants. Phyllosphere is constantly exposed to changes in light intensity, humidity, temperature, etc. directly affecting the nutrient composition whereas there is assured higher abundance of nutrients in rhizosphere due to uncommon changes in light and temperature conditions. Thus, numerous species of bacteria, nematodes, fungi, and protozoa colonize the rhizosphere. Mendes and colleagues

have summarized past studies that focused on identification of diversity of bacterial taxa present in different rhizosphere samples highlighting the presence of *Proteobacteria* as dominant phylum followed by *Firmicutes* (Mendes et al. 2013). Among various interactions of plants with microorganisms, the one beneficial interaction is that of N-fixing plant growth-promoting rhizobacteria (PGPR), which colonize rhizosphere. Some of these are diazotrophic bacteria.

Many microbial species capable of fixing N, both as free-living soil microorganisms (e.g., bacteria and blue-green algae (BGA)) or in symbiotic association with plants (e.g., rhizobia-legume; angiosperm-actinomycetes; and BGA associative systems, including rhizosphere, phyllosphere, and lichens), have been identified. N-fixing microorganisms are found in several phyla and representatives from the majority of these phyla are known to fix N either in nonsymbiotic or symbiotic association with plants (Boyd and Peters 2013; Hardoim et al. 2015). Plants have developed various ways to achieve a mutualistic relationship with diazotrophs to obtain atmospheric N (Mus et al. 2016). In all these associations and symbioses, the host plants get the benefit of the fixed N provided by the symbiotic partner, which, in turn, receives reduced C along with other nutrients it requires. Also, the symbiotic or endophytic plant structure colonized by the N-fixing microorganisms may offer the suitable conditions for shielding the nitrogenase enzyme complex from O_2 exposure (Santi et al. 2013).

15.1.2.1 Nonsymbiotic N Fixation

Free-living soil N-fixing microorganisms include *Archaea*, *Proteobacteria*, *Firmicutes*, and *Cyanobacteria* (Zhan and Sun 2012). *Archaea* form a monophyletic domain of remarkably diverse and successful organisms distinct from bacteria and eukarya. *Archaea* inhabits some of the most extreme environments on the planet, distributed over two main subdivisions: crenarchaeota (primarily hyperthermophiles and anaerobic respirers) and euryarchaeota (includes methanogens and extreme halophiles) based on 16S rRNA sequence comparisons (Woese et al. 1990; Brown and Doolittle 1997; Cabello et al. 2004). N_2 fixation in *Archaea* was first discovered in 1984 in *Methanosarcina barkeri* and *Methanococcus thermolithotrophicus* (Murray and Zinder 1984; Belay et al. 1984). Within the *Archaea*, N fixation has been found exclusively in the methanogenic euryarchaeota. However, N fixation is widespread within the methanogens, extending to all three orders, viz. Methanococcales (e.g., *Methanococcus thermolithotrophicus*, *Methanococcus maripaludis*), Methanomicrobiales (e.g., *Methanosarcina barkeri*, *Methanospirillum hungatei*), and Methanobacteriales (e.g., *Methanobacterium bryantii*) (Leigh 2000, Offre et al. 2013). Many bacteria reside in the soil and fix significant levels of N without the direct interaction with other organisms including *Azotobacter* (aerobic), *Klebsiella*, *Beijerinckia* (aerobic), *Bacillus*, *Clostridium* (anaerobic and non-photosynthetic), and *Rhodospirillum* (anaerobic and photosynthetic). Sergei Winogradsky in 1983 discovered the first N-fixing bacterium, *Clostridium pasteurianum*, followed by Martinus Beijerinck's description of *Azotobacter* in 1901. Following the discovery of *Clostridium* and *Azotobacter* an increasing number of N-fixing microorganisms were isolated. Within the domain bacteria, the phylum

Proteobacteria comprises the largest and phenotypically most diverse phylogenetic lineage scattered over five classes at present, viz. Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Deltaproteobacteria, and Epsilonproteobacteria (Kersters et al. 2006). The *Proteobacteria* include both free-living and symbiotic N-fixing bacteria. The genus *Azotobacter* belongs to the gamma-subclass of the *Proteobacteria* (Becking 2006). *Azotobacter* spp. are free-living, Gram-negative, aerobic bacteria primarily found in neutral to alkaline soils along with rhizosphere of many plants. They are nonsymbiotic heterotrophic bacteria capable of fixing an average 20 kg N/ha/per year and also produce plant growth-promoting substances. *Azotobacter chroococcum* is the most prevalent species found. The genus *Beijerinckia* belongs to the alpha-subclass of the *Proteobacteria*. *Beijerinckia* spp. are free-living, aerobic, chemoheterotrophic bacteria commonly found in acidic soils and within rhizosphere and phyllosphere environments of many higher plants (Kennedy 2005). Certain *Bacilli* comprise the known facultative, non-photosynthetic N-fixing bacteria. The genus *Klebsiella* belongs to the gamma-subclass of the *Proteobacteria. Klebsiella* spp. are Gram-negative, facultative anaerobic bacteria. Among the anaerobes, the most ubiquitous N-fixing genus is *Clostridium*, found in almost all kinds of soils. About 15 genera of O_2-evolving photosynthetic BGA (cyanobacteria) are found freely in the soil where they fix free atmospheric N_2 including *Nostoc*, *Anabaena*, and *Aulosira*. N_2-fixing cyanobacteria can be broadly divided into two groups: heterocystous forms and non-heterocystous forms. Heterocysts are specialized thick-walled cells, which occur at intervals along the cyanobacterial filaments and provide an environment suitable for the functioning of nitrogenase enzyme complex. In comparison to heterocystous N-fixing BGA, non-heterocystous N-fixing BGA are less in number, e.g., *Oscillatoria*, *Phormidium*, and *Gloeothece*.

15.1.2.2 Symbiotic N Fixation

On the basis of the level of proximity and interdependency of the plants and N-fixing microbes, their association could be broadly divided into three categories: loose associations with free-living N fixers, intercellular endophytic associations, and endosymbioses (Mus et al. 2016). Many free-living microorganisms are loosely associated with a variety of plants. These associations do not engage any kind of structural or morphological accommodation of the N-fixing organisms but involve more of close physical contacts in which reciprocal influence can be exerted between the symbionts (Hamdi 1982). N-fixing bacteria that live in loose associations with plant roots are usually designated as "associative" N-fixing bacteria (Elmerich 2007). An interaction between plant and associative N-fixing bacteria is one of the simplest types of N-fixing symbiosis. *Azospirillum* (α-subclass of proteobacteria), a Gram-negative free-living N-fixing bacteria, is one of the best studied associative diazotrophs that colonize the rhizosphere of many plants, but do not invade plant tissues. These PGPR positively influence the acquisition of N, P, and other essential minerals and thereby improve the yield and growth of several crops, such as wheat, maize, and rice (Steenhoudt and Vanderleyden 2000). The plant scientists are exploring PGPR and investigating their modes of action so as to use them commercially as biofertilizers. Leaf surfaces (phyllosphere) have also been shown to

support extensive surface populations of a range of microorganisms, including diazotrophic bacteria. Ruinen (1975) mentioned the common genera of microorganisms detected in the phyllosphere in different regions of the globe, including *Pseudomonas*, *Xanthomonas*, *Bacillus*, *Clostridium*, *Cyanophyceae*, *Chlorophyceae*, and lichens. In tropical forests, N-fixing cyanobacteria belonging to the genera *Scytonema*, *Oscillatoria*, *Microcoleus*, and *Stigonema* have been reported to colonize the phyllosphere of plants (Bentley 1987; Fürnkranz et al. 2008). *Azolla*, an aquatic fern, has been used by farmers as biofertilizers in rice fields since ages as heterocystous cyanobacterium *Nostoc azollae* is present within the specialized cavities on the underside of *Azolla* leaves.

In more developed associations, diazotrophic bacteria spread and multiply within plant tissues without causing any visible harmful effects, classified as endophytic N-fixing bacteria due to their firm association with plant tissues (Mus et al. 2016). Several diazotrophic endophytes have been identified in grass species (e.g., maize, rice, sorghum, sugarcane, and wheat) that are members of the alpha-, beta-, and gamma-subclasses of the proteobacteria including *Azoarcus*, *Herbaspirillum*, *Acetobacter diazotrophicus, Burkholderia, Serratia marcesens*, and *Gluconacetobacter* (Triplett 1996; Reinhold-Hurek and Hurek 1998; Pedraza 2008). Endophytic bacteria invade plant tissues; however, they do not dwell within living plant cells and does not stimulate the development of any differentiated plant structure like that of endosymbionts (Carvalho et al. 2014). Endophytic bacteria usually reside in root intercellular spaces, xylem vessels, and lignified xylem parenchyma, as well as in dead cells (James 2000). In order to uphold stable symbiosis, apart from N-fixation diazotrophic endophytes produce numerous compounds that support plant growth and help them acclimatize better to the environment. The most well-studied diazotrophic endophytes are *Gluconacetobacter diazotrophicus* and *Herbaspirillum* spp. in sugarcane; *Azoarcus* spp. in Kallar grass; and *Bacillus*, *Enterobacter*, *Herbaspirillum*, and *Pseudomonas* in rice and maize (James 2000). Inoculation experiments on sugarcane plants with different strains of endophytic diazotrophic bacteria reported an approximately 30% contribution of BNF, along with a 39% increase in total plant biomass (Oliveira et al. 2002). *Nostoc* is able to endophytically colonize the thallus of two genera of liverworts, viz. *Blasia* and *Cavicularia* (in dome-shaped auricles), and all hornworts (in slime cavities or mucilage-filled canals) along with the cortical layer of the specialized coralloid roots of cycads that arise from the lateral roots (Adams and Duggan 2008; Costa and Lindblad 2002). Cycads are the only known gymnosperms so far that have shown the ability to develop a N-fixing symbiosis with cyanobacteria through an endophytic association. *Calothrix* has also been reported to form endosymbiotic association with Cycadaceae occasionally (Costa et al. 1999; Thajuddin et al. 2010).

The highly specific, intimate, and most efficient form of N-fixing plant-microbe association is endosymbiosis. In some plants, diazotrophs elicit the formation of root nodules, specialized 2–5 mm diameter organs that are usually developed on roots to house N-fixing bacteria (Gage 2004). Diazotrophic endophytic bacteria and endosymbionts have an advantage over associative bacteria as they are in direct contact with plant cells, a better niche for N fixation and assimilation of fixed N by plants. The most well-studied plant endosymbioses are those between legumes

(family Fabaceae) and rhizobia, Gram-negative members of the alpha-subgroup of the phylum proteobacterial *Rhizobiaceae* family (including the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, *Bradyrhizobium*, and *Azorhizobium*), and between actinorhizal plants and *Frankia* bacteria (Santi et al. 2013). *Frankia* is able to fix N both under symbiotic and free-living conditions, unlike rhizobia.

In order to begin a fruitful mutualistic relationship with the host plant, rhizobia must first identify and then respond to the presence of host plant roots. Plants secrete certain flavonoids in the soil to attract the bacteria. These flavonoids induce the expression of signaling molecules called nodulation (Nod) factors which are important for nodule formation on the plant roots. The particular chemical structure of these Nod factors recognized by plant varies among bacterial species adding to host-symbiont specificity. Rhizobia enter the roots of most legume plants via root hair infection, which grows through the root cortex and branch repeatedly (Sprent et al. 2013). During growth in the rhizosphere, rhizobia recognize compounds secreted by the host root (e.g., flavonoids and betaines) and in turn respond by inducing *nod* genes (Gage 2004). The *nod* genes then encode around 25 proteins necessary for the bacterial synthesis and export of Nod factor, a lipo-oligosaccharide nodulation signal molecule that initiates many developmental changes in the host plant including root hair deformation, membrane depolarization, initiation of cell division in the root cortex, and initiation of nodule primordia (Gage 2004). The formation of infection thread is initiated when a deformed root hair quickly curls, and traps the bacteria bound to it between appressed cell walls. Afterwards, degradation of the cell wall and invagination of the cell membrane, followed by tip growth of the invagination, lead to extension of infection thread that grows down the inside of the root hair and into the body of the epidermal cell (Callaham and Torrey 1981). The rhizobia keep on dividing and growing inside the thread, thereby keeping the tubule filled with bacteria and infection thread propagated further towards the root interior. Rhizobia eventually enter cortical cells of the roots via endocytosis. In cortical cells, they differentiate into N-fixing bacteroides within a unique plant organelle known as symbiosome and continue to produce proteins essential for N-fixation and for the maintenance of the symbiotic relationship (van Spronsen et al. 1994; Andrews and Andrews 2017). The symbiosome is delimited by a plant-derived membrane that controls the nutrient exchange between the symbionts (Mus et al. 2016). Other than the usual hosts of rhizobia–legumes (family Fabaceae), the only non-legume host known is *Parasponia* species (family Cannabaceae) (Akkermans et al. 1978). A similar Nod-dependent symbiotic interaction has been observed for *Parasponia*.

Frankia, a filamentous Gram-positive actinomycete, induces root nodules on a variety of woody plants belonging to eight families distributed over three orders, viz. Fagales (Betulaceae, Casuarinaceae, and Myricaceae), Rosales (Rosaceae, Elaeagnaceae, and Rhamnaceae), and Cucurbitales (Datiscaceae and Coriariaceae), collectively called actinorhizal plants (Wall 2000; Pawlowski 2009; Franche and Bogusz 2011; Berry et al. 2011). The strategies used by *Frankia* spp. to infect actinorhizal plants are somewhat alike to those used by rhizobia. *Frankia* infects the root cells of actinorhizal plants either through intercellular root invasion or through intracellular root-hair infection (Benson and Silvester 1993). Although factors

responsible for host-symbiont specificity and preliminary plant response induction are poorly understood for *Frankia*, it is known that *nodABC*-like genes are present with other genes such as *nif* (nitrogenase), *suf* (sulfur-iron cofactor synthesis), and *hup* (hydrogenase uptake), all known to be involved in symbiosis (Alloisio et al. 2010). In temperate climates, the actinorhizal plants inhabited by *Frankia* have been reported to fix N at comparable rates to those of legume symbioses, i.e., ~300 kg/ha/year (Hibbs and Cromack 1990; Wheeler and Miller 1990). Many actinorhizal plants are also competent of forming mycorrhizal associations, and this tripartite symbiosis (host plant–*Frankia*–mycorrhiza) helps actinorhizal plants to grow in degraded and disturbed soils and play important ecological roles to prevent land desertification (Dawson 2008).

N-fixing cyanobacteria *Nostoc* have also been found to colonize plants in the family Gunneraceae. The intimacy of the symbiosis *Gunnera-Nostoc* is greater than in any other cyanobacterial associations as here the cyanobacteria are hosted intracellularly. The cyanobiont enters nonlegume angiosperm Gunnera plants through specialized glands located on the stem in the axis of the leaves, which secrete carbohydrate-rich mucilage and attract specific symbiotic cyanobacteria (Silvester and McNamara 1976; Towata 1985; Bonnett 1990). After entering the gland, cyanobacteria induce divisions in the host cells and are accommodated within inner cortex cells (Bergman et al. 1992). *Nostoc* filaments are surrounded by the host's plasma membrane, which acts as the interface for nutrient exchange. Other diazotrophic bacteria with the common ability to fix N are mentioned in Table 15.1. Recent research and advances in the understanding of associative, endophytic, and endosymbiotic N-fixation with legumes and nonlegume plants may lead to the development of novel techniques for delivering N to cereals and other nonlegume crops which is crucial for the future of sustainable agriculture, including the reduction of soil pollution and ensuring of food security.

15.1.3 N Uptake and Its Regulation in N-Deficit Conditions

N is acquired by plants in both inorganic (ammonium and NO_3-) and organic (amino acids, urea, etc.) forms; however, ammonium and NO_3^- are universally favored and found in most soils. As a general practice, chemical fertilizers rich in N are used as additives for crops. This has significantly impacted the global N balance in a negative way contributing to pollution of lakes and rivers, accumulation of greenhouse gases, acidification of soil, and ozone-layer depletion. Most of these mentioned concerns root from the fact that plants utilize only a fraction of available N. The availability of N is low and variable in natural soils which create N-depletion zones (Jackson and Caldwell 1993). In response to low N availability plants enhance NUE (Nacry et al. 2013). NUE has been defined in many ways. To sum, it is the ratio of the amount of fertilizer used by the crop to the amount of fertilizer applied. N uptake and N utilization together contribute to plant NUE. The former is the N-acquiring capacity of the roots and the latter is the fraction of the acquired N that is converted to plant biomass (Xu et al. 2012). Achieving synchrony between the two under limiting N conditions is a challenge which plants face to survive.

Table 15.1 Non-nodulating and nodule-inducing diazotrophic bacteria from legume root nodules (source: Martínez-Hidalgo and Hirsch 2017)

Phylum/class	Genus	Host	Nodule inducing/non-nodulating
Alpha-proteobacteria			
	Agrobacterium	*Sebania, Glycine*	Nodule inducing
	Aminobacter	*Anthyllis*	Nodule inducing
	Bosea	*Ononis, Lupinus*	Nodule inducing
	Devosia	*Neptunia*	Nodule inducing
	Methylobacterium	*Crotalaria, Listia, Lotononis*	Nodule inducing
	Microvirga	*Listia, Lupinus, Vigna*	Nodule inducing
	Ochrobactrum	*Cytisus, Lupinus*	Nodule inducing
	Phyllobacterium	*Ononis, Sophora*	Nodule inducing
	Shinella	*Kummerowia*	Nodule inducing
	Azospirillum	*Trifolium, Phaseolus, Vicia, Medicago*	Non-nodulating
	Gluconacetobacter	*Glycine*	Non-nodulating
	Ochrobactrum	*Cicer, Glycyrrhiza, Pisum, Lupinus, Vigna*	Non-nodulating
	Methylobacterium	*Arachis*	Non-nodulating
Beta-proteobacteria			
	Burkholderia	*Papilionoid, Mimosoid*	Nodule inducing
	Cupriavidus	*Mimosa*	Nodule inducing
	Burkholderia	*Mimosa, Glycine, Arachis, Lespedeza*	Non-nodulating
	Variovorax	*Crotalaria, Acacia*	Non-nodulating
Gamma-proteobacteria			
	Klebsiella	*Arachis, Glycine, Vicia*	Nodule inducing
	Pseudomonas	*Hedysarum, Robinia*	Nodule inducing
	Klebsiella	*Vigna, Arachis*	Non-nodulating
	Pseudomonas	*Vigna, Arachis*	Non-nodulating
	Pantoea	*Mimosa, Lathyrus, Lotus, Medicago, Melilotus, Robinia, Trifolium, Vicia, Phaseolus*	Non-nodulating
Actinobacteria			
	Rhodococcus	*Anthyllis, Lotus*	Nodule inducing
	Arthrobacter	*Lespedeza, Pisum, Trifolium*	Non-nodulating
	Brevibacterium	*Cicer, Cajanus*	Non-nodulating
	Micromonospora	*Lupinus, Pisum, Medicago, Casuarina*	Non-nodulating
	Streptomyces	*Pisum, Cicer*	Non-nodulating
Firmicutes			
	Bacillus	*Calycotome, Cicer, Glycine, Oxytropis, Pisum, Sophora*	Non-nodulating
	Paenibacillus	*Cicer, Medicago, Lupinus, Prosopis*	Non-nodulating

15.1.3.1 Root Architecture

Due to the heterogeneous availability of resources, plant roots modulate its physiological structure to maximize resource capture. Root proliferation, that is, production of new roots (lateral initiation in particular), occurs in/towards nutrient-rich

patches (Hodge 2004). This phenomenon is enhanced when there is a scarcity of a particular nutrient. This holds true for NO_3^-, one of the most important growth-limiting nutrients. The strength of the N limitation and varied environmental cues depict the root modifications. For example, under the mild N limitation, the length of primary and lateral roots is increased, whereas during severe N limitation total root development is affected, the outcome being shortened primary roots (Lopez-Bucio et al. 2003). The heterogeneity in resource distribution in soil is a result of microbial decomposition. Microbial decomposition yields inorganic nutrients readily available for plant capture from both organic and inorganic sources. Root proliferation albeit a slow process eventually outcompetes the microbial population.

NO^{3-} is the major form of N present in aerobic soils and ammonium is the major form in flooded wetlands or waterlogged soils. NO3 and ammonium are taken up by the roots of plants via their specific promoters.

15.1.3.2 Nitrate Transporters

Nitrate transporter system can be divided into low-affinity transport system (LATS) and high-affinity transport system (HATS) based on the supply of cellular energy (Miller et al. 2007). There are four gene families of nitrate transporters, of which *nitrate transporter 1* (NRT1)/*peptide transporter* (PTR) family (now renamed as NPF family) and *nitrate transporter 2* (NRT2) are involved in NO_3^- uptake (Nacry et al. 2013; Krapp et al. 2014; Fan et al. 2017). NPF family comprises 53 members in *Arabidopsis* and 93 members in rice (*Oryza sativa* L.) (Léran et al. 2014; von Wittgenstein et al. 2014). Except for AtNPF6.3 (NRT1.1) in *Arabidopsis* (Ho et al. 2009) and MtNRT1.3 in *Medicago truncatula* (Morere-Le Paven et al. 2011), which function as dual-affinity transporters, NPF members suggestively belong to LATS (Léran et al. 2014). On the other hand, all the NRT2 family members belong to HATS and generally require a partner protein, namely NAR2 (nitrate assimilation-related protein), to transport NO_3^- (Fan et al. 2017). Notably, there are about seven members of the NRT2 family in *Arabidopsis* (Miller et al. 2007) and five in rice genome (Feng et al. 2011).

Under high-NO_3^- conditions, both the dual-affinity transporters and low-affinity transporters have been shown to function. However, under N-limiting conditions, high-affinity transporters of NRT2 family play a major role in NO_3^- influx through roots (Fan et al. 2016a, b; O'Brien et al. 2016). Among the seven genes of NRT2 family in *Arabidopsis*, four, namely *AtNRT2.1*, *AtNRT2.2*, *AtNRT2.4*, and*AtNRT2.5*, are predominantly expressed under N-deprived conditions. These transporters contribute to 95% of NO_3^- influx (Lezhneva et al. 2014). AtNPF6.3, a dual-affinity transporter, is also expressed under N-limiting conditions; however, its contribution towards NO^{3-} uptake is almost nonexistent (Glass and Kotur 2013).

The role of nitrate transceptor (transporter/sensor) NRT1.1 is well established in NO_3^- sensing (Remans 2006; Ho et al. 2009; Gojon et al. 2011). It works as a dual transporter of $NO3^-$ and auxin in NO_3^--rich conditions. There are numerous other regulatory molecules that are involved in NO_3^- perception and transportation and control root developmental activity accordingly. For example, in calcineurin B-like

(CBL) interacting protein kinase (CIPK) gene, namely *CIPK8*, the expression is NO^{3-} induced. CIPK8 induces downstream nitrate transporter genes, genes required for nitrate assimilation and also nitrate-induced primary root growth. Another kinase, CIPK23, phosphorylates NRT1.1, which acts as a high-affinity transporter when phosphorylated and as a low-affinity transporter when dephosphorylated, contributing to its dual nature. It is also responsible for localized root proliferation. This highlights a faint corner of the picture, giving us an idea of the complex nature of NO_3^- sensing and how plants are able to actively forage NO_3^- in a heterogeneous N environment by transcriptome reprogramming.

15.1.3.3 Ammonium Transporters

Uptake of ammonium is mediated by ammonium transporters (AMTs) or methylammonium permeases (MEPs). In *Arabidopsis*, AtAMT family comprises six members, all of which are high-affinity transporters (Loque et al. 2006), and in rice, at least ten AMT homologues are present designated as OsAMT-like transporters further subgrouped into two families—OsAMT1 and OsAMT2 (Sonoda et al. 2003; Suenaga et al. 2003).

In *Arabidopsis*, under N-limiting conditions, it has been observed that the transcript levels of five out of six AMT genes are upregulated. Of these, the first three genes mentioned account for 90% of high-affinity ammonium uptake from soil under the same conditions (Yuan et al. 2007). While AMT expression is activated under N-limited conditions, in rice, maize, and tomato an "ammonium-inducible" expression is observed (Sonoda et al. 2004; Gu et al. 2013).

15.1.4 Nitrate Sensing and Signaling

Plants often grow in N-deficient soils and they must sense changes in external and internal concentrations of metabolites and adjust to the nutrient availability for survival in such nutrient-limited environments. NO^{3-} is the most common form of nitrogen available to plants; sensing and signal transduction networks that control plant responses to nutrient deprivation are not well characterized for N. The N and carbon (C) metabolisms are linked and therefore cross talk between the signal transduction pathways that regulate N assimilation and C metabolism is expected. Though many studies have been done in the past few decades, the mechanisms by which N and C signaling is integrated into plants are still not clear. NO_3^- sensing is very complex, necessitating the study of the coordinated regulation of multiple metabolic and regulatory pathways (including N and C) by NO_3^- as a signal (Zheng 2009; Nunes-Nesi et al. 2010). Recently, HY5 has been identified as a novel regulatory molecule with the ability to maintain C/N status in the plants (Chen et al. 2016).

15.1.4.1 How Plants Perceive N Stress

Nitrate absorbed by NO_3^- transporters in plants is transported to other organs and it is also involved in sensing as well as integrated plant responses. The role of NO_3^- in modulating a wide range of processes including plant growth, root system

architecture, leaf development, seed dormancy, and flowering time is very well documented in literature (Alvarez et al. 2012; Krouk et al. 2010; Rahayu et al. 2005; Alboresi et al. 2005; Marín et al. 2011). Under N deficiency, activities of nitrate/ammonium transporters can be modulated at both the transcriptional and posttranslational levels by signaling molecules such as Ca^{2+}, ROS, and phytohormones in plants (Schachtman and Shin 2007). In addition, light, regulatory proteins, downstream N metabolites, and epigenetic mechanisms are also shown to be involved in NO_3^- sensing and regulation as discussed below. The comprehensive network established by the integration of various signaling pathways and the mechanism of regulation need to be investigated further.

15.1.4.2 Candidates in Nitrate Sensing and Signaling

Nitrate as a signal has been proposed for a long time, yet there is still much less clarity about how the plant monitors its N status and the downstream signaling events associated with it. Much work has focused on the immediate response of the addition of nitrate and the induction of nitrogen metabolism. The root responses to nitrate are well documented, and the transporter NRT2.1 was reported to be involved in nitrate sensing or transduction in roots. The activity of the enzyme nitrate reductase (NR), involved in conversion of nitrate to nitrite, is controlled by both its steady-state levels and reversible protein phosphorylation by the action of protein phosphatases and kinases. NR activity responds rapidly to light/dark signals. NR is active and dephosphorylated in the light by protein phosphatases, while it is inactive and phosphorylated in the dark by sucrose nonfermenting-related kinase (SnRK) and calcium-dependent protein kinase (CDPK). The light-mediated regulation of NR gene expression has been studied in depth by Raghuram and Sopory (1995a); and Lillo and Appenroth (2001). More studies on the phytochrome-mediated regulation of NR gene expression in maize and rice mediated through G-protein (Raghuram et al. 1999), PI cycle, and protein kinase C (Raghuram and Sopory 1995b) have been reported. The posttranslational regulation of metabolic activity of nitrate reductase by 14-3-3 is studied in detail (Shin et al. 2011,). Moreover, the central role of 14-3-3 in maintaining metabolic coordination between multiple enzymes such as glutamine synthetase, sucrose-phosphate synthase, trehalose-phosphate synthase, glutamyl-tRNA synthetase, and various signaling molecules of C/N metabolism also needs to be investigated further (Pathak et al. 2008, 2011).

Similarly, much work has been done on the downstream metabolites of nitrate assimilation pathway such as nitrite, ammonium ions, glutamine, and other amino acids, which is beyond the scope of this review. Numerous nitrogen metabolites have been shown to regulate carbon metabolism to meet the demands of increasing N flux through 2-OG. Glutamine is another key metabolite linked to nitrogen metabolism that plays a role in metabolic regulation and possibly signal transduction (Fallon and Weng 2014). In addition to sensing nitrate, plant cells may also sense carbon status, which leads to the regulation of key nitrate transporters NRT2.1 and NRT1. Another key metabolite involved in nitrogen metabolism is glutamate and the presence of multiple glutamate receptors in plant genomes (Price et al. 2012) may indicate the potential importance of this metabolite in initiating signal transduction cascades.

Nitrogen sensing and plant response are also mediated through plant hormones, though the signaling pathways need to be characterized in detail further. The levels of ABA decrease under nitrogen sufficiency, while nitrogen deficiency leads to decreased cytokinin levels to facilitate biomass allocation between roots and shoots. Auxin synergistically affects cytokinin activity on cell division and organogenesis (Soni et al. 1995), while ABA antagonizes the cytokinin-mediated nitrogen signaling by negatively regulating cytokinin-inducible response regulator genes.

With the recent whole-transcriptomics studies, more than a thousand genes are shown to be nitrate responsive and the presence of nitrate response elements (NRE) has been proposed in nitrate reductase and nitrite reductase from *Arabidopsis thaliana* and birch (Hwang et al. 1997; Warning and Hatchel, 2000). But later they were shown to be randomly distributed throughout the genome (Raghuram et al. 2006). There are no further reports of new cis-regulatory elements working independently or in integration with carbon metabolism (Pathak et al. 2011).

Global transcriptomic studies have also revealed several MADS-box transcription factors like *ANR1* and a few Myb transcription factors which were found to be upregulated by nitrogen deprivation and are thought to play important roles in the signaling of nitrogen deprivation (Schachtman and Shin 2007) but their targets are yet unknown. There are few other candidates that have been proposed in N signaling such as Ca^{2+} and protein kinases/phosphatases in the expression of NR, NiR, and GS2 transcripts (Sakakibara et al. 1998; Sueyoshi et al. 1999); the target of rapamycin (TOR) signaling pathway; the general control non-derepressible 2 (GCN2) pathway; the plastidic PII-dependent pathway; and family of plant glutamate receptors (GLR); however, their specific role in N sensing is not elucidated yet. GLR is proposed to act upstream of a Ca^{2+} regulated signaling pathway to regulate the plant's response to changes in N status (Gent and Forde 2017).

15.1.5 Epigenetic Regulation in N Homeostasis

Many miRNAs are constantly being implicated in novel cellular responses, including nutrient response in plants. These are a part of complex epigenetic mechanisms involved in sensing nutrient limitation and maintaining homeostasis (Paul et al. 2015; Chiou 2007). Till now, very few studies have focused on the role of chromatin regulation in response to changes in nutrient availability, and thus its potential role in regulating nutrient homeostasis mainly in P, N, and sulfur (S). Kou et al. (2011) showed enhanced tolerance to the N-deficiency stress in rice by DNA methylation. Recently, two nitrate-responsive miRNA, miR393 and miRNA167, along with their target, auxin receptors, AFB3 and ARF8, respectively, have been shown to regulate root growth in response to NO_3^- availability in soils (Vidal et al. 2010). Moreover, the role of posttranslational chromatin modification in N acquisition in *Arabidopsis* has been reported by Widiez et al. (2011) in case of high N-insensitive 9 (HNI9) which represses NRT2.1 expression by DNA methylation in roots.

The signaling mechanisms also synchronize the mineral status of the plant in response to the nutrient availability at the root level by altering the expression and/or activity of the membrane transporters (Liu et al. 2009). Epigenetic mechanisms

involved in this adaptive response definitely will provide new targets for plant breeding and gene editing.

15.1.6 Cross Talk Between Nitrate and Other Mineral Nutrients

Recently, many reports have indicated that nitrate signaling has a close relationship with other nutrients at the transcriptional as well as posttranslational level. Initially, ROS signaling may be induced in any of the nutrient deficiencies involving N, P, K, or S (Schachtman and Shin 2007). Lin et al. (2008) have reported that in *Arabidopsis*, K^+ deficiency not only induces the expression of K^+ transporter genes but also alters the transcription of nitrate transporter genes. This indicates that a cross talk may exist among different signaling pathways for plant responses to different nutrient deficiencies.

15.1.7 Use of Microbiome as Bioinoculants or Biofertilizers

There has been a constant demand to increase agricultural productivity due to increase in human population and environmental pressure. Green revolution paved the road to modern practices involving the use of N fertilizers. The use of N fertilizers has increased from 10 to 100 million metric tons from 1960 to 2005 in developed and developing nations (Ogburn 2010). Despite evident advantages of N fertilizers, today a situation has come where this abuse has influenced the global N balance and placed our ecosystems into a precarious condition. It is a threat to climate, human health, fish, and other wildlife through run off, and not only this, there are associated risks of explosion with storage of N fertilizers. A lot of money and energy are invested each year in research to minimize the use or get rid of these fertilizers and there are not many alternatives. Improving plant N uptake and assimilation by genetic improvement (through conventional breeding and indirect manipulation of quantitative trait loci) seemingly over simplistic is rather unrealistic as not every plant can be modified and there is a threshold limit to improvement. The other suitable way is to modify soil microflora.

There are many reports where beneficial effects of PGPR have been shown in improving plant growth and yield, even though the mechanisms used to directly or indirectly promote plant growth are not fully understood yet. PGPR can be used as either biofertilizers, phytostimulants, or biopesticides based on their mode of action. It is known that many PGPR often use more than one mode for improving plant growth and the utilization of microbial inoculants can play an important role in sustainable agriculture (Bhattacharyya and Jha 2012; Glick 2012; Nehra and Choudhary 2015). Many of the PGPRs like *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Rhizobium*, and *Serratia* have been used for large scale commercially yet successful commercial-scale production of numerous new PGPR like *Azoarcus*, *Exiguobacterium*, *Methylobacterium*, *Paenibacillus*, and *Pantoea* with substantial beneficial activities is yet to be achieved. These PGPR can offer excellent solutions for enhanced yields and production of staple food crops. The use of PGPR for enhanced plant growth is summarized in Table 15.2.

Table 15.2 Effects of addition of PGPPR as biofertilizers, phytostimulants, and biopesticides

S. no.	Plant	Bacterial consortium	Role/abilities	Effect on plant growth	References
1.	*M. truncatula*	*Pseudomonas brassicacearum* KK 5 and consortium of *Pseudomonads*, B*acillus* sp, *Xanthomonas* sp.	Biofertilizer	Significantly enhance fresh and dry weight of root, shoots, and whole 5-week-old seedlings	Kisiel and Kępczyńska (2016)
2.	Maize	*Serratia liquefaciens*, *Bacillus* sp. *Pseudomonas* sp. (individual and consortia experiments)	Biofertilization (N), biocontrol (several root pathogens)	Increase in maize yield by 14% (dry wt.) when inoculated as consortia, *S. liquefaciens* increased the dry weight of maize by more than 10%, *Bacillus* sp. by more than 7% and *Pseudomonas* sp. by more than 10%	Lalande et al. (1989)
3.	Cotton	*Klebsiella oxytoca*	Seeds, Rhizosphere	In addition to significant increase in height and dry weight of cotton plants, inoculation with PGPR uptake of major nutrients like N, P, K, and Ca increased while Na deceased	Yue et al. (2007)
4.	Wheat	*Pseudomonas putida, P. aeruginosa, S. proteamaculans*	Rhizosphere	Significant increase in plant height, root length, and chlorophyll content	Zahir et al. (2009)
5.	*Glycine max, Zea mays L.*	*B. japonicum, Azospirillum brasilense, Pseudomonas, Bacillus subtilis, B. thuringiensis, Aeromonas sp, Serratia sp*	Rhizosphere	BNF, promote seed germination and early seedling growth	Bai et al. (2003)

(continued)

Table 15.2 (continued)

S. no.	Plant	Bacterial consortium	Role/abilities	Effect on plant growth	References
6.	*Pinus sabiniana*, *Solanum lycopersicum*, *Lactuca sativa*, and *Zea mays*	AM fungi, free-living N_2 fixing bacteria like *Azospirillum brasilense* or *Azotobacter*	Roots	Stimulates root colonization, BNF, increases biomass, limits soil salinity stress, and affects plant yield	Kohler et al. (2010)
7	*Zea mays*	*Pseudomonas* sp, *Bacillus*, *Mycobacterium*	Roots	Stimulates plant growth, N, P, and K uptake in nutrient-deficient soil	Egamberdiyeva (2007)
8	*Lycopersicon esculentum*	*Bacillus subtilis* strain 101 *Azospirillum brasilense* sp. 245	Rhizosphere	Co-inoculation shows more plant height, node number, and total biomass	Felici et al. (2008)
9.	*Artichoke (Cynara scolymus)*	*Pseudomonas putida*, *Azospirillum*, *Azotobacter*	Rhizosphere	Phosphate-solubilizing bacteria along with nitrogen-fixing bacteria led to significant increase in radicle and shoot length, shoot weight, coefficient of velocity of germination, seedling, vigority index, and significant decrease in mean time of germination	Jahanian et al. (2012)
10.	*Brassica juncea*, *Alyssum serpyllifolium*	*Pseudomonas* sp. A3R3	Rhizosphere	Increased significantly the biomass (*B. juncea*) and Ni content (*A. serpyllifolium*) in plants grown in Ni-stressed soil	Ma et al. (2011)

(continued)

Table 15.2 (continued)

S. no.	Plant	Bacterial consortium	Role/abilities	Effect on plant growth	References
11.	*Lupinus luteus*	*Bradyrhizobium* sp. 750 *Pseudomonas* sp., *Ochrobactrum cytisi*	Rhizosphere	Increased both biomass and N content, accumulation of metals (phytostabilization potential)	Dary et al. (2010)
12.	Mung bean	*Ochrobactrum Bacillus cereus*	Rhizosphere	Lower the toxicity of cadmium to seedlings by reducing Cr (VI) to Cr (III)	Faisal and Hasnain (2005)
13.	*Brassica napus*	*Xanthomonas* sp. RJ3, *Azomonas* sp. RJ4 *Pseudomonas* sp. RJ10 *Bacillus* RJ31	Rhizosphere	Stimulated plant growth and increased cadmium accumulation	Sheng and Xia (2006)
14.	Peanut (*Arachis hypogaea* L.)	*P. fluorescens* strains PGPR1, PGPR2, PGPR4	Rhizosphere	Enhanced pod yield (23–26%), haulm yield and nodule dry weight, increased root length, pod number	Dey et al. (2004)
15.	*Brassica juncea, B. oxyrrhina*	*Pseudomonas sp. SRI2, Psychrobacter* sp. SRS8, *Bacillus* sp. SN9	Rhizosphere	Increased the biomass of the test plants and enhanced Ni accumulation in plant tissues	Ma et al. (2009a)
16.	*B. juncea, B. oxyrrhina*	*Psychrobacter* sp. SRA1, *Bacillus cereus* SRA10	Rhizosphere	Enhance the metal accumulation in plant tissue by facilitating the release of Ni from non-soluble phase in the soil	Ma et al. (2009b)
17.	*Maize*	*Pseudomonas aeruginosa, P. fluorescens, Ralstonia metallidurans*	Rhizosphere	Promoted plant growth, facilitated soil metal mobilization, enhanced Cr and Pb uptake	Braud et al. (2009)

(continued)

Table 15.2 (continued)

S. no.	Plant	Bacterial consortium	Role/abilities	Effect on plant growth	References
18.	*Lupinus luteus*	*Bradyhizobium* sp. 750 *Pseudomonas* sp., *Ochrobactrum cytisi*	Rhizosphere	Increased both biomass and N content, accumulation of metals	Dary et al. (2010)
19.	*L. sativa*	*P. putida CCR2–4, Bacillus subtilis* CC-pg104	Rhizosphere	Significant increase in shoot length and root length achieved through encapsulated inoculant	Rekha et al. (2007)
20.	Maize	*P. putida* strain R-168, *P. fluorescens* strain R-93, *P. fluorescens*, DSM 50090, *P. putida* DSM291, *Azospirillum lipoferum* DSM 1691, *A. brasilense* DSM 1690	Rhizosphere	Significant increase in plant height, shoot dry weight, seed weight, number of seeds per ear and leaf area	Gholami et al. (2009)
21.	*Cicer arietinum* L.	*Escherichia coli, P. fluorescens, Burkholderia sp*	Rhizosphere	Increased plant height and leaf numbers and biomass	Dasgupta et al. (2015)
22.	*Solanum lycopersicum, Abelmoschus esculentus*	*Bacillus subtilis, Pseudomonas*	Rhizosphere	Dry biomass increased 31% for tomato, 36% for okra, and 83% for African spinach	Adesemoye et al. (2008)
23.	Cotton	*Azotobacter, Chroococum, Azospirillum lipoferum*	Rhizosphere	Seed yield (21%), plant height (5%), and microbial population in soil (41%) increased over their respective controls while boll weight and staple length remained statistically unaffected	Anjum et al. (2007)

(continued)

Table 15.2 (continued)

S. no.	Plant	Bacterial consortium	Role/abilities	Effect on plant growth	References
24.	*Cajanus cajan*	*Bacillus subtilis, Bacillus pumilus, Rhizobium sp. IC3 123*	Rhizosphere	Increase in plant fresh weight, chlorophyll content, nodule	Rajendran et al. (2008)
25.	*Rice*	*Bacillus sp. Paenibacillus sp.*	*Roots*	Promote root and shoot growth significantly	Beneduzi et al. (2008)
26.	Wheat, spinach	*Bacillus cereus* RS18 *B. licheniformis* RC08	Roots	All bacterial strains were effective in IAA production	Cakmakci et al. (2007)
27.	Mustard	*Pseudomonas sp., Bacillus sp.*	Rhizosphere	Rhizosphere stimulated plant growth and decreased CR (VI) content	Rajkumar et al. (2006)
28.	*O. sativa*	*Azospirillum brasilense, Bacillus pantothenticus*	Rhizosphere	Increased rice grain yield, maximum up to 76.9%	Thakuria et al. (2004)
29	*T. aestivum*	*K. pneumonia* strains K11 and K42	Rhizosphere	Significantly increased root length and shoot height of inoculated wheat seedlings over the control	Sachdev et al. (2009)
30.	*Achyranthes aspera* L.	*P. aeruginosa* AL2-14B	Endophytic	Increased N, P, and K contents in plant by 3.8, 12.59, and 19.15%, respectively. Significant enhancement of shoot and root length, dry leaf, dry shoot and dry root weight, and leaf surface area as compared to control	Devi et al. (2017)

15.2 Conclusion

The knowledge of phytomicrobiome is essential and is necessary to enhance sustainable food production and mitigate environmental challenges. The excessive use of fertilizers is disturbing the natural balance between phytomicrobiome and plants. Beneficial effects of phytomicrobiome in improving plant growth and yield have been shown, though the mechanisms are not fully understood yet. The immediate challenge is implementing sustainable cropping systems and practices with management of phytomicrobiome along with plant breeding efforts to support a new Green Revolution that ensures food security for future generations. The agronomic practices should focus on the management of soil microbes adjusted to specific plant genotypes in specific environments. The focus should be on designing new strategies to utilize beneficial contribution of phytomicrobiomes to improve the crop yields and also exhaustive screening of plant varieties which can be optimized for higher yield using defined microbial composition. A deeper understanding on the role of phytomicrobiomes of crops in nitrogen and other mineral stresses along with suitable crop as well as soil management practices will offer solutions for increased agricultural production.

References

Adams DG, Duggan PS (2008) Cyanobacteria-bryophyte symbioses. J Exp Bot 59:1047–1058

Adesemoye AO, Obini M, Ugoji EO (2008) Comparison of plant growth promotion with *Pseudomonas aeruginosa* and *Bacillus subtilis* in three vegetables. Braz J Microbiol 39:423–426

Ahemad M, Malik A (2011) Bioaccumulation of heavy metals by zinc resistant bacteria isolated from agricultural soils irrigated with wastewater. Bacteriol J 2:12–21

Akkermans ADL, Abdulkadir S, Trinick MJ (1978) N_2-fixing root nodules in Ulmaceae: *Parasponia* or (and) *Trema* spp.? Plant Soil 49:711–715

Alboresi A, Gestin C, Leydecker MT et al (2005) Nitrate, a signal relieving seed dormancy in Arabidopsis. Plant Cell Environ 28:500–512

Alloisio N, Queiroux C, Fournier P et al (2010) The Frankia alni symbiotic transcriptome. Mol Plant-Microbe Interact 23(5):593–607

Alvarez JM, Vidal EA, Gutierrez RA (2012) Integration of local and systemic signaling pathways for plant N responses. Curr Opin Plant Biol 15:185–191

Andrews M, Andrews ME (2017) Specificity in legume-rhizobia symbioses. Int J Mol Sci 18:705

Anjum MA, Sajjad MR, Akhtar N et al (2007) Response of cotton to plant growth promoting rhizobacteria (PGPR) inoculation under different levels of nitrogen. J Agric Res 45:135–143

Argudo M, Little R, Shearer N et al (2005) Nitrogen fixation: key genetic regulatory Mechanisms. Biochem Soc Trans 33:152–156

Bai YM, Zhou X, Smith DL (2003) Enhanced soybean plant growth resulting from coinoculation of *Bacillus* strains with *Bradyrhizobium japonicum*. Crop Sci 43:1774–1781

Becking J (2006) The family *Azotobacteraceae*. Prokaryotes 6:759–783

Belay N, Sparling R, Daniels L (1984) Dinitrogen fixation by a thermophilic methanogenic bacterium. Nature 312:286–288

Beneduzi A, Peres D, Vargas LK et al (2008) Evaluation of genetic diversity and plant growth promoting activities of nitrogen-fixing Bacilli isolated from rice fields in South Brazil. Appl Soil Ecol 39:311–320

Benson DR, Silvester WB (1993) Biology of Frankia strains, actinomycete symbionts of actinorhizal plants. Microbiol Mol Biol Rev 57:293–319

Bentley BL (1987) Nitrogen fixation by epiphylls in a tropical rainforest. Ann Mo Bot Gard 74:234–241

Bergman B, Johansson C, Soderback E (1992) The *Nostoc-Gunnera* symbiosis. New Phytol 122:379–400

Berry A, Mendoza-Herrera A, Guo YT et al (2011) New perspectives on nodule nitrogen assimilation in actinorhizal symbioses. Funct Plant Biol 38:639–644

Bhattacharyya PN, Jha DK (2012) Plant growth -promoting rhizobacteria (PGPR): emergence in agriculture. World J Microbiol Biotechnol 28:1327–1350

Bloom AJ (2015) The increasing importance of distinguishing among plant nitrogen sources. Curr Opin Plant Biol 25:10–16

Bonnett HT (1990) The Nostoc–Gunnera association. In: Rai AN (ed) Handbook of symbiotic cyanobacteria. CRC Press, Boca Raton, FL, pp 161–171

Boyd ES, Peters JW (2013) New insights into the evolutionary history of biological nitrogen fixation. Front Microbiol 4:201

Braud A, Jézéquel K, Bazot S et al (2009) Enhanced phytoextraction of an agricultural Cr-, Hg and Pb-contaminated soil by bioaugmentation with siderophore producing bacteria. Chemosphere 74:280–286

Brown JR, Doolittle WF (1997) Archaea and the prokaryote-to-eukaryote transition. Microbiol Mol Biol Rev 61:456–502

Cabello P, Roldán MD, Moreno-Vivián C (2004) Nitrate reduction and the nitrogen cycle in archaea. Microbiology 150:3527–3546

Cakmakci R, Erat M, Erdogan UG et al (2007) The influence of PGPR on growth parameters, antioxidant and pentose phosphate oxidative cycle enzymes in wheat and spinach plants. J Plant Nutr Soil Sci 170:280–295

Callaham DA, Torrey JG (1981) The structural basis for infection of root hairs of *Trifolium repens* by *Rhizobium*. Can J Bot 59:1647–1664

Carvalho TLG, Balsemão-Pires E, Saraiva RM et al (2014) Nitrogen signalling in plant interactions with associative and endophytic diazotrophic bacteria. J Exp Bot 65:5631–5642

Chen H, Yang LQ, Wen L et al (2016) Effects of nitrogen deposition on soil sulfur cycling. Glob Biogeochem Cycles 30:1568–1577

Chiou TJ (2007) The role of microRNAs in sensing nutrient stress. Plant Cell Environ 30:323–332

Costa JL, Lindblad P (2002) Cyanobacteria in symbiosis with cycads. In: Rai AN et al (eds) Cyanobacteria in symbiosis. Springer, Dordrecht, The Netherlands, pp 195–205

Costa JL, Paulsrud P, Lindblad P (1999) Cyanobiont diversity within coralloid roots of selected cycad species. FEMS Microbiol Ecol 28:85–91

Crawford NM (1995) Nitrate: nutrient and signal for plant growth. Plant Cell 71(1):859–868

Dary M, Chamber-Pérez MA, Palomares AJ et al (2010) In situ phytostabilisation of heavy metal polluted soils using *Lupinus luteus* inoculated with metal resistant plant-growth promoting rhizobacteria. J Hazard Mater 177:323–330

Dasgupta D, Ghati A, Sarkar A (2015) Application of plant growth promoting rhizobacteria (PGPR) isolated from the rhizosphere of *Sesbania bispinosa* on the growth of chickpea (*Cicer arietinum* L.). Int J Curr Microbiol App Sci 4(5):1033–1042

Dawson JO (2008) Ecology of actinorhizal plants. In: Pawlowski K, Newton WE (eds) Nitrogen-fixing actinorhizal symbioses. Nitrogen fixation: origins, applications, and research progress. Springer, Dordrecht, pp 199–234

Dean DR, Jacobsen MR (1992) Biochemical genetics of nitrogenase. In: Stacey G et al (eds) Biological Nitrogen Fixation. Chapman and Hall, New York, NY, pp 763–834

Devi KA, Pandey G, AKS R et al (2017) The endophytic symbiont-*Pseudomonas aeruginosa* stimulates the antioxidant activity and growth of *Achyranthes aspera* L. Front Microbiol 8:1897

Dey R, Pal KK, Bhatt DM et al (2004) Growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) by application of plant growth-promoting rhizobacteria. Microbiol Res 159(4):371–394

Egamberdiyeva D (2007) The growth and nutrient uptake of maize inoculated with plant growth promoting bacteria affected by different soil types. Appl Soil Ecol 36:184–189
Elmerich C (2007) Historical Perspective: From Bacterization to Endophytes. In: Elmerich C, Newton WE (eds) Associative and endophytic nitrogen-fixing bacteria and cyanobacterial associations. Springer, Dordrecht, pp 1–20
Erisman JW, Sutton M, Galloway J, Klimont Z, Winiwarter W (2008) How a century of ammonia synthesis changed the world. Nat Geosci-NAT GEOSCI 1:636–639
Epstein E, Bloom AJ (2005) Mineral nutrition of plants: principles and perspectives, 2nd edn. Sinauer Associates, Inc., Sunderland
Faisal M, Hasnain S (2005) Bacterial Cr (VI) reduction concurrently improves sunflower (*Helianthus annuus* L.) growth. Biotechnol Lett 27:943–947
Fallon TR, Weng JK (2014) A molecular gauge for nitrogen economy in plants. Cell 159(5):977–978
Fan X, Feng H, Tan Y et al (2016a) A putative 6-transmembrane nitrate transporter OsNRT1.1b plays a key role in rice under low nitrogen. J Integr Plant Biol 58:590–599
Fan X, Naz M, Fan X et al (2017) Plant nitrate transporters: from gene function to application. J Exp Bot 68(10):2463–2475
Fan X, Tang Z, Tan Y et al (2016b) Over expression of a pH sensitive nitrate transporter in rice increases crop yields. Proc Natl Acad Sci U S A 113:7118–7123
Felici C, Vettori L, Giraldi E et al (2008) Single and coinoculation of *Bacillus subtilis* and *Azospirillum brasilense* on *Lycopersicon esculentum*: Effects on plant growth and rhizosphere microbial community. Appl Soil Ecol 10:260–270
Feng H, Yan M, Fan X et al (2011) Spatial expression and regulation of rice high-affinity nitrate transporters by nitrogen and carbon status. J Exp Bot 62:2319–2332
Franche C, Bogusz D (2011) Signalling and communication in actinorhizal symbiosis. In: Perotto S, Baluska F (eds) Signalling and communication in plant symbiosis. Springer, Berlin, pp 73–92
Fürnkranz M, Wanek W, al RA e (2008) Nitrogen fixation by phyllosphere bacteria associated with higher plants and their colonizing epiphytes of a tropical lowland rainforest of Costa Rica. ISME J 2:561–570
Gage DJ (2004) Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol Mol Biol Rev 68:280–300
Galloway JN, Cowling EB (2002) Reactive nitrogen and the world: 200 years of change. Ambio 31:64–71. https://doi.org/10.1579/0044-7447-31.2.64
Gent L, Forde BG (2017) How do plants sense their nitrogen status? J Exp Bot 68(10):2531–2539
Gholami A, Shahsavani S, Nezarat S (2009) The effect of plant growth promoting rhizobacteria (PGPR) on germination, seedling growth and yield of maize. Int J Biol Life Sci 1:35–40
Glass ADM, Kotur Z (2013) A reevaluation of the role of Arabidopsis NRT1.1 in high-affinity nitrate transport. Plant Physiol 163:1103–1106
Glick BR (2012) Plant growth-promoting bacteria: mechanisms and applications. Hindawi Publishing Corporation, Scientifica
Gojon A, Krouk G, Perrine-Walker F et al (2011) Nitrate transceptor(s) in plants. J Exp Bot 62:2299–2308
Good AG, Shrawat AK, Muench DG (2004) Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production? Trends Plant Sci 9:597–605
Gu R, Duan F, An X et al (2013) Characterization of AMT-mediated high-affinity ammonium uptake in roots of maize (*Zea mays* L.). Plant Cell Physiol 54:1515–1524
Hageman RV, Burris RH (1978) Dinitrogenase and nitrogenase reductase associate and dissociate with each catalytic cycle. Proc Natl Acad Sci USA 75:2699–2702
Halbleib CM, Ludden PW (2000) Regulation of biological nitrogen fixation. J Nutr 130:1081–1084
Hamdi YA (1982) Application of nitrogen-fixing systems in soil improvement and management. FAO Soils Bull. 49. Food and Agriculture Organization, Rome
Hardoim PR, van Overbeek LS, Berg G et al (2015) The hidden world within plants: ecological and evolutionary considerations for defining functioning of microbial endophytes. Microbiol Mol Biol Rev 79:293–320

Hibbs DE, Cromack K (1990) Actinorhizal plants in Pacific Northwest forests. In: Schwintzer CR, Tjepkema JD (eds) The biology of Frankia and Actinorhizal plants. Academic Press, San Diego, pp 343–363
Hirel B, Gouis J, Ney B, Gallais A (2007) The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches. J Exp Bot 58:2369–2387
Ho CH, Lin SH, Hu HC et al (2009) CHL1 functions as a nitrate sensor in plants. Cell 138:1184–1194
Hodge A (2004) The plastic plant: root responses to heterogeneous supplies of nutrients. New Phytol 162(1):9–24
Hwang CF, Lin Y, D'Souza T, Cheng CL (1997) Sequences necessary for nitrate-dependent transcription of Arabidopsis nitrate reductase genes. Plant Physiol 113:853–862
Jackson RB, Caldwell MM (1993) The scale of nutrient heterogeneity around individual plants and its quantification with geostatistics. Ecology 74:612–614
Jahanian A, Chaichi MR, Rezaei K et al (2012) The effect of plant growth promoting rhizobacteria (PGPR) on germination and primary growth of artichoke (*Cynara scolymus*). Int J Agric Crop Sci 4:923–929
James EK (2000) Nitrogen fixation in endophytic and associative symbiosis. Field Crop Res 65:197–209
Kennedy C (2005) Genus I *Beijerinckia*. In: Brenner DJ et al (eds) Bergey's manual of systematic bacteriology, vol 2., part C, 2nd edn. Springer, New York, NY, pp 423–432
Kersters K, De Vos P, Gillis M et al (2006) Introduction to the proteobacteria. The Prokaryotes 5:3–37. Springer, New York
Kim J, Rees DC (1992) Structural models for the metal centers in the nitrogenase molybdenum-iron protein. Science 257:1677–1682
Kisiel A, Kępczyńska E (2016) *Medicago truncatula* Gaertn. as a model for understanding the mechanism of growth promotion by bacteria from rhizosphere and nodules of alfalfa. Planta 243:1169–1189
Kohler J, Caravaca F, Roldan A (2010) An AM fungus and a PGPR intensify the adverse effects of salinity on the stability of rhizosphere soil aggregates of *Lactuca sativa*. Soil Biol Biochem 42(3):429–434
Kou HP, Li Y, Song XX et al (2011) Heritable alteration in DNA methylation induced by nitrogen-deficiency stress accompanies enhanced tolerance by progenies to the stress in rice (*Oryza sativa* L.). J Plant Physiol 168:1685–1693
Krapp A, David LC, Chardin C et al (2014) Nitrate transport and signalling in Arabidopsis. J Exp Bot 65:789–798
Kraiser T, Gras D, Gutierrez A, Gonzalez B, Gutierrez R (2011) Holistic view of nitrogen acquisition in plants. J Exp Bot 62:1455–1466
Krouk G, Crawford NM, Coruzzi GM et al (2010) Nitrate signaling: adaptation to fluctuating environments. Curr Opin Plant Biol 13:266–273
Lalande R, Bissonnette N, Coutlée D et al (1989) Identification of rhizobacteria from maize and determination of their plant-growth promoting potential. Plant Soil 115:7–11
Leigh JA (2000) Nitrogen fixation in methanogens: the archaeal perspective. Curr Issues Mol Biol 2(4):125–131
Léran S, Varala K, Boyer JC et al (2014) A unified nomenclature of nitrate transporter 1/peptide transporter family members in plants. Trends Plant Sci 19:5–9
Lezhneva L, Kiba T, Feria-Bourrellier AB et al (2014) The Arabidopsis nitrate transporter NRT2.5 plays a role in nitrate acquisition and remobilization in nitrogen-starved plants. Plant J 80:230–241
Lillo C, Appenroth KJ (2001) Light regulation of nitrate reductase in higher plants: Which photoreceptors are involved? Plant Biol 3:455–465
Lin SH, Kuo HF, Canivenc G et al (2008) Mutation of the Arabidopsis NRT1.5 nitrate transporter causes defective root-to-shoot nitrate transport. Plant Cell 20:2514–2528
Liu TY, Chang CY, Chiou TJ (2009) The long-distance signaling of mineral macronutrients. Curr Opin Plant Biol 12:312–319

Lopez-Bucio J, Cruz-Ramirez A, Herrera-Estrella L (2003) The role of nutrient availability in regulating root architecture. Curr Opin Plant Biol 6:280–287
Loque D, Yuan L, Kojima S et al (2006) Additive contribution of AMT1; 1 and AMT1; 3 to high-affinity ammonium uptake across the plasma membrane of nitrogen-deficient Arabidopsis roots. Plant J 48:522–534
Ma Y, Rajkumar M, Freitas H (2009a) Isolation and characterization of Ni mobilizing PGPB from serpentine soils and their potential in promoting plant growth and Ni accumulation by *Brassica* spp. Chemosphere 75(6):719–725
Ma Y, Rajkumar M, Freitas H (2009b) Improvement of plant growth and nickel uptake by nickel resistant -plant-growth promoting bacteria. J Hazard Mater 166:1154–1161
Ma Y, Rajkumar M, Luo Y et al (2011) Inoculation of endophytic bacteria on host and non-host plants-effects on plant growth and Ni uptake. J Hazard Mater 195:230–237
Mancinelli RL (1996) The nature of nitrogen: an overview. Life Support Biosph Sci 3:17–24
Marín IC, Loef I, BartetzkoIain L et al (2011) Nitrate regulates floral induction in Arabidopsis, acting independently of light, gibberellin and autonomous pathways. Plants 233(3):539–552
Martínez-Hidalgo P, Hirsch AM (2017) The nodule microbiome: N_2-fixing rhizobia do not live alone. Phytobiomes J 1(2):70–82
Marschner H (1995) Mineral nutrition of higher plants, 2nd edn. Academic, Boston, MA
Marschner P (2011) Marschner's mineral nutrition of higher plants, 3rd edn. Academic, London
Mendes R, Garbeva P, Raaijmakers JM (2013) The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiol Rev 37(5):634–663
Miller AJ, Fan X, Orsel M et al (2007) Nitrate transport and signalling. J Exp Bot 58:2297–2306
Miransari M (2011) Arbuscular mycorrhizal fungi and nitrogen uptake. Arch Microbiol 193(2):77–81
Morere-Le Paven MC, Viau L, Hamon A et al (2011) Characterization of a dual-affinity nitrate transporter MtNRT1.3 in the model legume *Medicago truncatula*. J Exp Bot 62:5595–5605
Murray PA, Zinder SH (1984) Nitrogen fixation by a methanogenic archaebacterium. Nature 312:284–286
Mus F, Crook MB, Garcia K, Garcia et al (2016) Symbiotic nitrogen fixation and the challenges to its extension to nonlegumes. Appl Environ Microbiol 82:3698–3710
Nacry P, Bouguyon E, Gojon A (2013) Nitrogen acquisition by roots: physiological and developmental mechanisms ensuring plant adaptation to a fluctuating resource. Plant Soil 370:1–29
Nehra V, Choudhary M (2015) A review on plant growth promoting rhizobacteria acting as bioinoculants and their biological approach towards the production of sustainable agriculture. J Appl Nat Sci 7(1):540–556
Nunes-Nesi A, Fernie AR, Stitt M (2010) Metabolic and signaling aspects underpinning the regulation of plant carbon nitrogen interactions. Mol Plant 3:973–996
O'Brien JA, Vega A, al BE e (2016) Nitrate transport, sensing, and responses in plants. Mol Plant 9:837–856
Offre P, Spang A, Schleper C (2013) Archaea in Biogeochemical Cycles. Annu Rev Microbiol 67:437–457
Ogburn S (2010) The dark side of nitrogen. Grist. Retrieved from http://grist.org/article/2009-11-11-the-dark-side-of-nitrogen/
Oliveira ALM, Urquiaga S, Döbereiner J (2002) The effect of inoculating endophytic N2-fixing bacteria on micropropagated sugarcane plants. Plant Soil 242:205–215
Pathak RR, Ahmad A, Lochab S et al (2008) Molecular physiology of plant N-use efficiency and biotechnological options for its enhancement. Curr Sci 94(11):1394–1403
Pathak RR, Lochab S, Raghuram N (2011) Improving plant nitrogen-use efficiency. In: M-Y M (ed) Comprehensive biotechnology, vol 4, 2nd edn. Elsevier, pp 209–218
Paul S, Datta SK, Datta K (2015) miRNA regulation of nutrient homeostasis in plants. Frontiers in. Plant Sci 6:232
Pawlowski K (2009) Induction of actinorhizal nodules by Frankia. In: Pawlowski K (ed) Prokaryotic symbionts in plants. Springer, Berlin, pp 127–154

Pedraza RO (2008) Recent advances in nitrogen-fixing acetic acid bacteria. Int J Food Microbiol 125:25–35

Peters JW, Boyd ES, Hamilton TL et al (2011) Biochemistry of Mo-nitrogenase. In: Moir JWB (ed) Nitrogen cycling in bacteria: molecular analysis. Caister Academic Press, Norfolk, pp 59–100

Price MB, Jelesko J, Okumoto S (2012) Glutamate receptor homologs in Plants: Functions and evolutionary origins. Front Plant Sci 3:235

Raghuram N, Chandok MR, Sopory SK (1999) Light regulation of nitrate reductase gene expression in maize involves a G-protein. Mol Cell Biol Res Commun 2:86–90

Raghuram N, Sopory SK (1995a) Evidence for some common signal transduction events for opposite regulation of nitrate reductase and phytochrome I gene expression in maize. Plant Mol Biol 29:25–35

Raghuram N, Sopory SK (1995b) Light regulation of NR gene expression: Mechanism and signal-response coupling. Physiol Mol Biol Plants 1:103–114

Raghuram N, Pathak RR, Sharma P (2006) Signalling and the molecular aspects of N-use eYciency in higher plants. In: Singh RP, Jaiwal PK (eds) Biotechnological approaches to improve nitrogen use eYciency in plants. Studium Press LLC, Houston, pp 19–40

Rahayu YS, Walch-Liu P, Neumann G et al (2005) Root-derived cytokinins as long-distance signals for NO_3−-induced stimulation of leaf growth. J Exp Bot 56(414):1143–1152

Rajendran G, Sing F, Desai AJ et al (2008) Enhanced growth and nodulation of Pigeon Pea by co-inoculation of *Bacillus* strains with *Rhizobium* spp. Biosour Technol 99(11):4544

Rajkumar M, Nagendran R, Kui JL et al (2006) Influence of plant growth promoting bacteria and Cr (VI) on the growth of Indian mustard. Chemosphere 62:741–748

Raymond J, Siefert JL, Staples CR et al (2004) The natural history of nitrogen fixation. Mol Biol Evol 21:541–554

Reinhold-Hurek B, Hurek T (1998) Life in grasses: diazotrophic endophytes. Trends Microbiol 6:139–144

Rekha PD, Lai W, Arun AB et al (2007) Effect of free and encapsulated *Pseudomonas putida* CC-R2-4 and *Bacillus subtilis* CC-pg104 on plant growth under gnotobiotic conditions. Bioresour Technol 98:447–451

Remans T (2006) The Arabidopsis NRT1.1 transporter participates in the signaling pathway triggering root colonization of nitrate-rich patches. Proc Natl Acad Sci U S A 103:19206–19211

Richardson AE, Barea JM, McNeill AM et al (2009) Acquisition of phosphorus and nitrogen in the rhizosphere and plant growth promotion by microorganisms. Plant Soil 321(1–2):305–339

Rubio LM, Ludden PW (2008) Biosynthesis of the iron-molybdenum cofactor of nitrogenase. Annu Rev Microbiol 62:93–111

Ruinen J (1975) Nitrogen-fixation in the phyllosphere. In: Stewart WDP (ed) Nitrogen-fixation by free-living microorganisms. Cambridge University Press, pp 85–100

Sachdev DP, Chaudhari HG, Kasture VM et al (2009) Isolation and characterization of indole acetic acid (IAA) producing *Klebsiella pneumoniae* strains from rhizosphere of wheat (*Triticum aestivum*) and their effect on plant growth. Indian J Exp Biol 47(12):993–1000

Sakakibara H, Suzuki M, Takei K et al (1998) A response-regulator homologue possibly involved in nitrogen signal transduction mediated by cytokinin in maize. Plant J 14:337–344

Santi C, Bogusz D, Franche C (2013) Biological nitrogen fixation in non-legume plants. Ann Bot 111:743–767

Sanhueza E (1982) The role of atmoshere in nitrogen cycling. Plant Soil 67:61–71

Schachtman DP, Shin R (2007) Nutrient sensing and signaling: NPKS. Annu Rev Plant Biol 58:47–69

Sheng X-F, Xia J-J (2006) Improvement of rape (*Brassica napus*) plant growth and cadmium uptake by cadmium-resistant bacteria. Chemosphere 64(6):1036–1042

Shin R, Joseph MJ, Basra A et al (2011) 14-3-3 Proteins fine-tune plant nutrient metabolism. FEBS Letters 585(1): 143–147

Silvester WB, McNamara PJ (1976) The infection process and ultrastructure of the *Gunnera-Nostoc* symbiosis. New Phytol 77:135–141

Simpson FB, Burris RH (1984) A nitrogen pressure of 50 atmospheres does not prevent evolution of hydrogen by nitrogenase. Science 224:1095–1097
Soni R, Carmichael JP, Shah ZH et al (1995) A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. Plant Cell 7:85–103
Sonoda Y, Ikeda A, Saiki S et al (2003) Distinct expression and function of three ammonium transporter genes (*OsAMT1;1–1;3*). Plant Cell Physiol 44:726–734
Sonoda Y, Ikeda A, Yamaya T (2004) Feedback regulation of the ammonium transporter gene family AMT1 by glutamine in rice. Plant Cell Physiol 45:S98–S98
Sprent JI, Ardley JK, James EK (2013) From north to south: A latitudinal look at legume nodulation processes. S Afr J Bot 89:31–41
Steenhoudt O, Vanderleyden J (2000) *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol Rev 24:487–506
Suenaga A, Moriya K, Sonoda Y et al (2003) Constitutive expression of a novel-type ammonium transporter OsAMT2 in rice plants. Plant Cell Physiol 44:206–211
Sueyoshi K, Mitsuyama T, Sugimoto T et al (1999) Effects of inhibitors of signalling components on the expression of genes for nitrate reductase and nitrite reductase in excised barley leaves. Soil Sci Plant Nutr 45:1015–1019
Thajuddin N, Muralitharan G, Sundaramoorthy M et al (2010) Morphological and genetic diversity of symbiotic cyanobacteria from cycads. J Basic Microbiol 50:254–265
Thakuria D, Talukdar NC, Goswami C, Hazarika S, Boro R, Khan M (2004) Characterization and screening of bacteria from rhizosphere of rice grown in acidic soils of Assam. Curr Sci 86:978–985
Towata EM (1985) Mucilage glands and cyanobacterial colonization in *Gunnera kaalensis* (Haloragaceae). Bot Gaz 146:56–62
Triplett E (1996) Diazotrophic endophytes: Progress and prospects for nitrogen fixation in monocots. Plant Soil 186:29–38
Tschoep H Gibon Y, Carilllo P et al (2009) Adjustment of growth and central metabolism to a mild but sustained nitrogen limitation in Arabidopsis. Plant Cell and Environment 32(3):300–318
Uchida R (2000) Essential nutrients for plant growth: nutrient functions and deficiency symptoms. In: Silva JA, Uchida R (eds) Plant nutrient management in hawaii's soils: approaches for tropical and subtropical agriculture. College of Tropical Agriculture and Human Resources, University of Hawaii, Manoa, pp 31–35
van Spronsen PC, Bakhuizen R, van Brussel AAN et al (1994) Cell-wall degradation during infection thread formation by the root nodule bacterium *Rhizobium leguminosarum* is a 2-step process. Eur J Cell Biol 64:88–94
Vidal EA, Araus V, Lu C et al (2010) Nitrate-responsive miR393/AFB3 regulatory module controls root system architecture in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A 107:4477–4482
von Wittgenstein NJ, Le CH, Hawkins BJ et al (2014) Evolutionary classification of ammonium, nitrate, and peptide transporters in land plants. BMC Evol Biol 14:11
Wall LG (2000) The actinorhizal symbiosis. J Plant Growth Regul 19:167–182
Warning HO, Hachtel W (2000) Functional analysis of a nitrite reductase promoter from birch in transgenic tobacco. Plant Sci 155:141–151
Wheeler CT, Miller IM (1990) Current potential uses of actinorhizal plants in Europe. In: Schwintzer RC, Tjepkema JD (eds) The biology of Frankia and actinorhizal plants. Academic Press, San Diego, CA, pp 365–389
Widiez T, El-Kafafiel S, Girin T et al (2011) High nitrogen insensitive 9 (HNI9)-mediated systemic repression of root NO_3- uptake is associated with changes in histone methylation. Proc Natl Acad Sci U S A 108:13329–13334
Woese CR, Kandler O, Wheelis ML (1990) Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eukarya. Proc Natl Acad Sci U S A 87:4576–4579
Xu G, Fan X, Miller AJ (2012) Plant nitrogen assimilation and use efficiency. Annu Rev Plant Biol 63:153–182

Yuan L, Loqué D, Kojima S et al (2007) The organization of high-affinity ammonium uptake in Arabidopsis roots depends on the spatial arrangement and biochemical properties of AMT1-type transporters. Plant Cell 19:2636–2652

Yue H, Mo W, Li C et al (2007) The salt stress relief and growth promotion effect of RS-5 on cotton. Plant Soil 297:139–145

Zahir ZA, Ghani U, Naveed M et al (2009) Comparative effectiveness of *Pseudomonas* and *Serratia* sp. containing ACC-deaminase for improving growth and yield of wheat (*Triticum aestivum* L.) under salt-stressed conditions. Arch Microbiol 191:415–424

Zhan J, Sun Q (2012) Diversity of free-living nitrogen-fixing microorganisms in the rhizosphere and non-rhizosphere of pioneer plants growing on wastelands of copper mine tailings. Microbiol Res 167:157–165

Zheng Z (2009) Carbon and nitrogen nutrient balance signaling in plants. Plant Signal Behav 4:584–591

16 Halotolerant Microbes for Amelioration of Salt-Affected Soils for Sustainable Agriculture

Sanjay Arora

Abstract

Soil salinity is one of the major abiotic stresses that adversely affect the sustainable agricultural production globally. About 20% of the total land area is affected by salinity, and the area is increasing at an alarming rate. There is a damaging effect of salinity on soil microbial communities, and their activities have been reported in majority of the studies. Excess accumulation of salts in the root zone often deteriorates the soil properties, viz. physical, chemical and biological to such an extent that crop production is adversely affected. Also, salt-affected soils are poor in organic matter content and thus the biomass as well as microbial activity, thereby affecting the microbiologically mediated processes required for plant growth. The methods available for reclamation of salt-affected soils are not cost effective, and further the availability of good-quality waters required for leaching salts in saline soils and mineral gypsum or organic amendments for sodic soils is scarce. Halotolerant and halophilic microorganisms having plant growth-promoting (PGP) traits have the potential to assuage salt stress and enhance plant growth and production in salt-affected soils. These plant growth-promoting rhizobacteria (PGPR) tolerate wide range of salt stress and thus enable plants to withstand salinity by different mechanisms such as hydraulic conductance, osmotic accumulation, sequestering toxic Na^+ ions, maintaining the higher osmotic conductance and photosynthetic activities. The halophilic microbes have the potential to influence direct growth promotion of plants by fixing atmospheric nitrogen, solubilizing insoluble nutrients and secreting hormones such as IAA, GAs and kinetins besides ACC deaminase production, which helps in regulation of ethylene. Some of the recent researchers have confirmed the possibility of using halophiles in recovery of salt-affected soils and sustain agricultural production in degraded lands. We also

S. Arora (✉)
ICAR-Central Soil Salinity Research Institute, Lucknow, Uttar Pradesh, India
e-mail: Sanjay.Arora@icar.gov.in

© Springer Nature Singapore Pte Ltd. 2020

M. Kumar et al. (eds.), *Phyto-Microbiome in Stress Regulation*, Environmental and Microbial Biotechnology, https://doi.org/10.1007/978-981-15-2576-6_16

observed beneficial effects of using PGP halophilic bacteria isolated from the native salty soils for enhancing crop production under salt stress conditions. For easy application in agriculture, liquid bioformulations have been prepared for efficient strains, and their use has enhanced the yield of rice and wheat by 11–14% and also for other crops like mustard, vegetables and fodder crops under salt stress conditions. Therefore, the bioremediation approach being cheap and eco-friendly is being promoted to optimize crop yields under sodic and saline-sodic soils of the Indo-Gangetic plains of north India.

Keywords
Soil salinity · Plant growth-promoting rhizobacteria (PGPR) · Halophiles

16.1 Introduction

The productivity of agricultural crops is constrained by several environmental biotic and abiotic stresses that have adversely affected the area under cultivation, crop productivity and quality. Amongst the different abiotic stresses, soil salinization, soil pH, acidification, drought and temperatures are the major restrictive factors in sustaining crop production. Soil salinity is a major issue for agriculture because high concentration of salt turns useful lands into unproductive areas. Thus, soil salinization has been recognized as one of the most devastating soil degradation threats on the Earth. Globally, soil salinity has affected almost 1 billion ha of land area, representing about 7% of Earth continental extent, with about 1–3 million ha land area in Europe, about 850 million ha in Asia and 104 million ha in Pacific subregion (Rengasamy 2006; Ladeiro 2012). In India, about 6.73 million ha of land is salt affected which spreads in 194 districts and represents 2.1% of the total geographical area of the country (Mandal et al. 2009). Out of these, 2.8 million ha of area is sodic in nature and mainly occurring in the Indo-Gangetic alluvial plains of north India. It has also been estimated that globally, about 20% of total agricultural land is affected by high salinity, and these saline areas are increasing at the rate of 10% annually due to low rainfall, high surface evaporation, weathering of native rocks, irrigation with poor (saline) quality water and poor cultural practices, especially in arid and semi-arid regions. Furthermore, it is expected that about more than 50% of the arable lands would turn salinized by 2050 (Jamil et al. 2011).

The agricultural crops under salinity exhibit a variety of responses that range from decline in crop yields due to alterations in physicochemical properties of the soil to the disturbance in ecological balance of the region. Salinity is thus the major cause of land abandonment and aquifers for agricultural purposes and a foremost factor for reducing agricultural productivity. The impacts include poor crop productivity, low economic returns and erosion of soils (Hu and Schmidhalter 2002). The poor crop productivities are due to complex interactions amongst morphological, physiological and biochemical processes because of salinity-induced limited water and nutrient uptakes throughout the crop growth cycle (Akbarimoghaddam et al. 2011; Singh and

Chatrath 2001). The limited uptake of water and nutrients affects almost every developmental stage of the crop plant with osmotic and oxidative stress, nutrient (N, Ca, K, P, Fe and Zn) deficiency and toxicity of ions (Munns 2002). Ion toxicity through the accumulation of excessive salt ions in the cell walls leads to osmotic stress, causing replacement of K^+ by Na^+ in biochemical reactions inducing conformational changes in proteins. Likewise, enzyme activities during the developmental stages get affected as K^+ ions, which act as cofactors and are required for binding tRNA to ribosomes. Substitution of K^+ by Na^+ also adversely affects the protein synthesis (Zhu 2002). Metabolic imbalance, caused by ion toxicity and osmotic stress, in turn, leads to oxidative stress (Chinnusamy et al. 2006), resulting in osmotic balance failure, loss of turgidity and dehydration of cell, and eventually culminating in cell death.

The major causes of naturally induced salinity are salt water intrusion and salt deposition through salt-laden winds. Salts also originate from weathering of minerals and anthropogenic factors like irrigation of crops with salty waters through which salt accumulates in soil and salinization gets accelerated. The other factors include injudicious use of inorganic fertilizers and soil amendments like gypsum, composts, manures, etc.

Salinization results in inhibition of plant growth that results due to accumulation of dissolved salts in soil water. The major water-soluble salts that accumulate in the soil include potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), chloride (Cl^-), sulphate (SO_4^{2-}), carbonate (CO_3^{2-}), bicarbonate (HCO_3^-) and sodium (Na^+) ions. The soil solutions differ in dissolved salt contents, and when the concentration of salts in terms of electrical conductivity (ECe) exceeds 4 dSm^{-1} in the soil, these are categorized as salt affected (Abrol et al. 1988). A saline soil is defined as the soil having a high concentration of soluble salts ($EC_e > 4$ dSm^{-1}) that are enough to affect the growth and development of plants. However, many crops are affected by soil with an $EC_e < 4$ dSm^{-1}. Excessive sodium (Na^+) salt accumulation destroys soil structure, deteriorates soil hydraulic properties, increases soil pH and reduces water infiltration and soil aeration, leading to compaction of soil and thereby increasing erosion and higher water run-off. Furthermore, sodium, being the most prominent destructor of secondary clay minerals by dispersion, replaces calcium (Ca^{2+}) and other coagulators like Mg^{2+} and gets adsorbed on the surface and/or interlayers of soil aggregates (Ondrasek et al. 2010). Dispersion of clay particles undergoes leaching through the soil to accumulate and block pore spaces, especially in fine-textured soil horizons. These lands are thus degraded in structural, chemical, nutritional, hydrological and microbiological characteristics. The degraded sodic soil thus becomes unsuitable for proper root growth and plant development. The secondary result is salinity-induced sodicity, where leaching either through natural or human-induced processes washes away the soluble salts into the subsoil and leaves negative charges of sodium bound to the clay.

16.2 Reclamation and Management of Salt-Affected Soils

To improve crop growth in saline soils, the excess salts need to be removed from the root zone. Leaching is one of the most effective methods for removing salts beyond the root zone. Leaching is accomplished by ponding fresh water on the soil surface

and allowing it to infiltrate, and it is effective only when the salty drainage water is discharged through drains out of the area under reclamation. The process of leaching may reduce salinity levels in the absence of artificial drains when there is sufficient natural drainage, i.e. the ponded water drains without raising the water table. It is preferred to leach when the soil moisture content is low and the groundwater table is deep.

Sodic or alkali soils are generally reclaimed using mineral gypsum along with organic amendments/manures. The availability of mineral gypsum and also manures is scarce these days. Also the estimation of gypsum requirement of sodic soil is tedious, and most of the soil testing laboratories either do not have facility for gypsum requirement or lack expertise in estimation, so a model was developed to estimate gypsum requirements based on soil pH value. The mobile application "GypCal" in Hindi and English was developed to promote judicious use of chemical amendment gypsum for reclamation of sodic soil using soil pH as input, and this application is made freely available for download through Google Play Store.

Both physical and chemical methods for reclamation of saline and sodic soils are not cost-effective, and on the other hand, organic crop production is being promoted. The microbial strains available as bio-fertilizers for different crops do not perform effectively under salt stress, and their activity decreases when used in salt-affected soils due to osmolysis. The soils of vast areas of Indo-Gangetic plains in north India are sodic or saline-sodic. The halophilic plant growth-promoting microbes have potential to ameliorate these soils. The halophilic bacterial strains can help in recovery of salt-affected soils by directly supporting vegetation growth thus indirectly increasing crop yields under salt stress conditions. Halophilic plant growth-promoting bacteria have high potential for remediation of salt-affected soils and enhancing productivity of crops especially paddy, mustard and wheat.

16.3 The Effect of Salinity on the Soil Microorganisms

The soil microbial communities perform a fundamental role in nutrient cycling, decomposition of organic matter in the soil and in maintaining plant productivity. It is therefore important to understand the microbial response to environmental stress. Stress can be damaging for sensitive microorganisms and decrease the activity of surviving cells, due to the metabolic load imposed by the need for stress tolerance mechanisms. In a dry hot climatic condition, the low humidity and soil salinity are the major stressful factors for the soil microbial communities, and these stresses frequently occur simultaneously. Excessive accumulation of salts in soil hampers the growth and activity of soil microflora thereby affecting the population of N_2-fixing and phosphate-solubilizing bacteria which lead to low soil fertility. Due to increased quantity of salts, the microbial flora is worst affected; this also interfered with nitrogen-fixing and phosphate-solubilizing ability of bacteria. The salinity effect is always more pronounced in the rhizosphere according to the increase in water absorption by the plants due to transpiration. This is so as life in high salt

concentrations has a high bioenergetic taxation, because the microorganisms need to maintain osmotic equilibrium between the cytoplasm and the contiguous medium, excluding sodium ions from inside the cell which require sufficient energy for osmo-adaptation.

16.3.1 Salinity Impacts on Rhizosphere Microbes

Soluble ion concentrations (especially sodium ion) greater than about 0.15 *M* ions in soil lead to hyperosmotic conditions which force water to diffuse out of a microbial cell. The cells will then shrink or plasmolyse. In addition, the high sodium ion concentration also causes the water associated with such solutes to become unavailable to microorganisms. Basically, the effect of sodium ion on the growth of microorganisms of different species will differ due to growing water activity of each microorganism.

Bacteria are adsorbed onto soil particles by ion exchange, and a soil is considered to be naturally fertile when the soil organisms are releasing inorganic nutrients from the organic reserves at a rate sufficient to sustain rapid plant growth. Since the soil organic matter and consequently the biomass and microbial activity are generally more relevant in the surface layer of the soil, salinization close to the surface significantly affects a series of microbiologically mediated processes. Along with it disturbs the natural ecosystem functioning and plant health. For rhizobacteria, life in high salt concentrations is difficult as they need to maintain an osmotic balance between their cytoplasm and the surrounding medium while excluding Na^+ ions for which sufficient energy is required for adaptation. Depletion of potassium ions by plants under saline conditions further reduces the ability of rhizobacteria to use potassium ions as a primary osmoregulator. Plant use of osmolytes under salt stress deprives rhizobacteria of osmolytes, which finally limits the bacterial growth. The salinity level above 5% thus reduces the total population of bacteria and actinobacteria drastically. In addition, it inhibits nitrogen fixation, root exudation and decomposition of organic matter. Negative correlations between soil electrical conductivity and carbon dioxide emission or microbial biomass C suggested that it has a severe adverse effect on microbial biomass and activities. Naturally occurring soil organic matter decomposers thus become sensitive to salt-induced stress, and the effect is always more pronounced in the rhizosphere pursuant to increased water uptake by the plants due to transpiration. Alteration of proteins, exo-polysaccharide and lipopolysaccharide composition of the bacterial cell surface, impairment of molecular signal exchange between bacteria and their plant host due to the alteration of membrane glucan contents and inhibition of bacterial mobility and chemotaxis towards plant roots significantly affect microbial diversity in the rhizosphere, under saline conditions. Overall, salinity has a negative impact on microbial abundance, diversity, composition and functions.

16.4 Soil Salinity Effects on Plant Growth and Development

All the major plant growth processes such as germination, cell division and elongation, leaf growth, leaf expansion, photosynthesis, protein synthesis, energy and lipid metabolism are adversely affected under salt stress. During the vegetative stages, salt stress induces stomatal closure, leading to reduction in CO_2 assimilation and transpiration. The reduced turgor potentials affect the leaf expansion and leaf area, which in turn reduces the light interception and photosynthetic rates, coupled with spurt in respiration resulting into reduced biomass accumulation. Water potential of the soil is reduced due to excessive salts, thus, making the soil solution unavailable to the plants and creating physiological drought. Further, osmotic pressure in the rhizosphere solution exceeds in root cells thereby reducing water and nutrient uptake. Salinity further creates nutritional imbalance through increase in uptake of Na^+ or decrease in uptake of Ca^{2+} and K^+ in leaves. Excess Na^+ causes metabolic disturbances in processes where low Na^+ and high K^+ or Ca^{2+} are required for optimum growth and developmental functions. Excess sodium and more importantly chlorides affect plant enzymes and cause cell swelling, resulting in reduced energy production and other physiological changes. The uptake and accumulation of Cl^- disrupt the photosynthetic function through inhibition of nitrate reductase activity. Under excessive Na^+ and Cl^- rhizosphere concentrations, competitive interactions with other nutrient ions (K^+, $NO_3^-$and $H_2PO_4^-$) occur for binding sites and transport proteins in root cells that have adverse effects on translocation, deposition and partitioning within the plant. Once the capacity of cells to store salts is exhausted, salt build-up in intercellular space leads to cell dehydration and death. Plants suffer from membrane destabilization and a general nutrient imbalance. All micro- and macronutrient contents decrease in roots and shoots with increasing NaCl concentration in the soil. Osmotic stress decreases cell growth and development, reduces leaf area and chlorophyll content, accelerates defoliation and senescence and reduces the yields. The primary salinity effects give rise to numerous secondary ones such as oxidative stress, characterized by accumulation of reactive oxygen species potentially harmful to bio-membranes, proteins, nucleic acids and enzymes. The plants with perturbed nutrients relations are more susceptible to invasion of different pathogenic microorganisms and physiological dysfunctions, whereas their edible parts have markedly less economic and nutritional value due to reduced fruit size and shelf life, non-uniform fruit shape and decreased vitamin contents.

16.5 Halotolerant Microbes

The halotolerant and halophilic microorganisms are those which can grow in hypersaline environments, while only halophiles specifically require at least 0.2 M of salt for their growth. Halotolerant microorganisms can only tolerate media containing <0.2 M of salt. Distinctions between different kinds of halophilic microorganisms are based on their level of salt requirement and salt tolerance. The halotolerant

microorganisms grow best in media containing <0.2 M (~1%) salt and may also tolerate high salt concentrations. This definition is widely referred to in many reports (Arahal and Ventosa 2002; Ventosa et al. 1998; Yoon et al. 2003).

Bacteria inhabiting soil play a role in conservation and restoration biology of higher organisms. The domain bacteria contain many types of halophilic and halotolerant microorganisms, spread over a large number of phylogenetic groups (Ventosa et al. 1998). The different branches of the Proteobacteria contain halophilic representatives often having close relatives that are nonhalophilic. Similarly, halophiles are also found amongst the cyanobacteria (Oren 1999), the *Flavobacterium*-Cytophaga branch, the Spirochetes and the Actinomycetes. Within the lineages of Gram-positive bacteria (*Firmicutes*), halophiles are found both within the aerobic branches (*Bacillus* and related organisms) and within the anaerobic branches. Most halophiles within the domain bacteria are moderate rather than extreme halophiles in general. However, there are a few types that resemble the archaeal halophiles of the family *Halobacteriaceae* in their salt requirements and tolerance. There is abundance of halophilic bacteria in saline soil, and the dominant types encountered in saline soil belong to genera of *Alcaligenes*, *Bacillus*, *Micrococcus* and *Pseudomonas* (Rodriguez-Valera 1988). It was reported that halotolerant Gram-positive endospore-forming rods isolated from saline soils and sediments of salterns located in different areas were assigned to the genus *Bacillus*. The majority of them were classified as extremely halotolerant microorganisms as they are able to grow in most cases in up to 20% or 25% salts (Garabito et al. 1998).

Many of the halotolerant microbial species have been isolated and identified such as *Azotobacter*, *Azospirillum*, *Phosphobacter* and blue-green algae from marine aquatic sediments. The bacterial sequences were assigned into 5784 operational taxonomic units (OTUs, based on ≥97% sequence identity), representing 24 known bacterial phyla, with maximum of Proteobacteria (44.9%) followed by Actinobacteria (12.3%), *Firmicutes* (10.4%), Acidobacteria (9.0%), Bacteroidetes (6.8%) and Chloroflexi (5.9%) being predominant. Bacterial genus *Lysobacter* (12.8%) was the dominant in saline soils followed by *Sphingomonas* (4.5%), *Halomonas* (2.5%) and Gemmatimonas (2.5%). Archaeal sequences were assigned to 602 OTUs, primarily from the phyla Euryarchaeota (88.7%) and Crenarchaeota (11.3%). Halorubrum and Thermofilum were the dominant archaeal genera in saline soils. Rarefaction analysis indicated less than 25% of bacterial diversity and approximately 50% of archaeal diversity, in saline soil.

These microorganisms have developed mechanisms to survive in such adverse media and many endemisms. The halophilic microorganisms are thus also called "salt-loving" microorganisms living in environments with high salt concentration that would kill most other microbes. Microorganisms under hypertonic environments (low water activity) either die or remain dormant except halotolerant and halophilic microorganisms that can overcome this problem. Generally, high salt concentration can interfere with the growth and activity of soil microbes; hence it indirectly affects the availability of nutrients to plants under salt stress.

16.5.1 Mechanisms for Halotolerance

Halotolerance is the adaptation of living organisms to conditions of high salinity. High osmolarity in hypersaline conditions can be deleterious to cells since water is lost to the external medium until osmotic equilibrium is achieved. Many microorganisms respond to enhanced osmolarity by accumulating osmotica in their cytosol, which protects them from cytoplasmic dehydration (Yancey et al. 1982). All microorganisms have to keep their cytoplasm at least isoosmotic with their environment to prevent loss of cellular water; when a turgor pressure is to be maintained, the cytoplasm should even be slightly hyperosmotic. Adaptation to conditions of high salinity has an evolutionary significance. The concentration of brines during prebiotic evolution suggests haloadaptation at earliest evolutionary times (Dundas 1998). Osmophily is related to the osmotic aspects of life at high salt concentrations, especially turgor pressure, cellular dehydration and desiccation. Halophily refers to the ionic requirements for life at high salt concentrations.

Salt-tolerant or halophilic microorganisms usually adopt either of the two strategies of survival in saline environments: 'compatible solute' strategy and 'salt-in' strategy (Ventosa et al. 1998). The cell volume is maintained when an isoosmotic balance with the medium is achieved. The majority of moderately halophilic and halotolerant bacteria, some yeasts, algae and fungi, employ the compatible solute strategy wherein the cells maintain low concentrations of salt in their cytoplasm by balancing osmotic potential through the synthesis or uptake of organic compatible solutes and exclusion of salts from the cytoplasm as much as possible. The compatible solutes or osmolytes, small organic molecules that are soluble in water to molar concentrations, that accumulate in halophiles are available in great spectrum and used in all three domains of life. These are assigned in two classes of chemicals, i.e. (1) the amino acids and their derivatives, such as glycine betaine, glutamine, glutamate, proline, ectoine or N-acetyl-β-lysine, and (2) polyols, e.g. glycine betaine, ectoine, sucrose, trehalose and glycerol, which do not disrupt metabolic processes and have no net charge at physiological pH. The accumulation can be accomplished either by uptake from the medium or by de novo synthesis (Shivanand and Mugeraya 2011).

The salt-in strategy is generally employed by true halophiles, including halophilic archaea and extremely halophilic bacteria. These microorganisms are adapted to high salt concentrations and cannot survive when the salinity of the medium is lowered (Arora et al. 2014a). They generally do not synthesize organic solutes to maintain the osmotic equilibrium. In this adaptation, the intracellular K^+ concentration is generally higher than that of the outside, the intracellular Na^+ concentration is generally lower than that in the medium, and the intracellular K^+ concentration increases with increasing external concentration of NaCl in a non-linear pattern. All the halophilic microorganisms contain potent transport mechanisms, which is generally based on Na^+/H^+ antiporters (Oren 1999).

Halobacillus is one of the first chloride-dependent bacteria reported, and several cellular functions depend on chloride ion for maximal activities, the most important being the activation of solute accumulation. *Halobacillus* switches its osmolyte strategy with the environmental salinity by the production of different compatible

solutes. Glutamate and glutamine dominate at intermediate salinities, and proline and ectoine dominate at high level of salinities. Chloride ion stimulates the glutamine synthetase thereby activating the enzyme and the product glutamate and then turns on the biosynthesis of proline by inducing the expression of the proline biosynthetic genes. *Halobacillus dabanensis* is used as a model organism to know about the genes involved in halotolerance, including genes encoding Na^+/H^+ antiporters as well as enzymes involved in osmotic solute metabolism and stress proteins.

16.5.2 Vesicular Arbuscular Mycorrhiza (VAM)

There are reports of common occurrence of vesicular arbuscular mycorrhizal fungi commonly called as VAM in natural and saline environment. Relationships between soil salinity and the presence of VAM have been investigated by several researchers. It has been reported that the number of VAM spores or infectivity of VAM fungi changed with change in salt concentration (Juniper and Abbott 1993). The stresses due to saline soils effect the growth of plants, fungus or both.

VA mycorrhizal fungi species that most commonly observed in saline soils are *Glomus* spp. (Juniper and Abbott 1993), and this suggests that this may be adapted to grow in saline conditions. There is evidence that the distribution of VAM species is markedly changed with increase in salt concentration (Stahl and Williams 1986). It has been observed by Aliasgharzadeh et al. (2001) that the most predominant species of arbuscular mycorrhizal fungi (AMF) in the severely saline soils of the Tabriz plains were *Glomus intraradices*, *G. versiform* and *G. etunicatum*. Few studies have indicated that the mycorrhizal fungi can increase growth of plants growing in saline habitats (Yadav et al. 2017). The VA mycorrhizal fungi have the ability to guard plants from salt stress although the mechanism is not fully known. At present it is suggested that fungi do have a potential to enhance plant growth by increasing nutrient uptake. The efficacy of three species of AMF—*Glomus mosseae*, *G. intraradices* and *G. claroideum*—was evaluated to alleviate salt stress in nursery of olive trees (Porras-Soriano et al. 2009). It was observed that *G. mosseae* was the most efficient fungus in terms of olive tree performance and protection against the detrimental effects of salinity. These findings suggest that the capability of AMF in protecting plants from the detrimental effects of salt stress may depend on the behaviour of the species.

16.6 Applications of Halophilic Bacteria

There is high potential for biotechnological applications of halophilic bacteria for two main reasons: (1) their participation in biogeochemical processes of C, N, S and P, the formation and dissolution of carbonates, immobilization of phosphate and production of growth factors and nutrients (Rodriguez-Valera et al. 1985) and (2) their simple nutritional requirements. The majority of halophilic bacteria can use a

wide range of compounds as their sole carbon and energy source. Most of them can grow at high salt concentrations, minimizing the risk of contamination. Moreover, several genetic tools developed for the nonhalophilic bacteria can be applied to the halophiles, and thus their genetic manipulation seems feasible (Ventosa et al. 1998).

Halophilic bacteria have the ability to produce compatible solutes, which are useful for the biotechnological production of the osmolytes. Some compatible solutes, especially glycine, betaines and ectoines, may be used as stress protectants (against high salinity, thermal denaturation, desiccation and freezing) and stabilizers of enzymes, nucleic acids, membranes and whole cells. There are many industrial applications of these compounds in enzyme technology. The other compatible solutes such as trehalose, glycerol, proline, ectoines, sugars and hydroxyectoine from halophilic bacteria showed the highest efficiency of protection of lactate dehydrogenase against freeze-thaw treatment and thermal stress. Halophilic bacteria can produce enzymes that have optimal activity at high salinity, which is advantageous for harsh industrial processes.

Also, halophilic bacteria produce a number of extra- and intracellular enzymes and antimicrobial compounds that are currently of commercial interest (Kamekura and Seno 1990). The application of halophilic bacteria in environmental biotechnology is possible for the (1) recovery of saline soil, (2) decontamination of saline or alkaline industrial wastewater and (3) degradation of toxic compounds in hypersaline environments. The use of halophilic bacteria in the recovery of saline soils is covered by the following hypotheses (Arora et al. 2014a, 2014b). The first hypothesis is that in saline soil, microbial activities may favour the growth of plants resistant to soil salinity. The second hypothesis is based on the utilization of these bacteria as bio-indicators as these microorganisms can be selected by their abilities to grow at different salt concentrations. These organisms could indicate that well water could be used with producing low saline contamination of plants or soils which could be alleviated by the desertification of soil. The last hypothesis is the application of halophilic bacteria using genetic manipulation technique to aid wild-type plants to adapt to grow in saline soil by giving them the genes for vital enzymes that are taken from halophiles (Arora et al. 2017).

16.7 Microbial Bioremediation

Utilization of microorganisms to the metabolically mediated desired chemical reactions or physical processes is a useful general definition of bioremediation (Skladany and Metting 1993). The use of selected symbiotic soil microorganisms to enhance plant growth, widely practised for some organisms and widely researched for others, fits at one end of the spectrum. These organisms include mycorrhizal fungi, *Rhizobium* and *Frankia*, which can enhance plant growth by increasing the supply of growth-limiting nutrients.

Bioremediation has been proposed as an economical, sustainable, effective and environmentally friendly alternative to conventional remediation technologies. Bioremediation is an expanding area of environmental biotechnology and can be

simply considered as the application of biological processes to the treatment of pollution. The metabolic usefulness of microorganisms underpins practically all bioremediation applications, and most work to date has concentrated on organic pollutants.

16.7.1 Plant Growth-Promoting Rhizobacteria (PGPR) for Bioremediation

The plant growth-promoting rhizobacteria (PGPR) can affect plant growth by different direct and indirect mechanisms (Glick 1995). PGPR influence direct growth promotion of plants by fixing atmospheric nitrogen, solubilizing insoluble phosphates and secreting hormones such as IAA, GAs and kinetins besides ACC deaminase production, which helps in regulation of ethylene (Glick et al. 2007; Glick 2014). Induced systemic resistance, antibiosis, competition for nutrients, parasitism and production of metabolites (hydrogen cyanide, siderophores) suppressive to deleterious rhizobacteria are some of the mechanisms that indirectly benefit plant growth. Numerous species of soil bacteria which flourish in the rhizosphere of plants may stimulate plant growth by a plethora of mechanisms (Vessey 2003). Soil bacteria are very important in biogeochemical cycles and have been used for crop production for decades. Plant bacterial interactions in the rhizosphere are the determinants of plant health and soil fertility (Vivekanandan et al. 2015). Interaction of plant growth-promoting rhizobacteria (PGPR) with host plants is an intricate and interdependent relationship involving not only the two partners but other biotic and abiotic factors of the rhizosphere region (Dutta and Podile 2010). PGPR bacteria are free-living soil bacteria that can either directly or indirectly facilitate root growth and development (Mayak et al. 1999) as well as overall growth of plants (Glick 1995). It is believed that generally about 2–5% of rhizosphere bacteria are PGPR (Antoun and Prevost 2005). PGPRs are the potential tools for sustainable crop production and trend for the future agriculture. The mechanism by which bacteria are adsorbed onto soil particles is by simple ion exchange, and a soil is said to be naturally fertile when the soil organisms are releasing inorganic nutrients from the organic reserves at a rate sufficient to sustain rapid plant growth.

In agriculture, the use of PGPR as inoculums to alleviate salt stress is the most promising approach to enhance production and yield in saline soils (Arora et al. 2012). These PGPR tolerate wide range of salt stresses and enable plants to tolerate salinity by hydraulic conductance, osmotic accumulation, sequestering toxic Na^+ ions, maintaining the higher osmotic conductance and photosynthetic activities (Dodd and Perez-Alfocea 2012). The bacteria isolated from saline environment (Moral et al. 1988) include *Flavobacterium*, *Azospirillum*, *Alcaligenes*, *Acinetobacterium*, *Pseudomonas* (Rodriguez-Valera et al. 1985; Reinhold et al. 1987; Moral et al. 1988; Ilyas et al. 2012), *Sporosarcina*, *Planococcus* (Ventosa et al. 1998), *Bacillus* (Upadhyay et al. 2009), *Thalassobacillus*, *Halomonas*, *Brevibacterium*, *Oceanobacillus*, *Terribacillus*, *Enterobacter*, *Halobacillus*,

Staphylococcus and *Virgibacillus* (Roohi et al. 2012). Halophilic bacteria strain (CSSRO2 *Planococcus maritimus*) and CSSRY1 (*Nesterenkonia alba*) having plant growth promotion properties were isolated from the rhizosphere of dominant halophytes from coastal ecosystem (Arora et al. 2012). Salt-tolerant *Rhizobium* species were isolated from the rhizosphere of legumes in coastal saline soils of India (Trivedi and Arora 2013).

The plant growth regulating hormone ethylene is produced in response to waterlogging (Grichko and Glick 2001), salinity and/or drought (Kausar and Shahzad 2006; Nadeem et al. 2007; Zahir et al. 2007). In the stressed environment, PGPR exhibit 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Arshad et al. 2007), and this reduces the level of ACC and endogenous ethylene (Glick et al. 1998; Yuhashi et al. 2000) thereby mitigating the deleterious effects of stress on overall plant growth (Ligero et al. 1991; Hirch and Fang 1994). Inoculation of plants with PGPR having ACC deaminase is relatively more tolerant to environmental stress (Singh and Jha 2015).

The inoculation with halophilic strains of PGPR will help to improve the plants tolerance in stress environment especially salinity and promote their growth particularly in food crops which is essentially required to meet the national food demands.

Plant growth-promoting rhizobacteria (PGPR) assist in diminishing the accumulation of ethylene levels and re-establish a healthy root system needed to cope with environmental stress. The primary mechanism includes the destruction of ethylene through the production of enzyme ACC deaminase. There are number of reports (Ghosh et al. 2003; Govindasamy et al. 2008; Duan et al. 2009) mentioning rhizosphere bacteria like *Achromobacter*, *Azospirillum*, *Bacillus*, *Enterobacter*, *Pseudomonas* and *Rhizobium* that exhibit ACC deaminase activity. Most of the researches have demonstrated the production of ACC deaminase gene in the plants treated with PGPR under environmental stress. It has been reported by Grichko and Glick (2001) that tomato seeds inoculated with *Enterobacter cloacae* and *Pseudomonas putida* express ACC deaminase activity and register an increase in plant resistance. Similarly, Ghosh et al. (2003) recorded ACC deaminase activity in three *Bacillus* species, namely, *Bacillus circulans* DUC1, *Bacillus firmus* DUC2 and *Bacillus globisporus* DUC3 that encouraged root elongation in *Brassica campestris*. Mayak et al. (2004) observed significant increase in fresh and dry weight of tomato plants inoculated with the bacterium *Achromobacter piechaudii* under water and salinity stress conditions.

Researchers have also demonstrated the feasibility of *Azospirillum* inoculation to mitigate negative effects of salt (NaCl) on plant growth parameters. This beneficial mitigating effect of *Azospirillum* inoculation that was previously observed in wheat (*Triticum aestivum*) seeds under salt stress was also evident (Creus et al. 1997). *Azospirillum* inoculated wheat (*T. aestivum*) seedlings subjected to osmotic stress developed significant higher coleoptiles, with higher fresh weight and better water status than non-inoculated seedlings (Alvarez et al. 1996; Creus et al. 1998).

16.8 Plant-Microbiome Interactions for Salt Stress Alleviation

The stress factors including salinity, drought, nutrient deficits, contamination, _diseases, pests, etc. can alter plant-microbe interactions in the plant rhizosphere. Researches evidencing that plant perception of environmental stress cues triggers the activation of signalling molecules, phytohormones play a key role (Barea 2015). This signal input is followed by a signal processing and finally by a signal output, which enables plants to respond to these environmental constraints. As the plants are exposed to multiple stresses simultaneously, appropriate meta-analyses reveal a complex regulation of the plant growth and immunity (Dimkpa et al. 2009). Understanding how phytohormones interact in the signalling network is fundamental to learn how plant-microbiome systems thrive and survive in stressed environments. This understanding is relevant to frame biotechnological strategies to optimize plant adaptation mechanisms and to improve the capability of soil microbes for stress alleviation in crop plants (Pozo et al. 2015), although mechanisms involved in plant-microbe interactions under stress situations are poorly understood. However, ongoing research is evidencing the involvement of changes in plant morphology, physiology, transporter activity and root exudation profiles, changes that can induce the plant to employ microbes with stress-alleviating capacities, a strategy that can help crop productivity under stress (Zolla et al. 2013).

As stress factors cause detrimental impacts on the functionality or productivity of agricultural systems, the role of rhizosphere microorganisms to enable plants to thrive in adverse conditions is important (Barea et al. 2013). Plants have highly beneficial interactions with their more mobile companions, microbes; and some of these interactions involve highly sophisticated symbioses that confer stress tolerance, such as with mycorrhizae and rhizobia that help ameliorate nutritional and water deficiencies under stress, while others are more transitory (Etesami and Beattie 2017).

The biotic and abiotic plant factors that shape the plant-associated microbiome through biasing the rhizosphere offer many challenges that current research is trying to envisage. The future work on plants must focus on reprogramming transport functions, while those on microorganisms have to focus on the uptake secreted nutrients and the time-course changes in the microbial community structure. A combination of these approaches can improve the understanding on how to enhance the competitiveness and persistence of bacteria in the influenced rhizosphere to finally improve plant growth and agroecosystem productivity (Savka et al. 2013).

16.9 Isolation of Halophilic Microbes from Rhizospheric Soils of Halophytes

The rhizospheric soil samples from halophyte plant species were collected in duplicate from coastal Gujarat, India. The area is affected by soil salinity due to seawater ingress. The soil pH of the rhizospheric soil varied from 7.3 to 8.8, and salinity

(electrical conductivity) varied from 2.7 to 39.6 dS m^{-1}. The isolation of bacteria was carried on nutrient agar medium and studied for colony and morphological characteristics in relation to soil biochemical properties. Salt tolerance of isolates was determined with varying NaCl concentrations of 0.5–20%. It was found that 7 out of 44 isolates were able to tolerate salt concentration up to 10%, while 29 isolates were able to tolerate salt concentration up to 5% NaCl. Thus, from the rhizosphere of dominant halophytes and other salt-tolerant plant species, various halotolerant bacteria, which were able to tolerate salt concentrations up to 10% NaCl, have been isolated. Out of 13 isolates that were able to tolerate salt concentration up to 15% NaCl, 3 were from the rhizospheric soil of *Capparis decidua*, 2 each from both rhizospheric soil of plants of *Capparis decidua* and *Salvadora oleoides* and 1 each from the rhizospheric soil of *Cressa cretica*, *Aeluropus lagopoides* and *Suaeda maritima* (Arora et al. 2014a).

16.9.1 Isolation of Halophilic Rhizobia spp.

Few Gram-negative bacteria, known as rhizobia, have been isolated from the saline soil samples. *Rhizobium spp.* can tolerate up to 500 mM of NaCl. It has been found out that some species of rhizobia adapt to saline conditions through the intracellular accumulation of low-molecular-weight organic solutes called osmolytes, such as glutamate, trehalose, glycine betaine and polyamines, or an accumulation of K^+ (Trivedi and Arora 2013). The ability of the isolates to grow in different concentrations of salt was tested by streaking isolates on YEM media containing 0.5%, 1%, 2%, 3%, 3.5%, 4% and 5% (w/v) NaCl. Twenty *Rhizobium* isolates could tolerate u pto 2% NaCl, while only 10 could tolerate up to 4% NaCl concentration.

16.9.2 Isolation of Halophilic Endophytic Bacteria

From the leaves of four dominant halophytes or salt-tolerant plants from coastal Gujarat, isolated halophilic bacteria. Twenty isolates were screened based on salt tolerance and their fast growth. All of the 20 endophytes showed superior growth at 2.5% NaCl concentration, while 18 (90%) endophytes sustain up to 5% NaCl, 17 (85%) isolates survived at 7.5% NaCl, and 15 (75%) tolerated up to 10% NaCl concentration (Arora et al. 2014a). *Bacillus foraminis* and *Bacillus gibsonii* were able to tolerate salt concentration up to 7.5% of NaCl, while *Acinetobacter baumannii* and *Paenibacillus xylanisolvens* could tolerate only up to 2.5% NaCl concentration and *Pseudomonas fluorescens* up to 5% NaCl level (Table 16.1). The other isolates were capable of tolerating 10% NaCl concentration in the media. Overall, the growth rate of endophytes showed decline with increasing NaCl concentration in the media. The bacterial counts were found maximum in *Sphaeranthus indicus* (40%) and were minimum in *Salicornia brachiata* (10%). Of the 20 endophyte isolates selected, 3 were pigmented, and 17 were non-pigmented isolates. Regarding cell shape and Gram's staining, seven were Gram-negative cocci, two Gram-positive

Table 16.1 Salt tolerance of endophytic bacteria from leaves of halophytes and salt-tolerant plant species

Isolate ID	Endophytic bacteria	Salt tolerance (NaCl %)			
		2.5%	5.0%	7.5%	10%
EB1	*Acinetobacter baumannii*	+	–	–	–
EB2	*Kocuria flavus*	+	+	+	+
EB3	*Bacillus cereus*	+	+	+	+
EB4	*Bacillus firmus*	+	+	+	+
EB5	*Staphylococcus pasteuri*	+	+	+	+
EB6	*Paenibacillus xylanisolvens*	+	–	–	–
EB7	*Bacillus horneckiae*	+	+	+	+
EB8	*Paenibacillus xylanisolvens*	+	+	+	+
EB9	*Bacillus licheniformis*	+	+	+	+
EB10	*Bacillus foraminis*	+	+	+	–
EB11	*Virgibacillus picturae*	+	+	+	+
EB12	*Oceanobacillus picturae*	+	+	+	+
EB13	*Bacillus subtilis*	+	+	+	+
EB14	*Bacillus aerius*	+	+	+	+
EB15	*Pseudomonas fluorescens*	+	+	–	–
EB16	*Bacillus subtilis*	+	+	+	+
EB17	*Bacillus aryabhattai /megaterium*	+	+	+	+
EB18	*Arthrobacter luteolus*	+	+	+	+
EB19	*Bacillus gibsonii*	+	+	+	–
EB20	*Paenibacillus sp.*	+	+	+	+

cocci, four Gram-negative bacilli and seven Gram-positive bacilli. Motility test results depicted that 18 isolates were motile, while only 2 isolates were nonmotile. In total, 11 isolates showed positive results for oxidase test, whereas all endophytic bacterial cultures showed negative catalase test. The enzymatic activity of endophytic isolates revealed that 50% isolates exhibited amylase activity, and only 15% isolates showed urease activity (Arora et al. 2014a).

Of the 20 endophytic bacteria screened for plant growth-promoting substances, six (30%) and two (10%) isolates showed positive test for ammonia production and phosphate solubilization activity. Only four (20%) were mixed acid fermenters, five (25%) showed the production of acetoin, and none of the isolates exhibited IAA production (Arora et al. 2014a). The selected bacterial isolates were submitted for 16S rRNA gene sequencing, and it was observed that *Acinetobacter baumannii*, *Bacillus cereus*, *Bacillus firmus*, *Bacillus aerius*, *Pseudomonas fluorescens* and *Bacillus subtilis* were positive for ammonia production, while phosphate solubilization was positive for *Acinetobacter baumannii* and *Pseudomonas fluorescens* (Arora and Vanza 2017).

16.10 Liquid Bioformulations Developed for Enhancing Crop Production in Salt-Affected Soils

Salt-tolerant (halophilic) bacterial strains of N-fixers and phosphate solubilization bacteria (PSB) were isolated from the salt-affected soils of Indo-Gangetic plains at ICAR-CSSRI, Regional Research Station, Lucknow (UP). These strains were characterized for plant growth promotion and tested for their efficacy under different levels of salt stress, Figs. 16.1 and 16.2. For seed application of these promising selected strains of beneficial soil microorganisms, these were cultured in laboratory and prepared in suitable standardized media as liquid bioformulations, viz. Halo-Azo and Halo-PSB. These can be used either for seed/seedling root treatment or soil application. These bioformulations when applied help to mobilize plant nutrients like nitrogen and phosphorous through their activities in the soil or rhizosphere and make available to plants in a gradual manner under salt stress. Also liquid formulations 'Halo-Zinc' and 'Halo-Rhizo' having salt-tolerant strains of zinc solubilizers and *Rhizobium* species, respectively, were developed and found to be effective under salt stress. These shall also help in maintenance of soil health, minimize environmental pollution and cut down on the use of chemicals in agriculture. The bioformulations are affordable for most of the small and marginal farmers. These bioformulations are also an ideal input for reducing the cost of cultivation and for promoting organic farming on salt-affected soils.

In sodic and saline-sodic soils, the bioformulation has been tested at farm, validated at different farmers' fields in five salt-affected districts of Indo-Gangetic plains. The seedling dip or seed inoculation with the bioformulation resulted in enhanced crop yields, management of soil health and stress regulation.

These liquid bioformulations are very beneficial for enhancing the production of cereal crops mainly rice and wheat as well as vegetable crops. These can be easily used as seed treatment, seedling dip and soil application with farmyard manure or compost. The packing of 100 ml bottle is sufficient for treating seeds of 1 acre land or root dip. It has been found to be very effective in sodic soil, and multilocation testing of these bioformulations was done in diverse sodic soils of Indo-Gangetic plains. There was increase in rice and wheat yield by 11.5–14% under salt stress conditions in Indo-Gangetic plains (Fig. 16.1). This is the cheap and eco-friendly approach for bioremediation of salt-affected soils and to optimize agricultural crop yields in the degraded lands.

Fig. 16.1 Liquid bioformulations including salt-tolerant bacterial strains

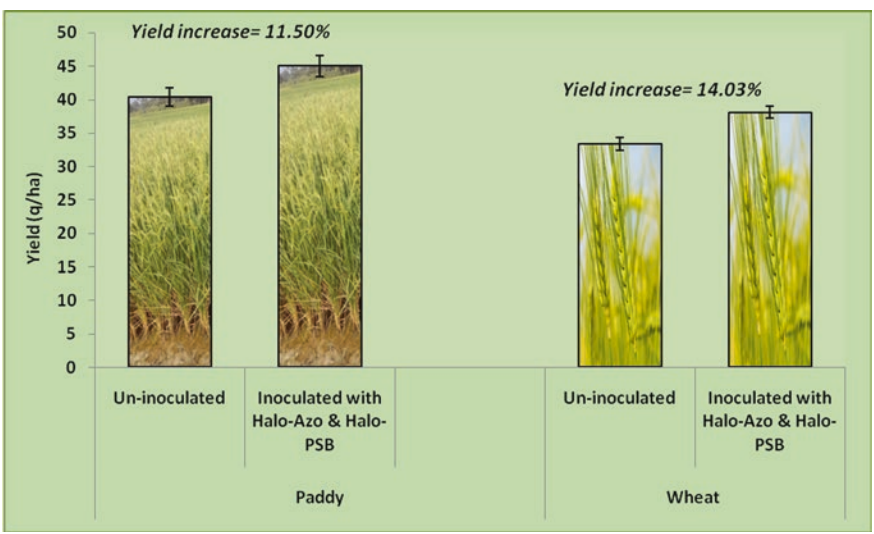


Fig. 16.2 Efficacy of liquid bioformulations of halophilic PGP strains on wheat and rice on sodic soils

Table 16.2 Effect of bioformulation use on sodic soil properties after harvest (initial soil pH = 9.42)

Treatment	pH (1:2)	EC (dS/m)	OC (%)	Exch. Na (mg/kg)	ESP	Av N (kg/ha)	Av P (kg/ha)	MBC (μg/g)	DHA (μgTPF/g/d)
Control (FYM)	9.24	0.432	0.28	338	44	103	10.8	44	10
FYM + halo azo	8.94	0.318	0.35	266	42	119	11.4	55	13.9
FYM + halo PSB	9.12	0.364	0.33	272	43	113	15.1	52	12.2
FYM + halo Azosp	9.18	0.385	0.31	282	43	121	14.4	58	13.2
FYM + consortia	8.91	0.322	0.38	238	41	123	15.6	61	14.8

The application of liquid bioformulations has the potential to improve the growth and yield of crops under salt stress, and they were also found to play a role in soil health improvement as observed in soil after harvest of the crop (Table 16.2).

16.11 Ameliorative Potential of Halophilic Microbes

There was substantial improvement in soil pH and exchangeable sodium content. It was observed that after 2 years of continuous rice-wheat with inoculation of halophilic plant growth-promoting microbial formulations, soil pH reduced from initial value of 9.42–8.91 and 8.94. Similarly reduction of exchangeable sodium from 416 to 238 mg/kg was noticed. The build-up of soil organic C and available N apart from

improvement in soil microbial biomass C and dehydrogenase activity was observed with application of liquid bioformulations. Soil microbial biomass carbon enhanced to 61 μg/g with the application of consortia over 41 μg/g where no bioformulations were used (Table 16.2).

References

Abrol IP, Yadav JSP, Massoud FI (1988) Salt-affected soils and their management. Food and Agriculture Organization of the United Nations, Soils Bull. 39, Rome, Italy

Akbarimoghaddam H, Galavi M, Ghanbari A, Panjehkeh N (2011) Salinity effects on seed germination and seedling growth of bread wheat cultivars. Trakia J Sci 9(1):43–50

Aliasgharzadeh N, Saleh Rastin N, Towfighi H, Alizadeh A (2001) Occurrence of arbuscular mycorrhizal fungi in saline soils of the Tabriz plain of Iran in relation to some physical and chemical properties of soil. Mycorrhiza 11:119–122

Alvarez MI, Sueldo RJ, Barassi CA (1996) Effect of *Azospirillum* on coleoptiles growth in wheat seedlings under water stress. Cereal Res Commun 24:101–107

Antoun H, Prevost D (2005) Ecology of plant growth promoting rhizobacteria. In: Siddiqui ZA (ed) PGPR: biocontrol and biofertilization. Springer, The Netherlands, pp 1–39

Arahal DR, Ventosa A (2002) Moderately halophilic and halotolerant species of *Bacillus* and related genera. In: Berkeley R, Heyndrickx M, Logan N, De Vos P (eds) Applications and systematics of *Bacillus* and relatives. Blackwell, Oxford, pp 83–99

Arora S, Vanza M (2017) Microbial approach for bioremediation of saline and sodic soils. In: Arora SS, Singh AK, Singh YP (eds) Bioremediation of salt affected soils: an Indian perspective. Springer International Publishing, Switzerland, pp 87–100

Arora NK, Tewari S, Singh S, Lal N, Maheshwari DK (2012) PGPR for protection of plant health under saline conditions. In: Maheshwari DK (ed) Bacteria in agrobiology: stress management. Springer, Berlin. https://doi.org/10.1007/978-3-642-23465-1_12

Arora S, Patel P, Vanza M, Rao GG (2014a) Isolation and characterization of endophytic bacteria colonizing halophyte and other salt tolerant plant species from coastal Gujarat. Afr J Microbiol Res 8(17):1779–1788

Arora S, Vanza M, Mehta R, Bhuva C, Patel P (2014b) Halophilic microbes for bio-remediation of salt affected soils. Afr J Microbiol Res 8(33):3070–3078

Arora S, Singh AK, Sahni D (2017) Bioremediation of salt-affected soils: challenges and opportunities. In: Arora S, Singh AK, Singh YP (eds) Bioremediation of salt affected soils: an Indian perspective. Springer International Publishing, Switzerland, pp 275–302

Arshad M, Saleem M, Hussain S (2007) Perspectives of bacterial ACC-deaminase in phytoremediation. Trends Biotechnol 25:356–362

Barea JM (2015) Future challenges and perspectives for applying microbial biotechnology in sustainable agriculture based on a better understanding of plant-microbiome interactions. J Soil Sci Plant Nutr 15(2):261–282

Barea JM, Pozo MJ, López-Ráez JA, Aroca R, Ruíz-Lozano JM, Ferrol N, Azcón R, Azcón-Aguilar C (2013) Arbuscular mycorrhizas and their significance in promoting soil-plant systems sustainability against environmental stresses. In: Rodelas B, González-López J (eds) Beneficial plant-microbial interactions: ecology and applications. CRC Press, Boca Raton, FL, pp 353–387

Chinnusamy V, Zhu J, Zhu J-K (2006) Gene regulation during cold acclimation in plants. Physiol Plant 126(1):52–61

Creus CM, Sueldo RJ, Barassi CA (1997) Shoot growth and water status in *Azospirillum*-inoculated wheat seedlings grown under osmotic and salt stresses. Plant Physiol Biochem 35:939–944

Creus CM, Sueldo RJ, Barassi CA (1998) Water relations in *Azospirillum* inoculated wheat seedlings under osmotic stress. Can J Bot 76:238–244

Dimkpa C, Weinand T, Asch F (2009) Plant rhizobacteria interactions alleviate abiotic stress conditions. Plant Cell Environ 32:1682–1694

Dodd IC, Perez-Alfocea F (2012) Microbial alleviation of crop salinity. J Exp Bot 63:3415–3428

Duan J, Muller KM, Charles TC, Vesely S, Glick BR (2009) 1-Aminocyclopropane-1-carboxylate (ACC) deaminase genes in rhizobia from southern Saskatchewan. Microb Ecol 57:423–436

Dundas I (1998) Was the environment for primordial life hypersaline? Extremophiles 2:375–377

Dutta S, Podile AR (2010) Plant growth promoting rhizobacteria (PGPR): the bugs to debug the root zone. Crit Rev Microbiol 36:232–244

Etesami H, Beattie GA (2017) Plant-microbe interactions in adaptation of agricultural crops to abiotic stress conditions. In: Kumar V et al (eds) Probiotics and plant health. Springer Nature Singapore Pte Ltd., Singapore, pp 163–200

Garabito MJ, Marquez MC, Ventosa A (1998) Halotolerant *Bacillus* diversity in hypersaline environments. Can J Microbiol 44:95–102

Ghosh S, Penterman JN, Little RD, Chavez R, Glick BR (2003) Three newly isolated plant growth-promoting bacilli facilitate the seedling growth of canola, *Brassica campestris*. Plant Physiol Biochem 41:277–281

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

Glick BR (2014) Bacteria with ACC deaminase can promote plant growth and help to feed the world. Microbiol Res 169:30–39

Glick BR, Cheng Z, Czarny J, Duan J (2007) Promotion of plant growth by ACC deaminase-producing soil bacteria. Eur J Plant Pathol 119(3):329–339

Govindasamy V, Senthilkumar M, Gaikwad K, Annapurna K (2008) Isolation and characterization of ACC deaminase gene from two plant growth-promoting rhizobacteria. Curr Microbiol 57(4):312–317

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

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

Hu Y, Schmidhalter U (2002) Limitation of salt stress to plant growth. In: Hock B, Elstner CF (eds) *Plant toxicology*. Marcel Dekker Inc., New York, pp 91–224

Ilyas N, Bano A, Iqbal S, Raja NI (2012) Physiological, biochemical and molecular characterization of *Azospirillum* spp. isolated from maize under water stress. Pak J Bot 44:71–80

Jamil A, Riaz S, Ashraf M, Foolad MR (2011) Gene expression profiling of plants under salt stress. Crit Rev Plant Sci 30(5):435–458

Juniper S, Abbott L (1993) Vesicular and arbuscular mycorrhizae and soil salinity. Mycorrhizae 4:45–57

Kamekura M, Seno Y (1990) A halophilic extracellular protease from a halophilic archaebacterium strain 172 P1. Biochem Cell Biol 68(1):352–359

Kausar R, Shahzad SM (2006) Effect of ACC-deaminase containing rhizobacteria on growth promotion of maize under salinity stress. J Agri Soc Sci 2:216–218

Ladeiro B (2012) Saline agriculture in the 21 st century : using salt contaminated resources to cope food requirement. J Bot 2012:1. https://doi.org/10.1155/201/310705

Ligero F, Caba JM, Lluch C, Oliverase J (1991) Nitrate inhibition of nodulation can be overcome by ethylene inhibitor amino ethoxy vinyl glycine. Plant Physiol 97(3):1221–1225

Mandal AK, Sharma RC, Singh G (2009) Assessment of salt affected soils in India using GIS. Geocarto Int 24(6):437–456

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

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

Moral AD, Prado B, Quesda E, Gacria T, Ferrer R, Ramos-Comenzana A (1988) Numerical taxonomy of moderately halophilic Gram-negative rods from an inland saltern. J Gen Microbiol 134:733–741

Munns R (2002) Comparative physiology of salt and water stress. Plant Cell Environ 25:239–250
Nadeem SM, Zahir ZA, Naveed M, Arshad M (2007) Preliminary investigation on inducing salt tolerance in maize through inoculation with rhizobacteria containing ACC-deaminase activity. Can J Microbiol 53:1141–1149
Ondrasek G, Rengel Z, Romic D, Savic R (2010) Environmental salinization processes in agro-ecosystem of neretva river estuary. Novenytermeles 59:223–226
Oren A (1999) Bioenergetic aspects of halophilism. Microbiol Mol Biol Rev 63:334–348
Porras-Soriano A, Soriano-Martin ML, Porras-Piedra A, Azcon R (2009) Arbuscular mycorrhizal fungi increased growth, nutrient uptake and tolerance to salinity in olive trees under nursery conditions. J Plant Physiol 166:1350. https://doi.org/10.1016/j.jplph.2009.02.010
Pozo M, López-Ráez J, Azcón-Aguilar C, García-Garrido J (2015) Phytohormones as integrators of environmental signals in the regulation of mycorrhizal symbioses. New Phytol 205:1431–1436
Reinhold B, Hurek T, Fendrik I, Pot B, Gillis M, Kersters K, Thielemans S, De L (1987) *Azospirillum halopraeferens* sp. nov., a nitrogen fixing organism associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth.). Int J Syst Bacteriol 37:43–51
Rengasamy P (2006) World salinization with emphasis on Australia. J Exp Bot 57:1017–1023
Rodriguez-Valera F (1988) Characteristics and microbial ecology of hypersaline environments. In: Rodriguez-Valera F (ed) Halophilic bacteria, vol 1. CRC Press, Boca Raton, FL, pp 3–30
Rodriguez-Valera F, Ventosa A, Juez G, Imhoff LF (1985) Variation of environmental features and microbial populations with the salt concentrations in a multi-pond saltern. Microb Ecol 11:107–111
Roohi A, Ahmed I, Iqbal M, Jamil M (2012) Preliminary isolation and characterization of halotolerant and halophilic bacteria from salt mines of Karak, Pakistan. Pak J Bot 44:365–370
Savka MA, Dessaux Y, McSpadden Gardener BB, Mondy S, Kohler PRA, de Bruijn FJ, Rossbach S (2013) The "biased rhizosphere" concept and advances in the omics era to study bacterial competitiveness and persistence in the phytosphere. In: de Bruijn FJ (ed) Molecular microbial ecology of the rhizosphere, vol 2. Wiley Blackwell, Hoboken, NJ, pp 1147–1161
Shivanand P, Mugeraya G (2011) Halophilic bacteria and their compatible solutes – osmoregulation and potential applications. Curr Sci 100(10):1516–1521
Singh KN, Chatrath R (2001) Salinity tolerance. In: Reynolds MP, Monasterio JIO, McNab A (eds) Application of physiology in wheat breeding. CIMMYT, Mexico, DF, pp 101–110
Singh RP, Jha PN (2015) Plant growth potential of ACC deaminase rhizospheric bacteria isolated from *Aerva javanica*: a plant adapted to saline environments. Int J Curr Microbiol Appl Sci 4(7):142–152
Skladany GJ, Metting FB (1993) Bioremediation of contaminated soil. In: Metting FB (ed) Soil microbial ecology: applications in agricultural and environmental management. Marcel Dekker, New York, pp 483–513
Stahl PO, Williams SE (1986) Oil shale process water affects activity of vesicular-arbuscular fungi and *Rhizobium* four years after application to soil. Soil Biol Biochem 18:451–455
Trivedi R, Arora S (2013) Characterization of acid and salt tolerant *Rhizobium* sp. isolated from saline soils of Gujarat. Int Res J Chem 3(3):8–13
Upadhyay SK, Singh DP, Saikia R (2009) Genetic diversity of plant growth promoting rhizobacteria isolated from rhizosphere soil of wheat under saline condition. Curr Microbiol 59:489–496
Ventosa A, Nieto JJ, Oren A (1998) Biology of moderately halophilic aerobic bacteria. Microbiol Mol Biol Rev 62(2):504–544
Vessey JK (2003) Plant growth promoting rhizobacteria as biofertilizer. Plant Soil 255:571–586
Vivekanandan M, Karthik R, Leela A (2015) Improvement of crop productivity in saline soils through application of saline-tolerant rhizosphere bacteria - current perspective. Int J Adv Res 3(7):1273–1283
Yadav RS, Mahatma MK, Thirumalaisamy PP, Meena HN, Bhaduri D, Arora S, Panwar J (2017) Arbuscular mycorrhizal fungi (AMF) for sustainable soil and plant health in salt-affected soils. In: Arora S, Singh AK, Singh YP (eds) Bioremediation of salt affected soils: an Indian perspective. Springer International Publishing, Switzerland, pp 133–156

Yancey PH, Clark ME, Hand SC, Bowlus RD, Somero GN (1982) Living with water stress: evolution of osmolyte systems. Science 217:1214–1216

Yoon JH, Kim IG, Kang KH, Oh TK, Park YH (2003) *Bacillus marisflavi* sp. nov. and *Bacillus aquimaris* sp. nov., isolated from sea water of a tidal flat of the yellow sea in Korea. Int J Syst Evol Microbiol 53:1297–1303

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

Zahir ZA, Munir A, Asghar HN, Shahroona B, Arshad M (2007) Effectiveness of *Rhizobacterium* containing ACC-deaminase for growth promotion of pea (*Pisum sativum*) under drought conditions. J Microbiol Biotechnol 18:958–963

Zhu JK (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247–273

Zolla G, Bakker MG, Badri DV, Chaparro JM, Sheflin AM, Manter DK, Vivanco J (2013) Understanding root-microbiome interactions. In: de Bruijn FJ (ed) Molecular microbial ecology of the rhizosphere, vol 2. Wiley Blackwell, Hoboken, NJ, pp 745–754