Jay Shankar Singh · Gamini Seneviratne
Editors

Agro-Environmental Sustainability

Volume 1: Managing Crop Health

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Editors
Jay Shankar Singh
Department of Environmental Microbiology
Babashaeb Bhimrao Ambedkar (Central) University
Lucknow, Uttar Pradesh, India

Gamini Seneviratne
Microbial Biotechnology Unit
National Institute of Fundamental Studies
Kandy, Sri Lanka

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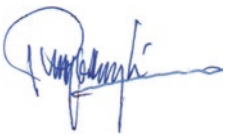
Foreword

There are enormous data and literature bases on microbes in soil and agricultural health. They have resulted in various concepts and applications in agricultural production in addressing future food security. However, mechanisms involved in the action of microbes in soil ecosystems are understudied. It is important to understand unified processes that coordinate microbes and agricultural sustainability. Therefore, this book basically addresses the above point with special reference to unique topics like microbial signaling and agro-ecosystem sustainability, which describes the role of soil food web signaling networks in the unification of ecosystems for long-lasting existence with good health and productivity. Developing new knowledge on this and its applications is a must in reinstating degraded ecosystems and establishing sustainable croplands.

Some chapters of the book explain how to exploit beneficial traits of plant-associated microbes for plant growth, health, and sustainable crop production. Chapters describing ecological applications of microbes in restoration, carbon sequestration, and soil fertility management are also included. Topics related to commercial use of biofertilizers based on beneficial microbes in agriculture strengthen their potential applications in addressing food demand in the future. Thus, the book attempts to

close the knowledge gap between mechanisms of the ecosystems and sustainable agriculture. The book is very useful for academics, students, policy makers, and researchers in understanding the impetus of agro-environmental sustainability.

I am pleased to see the contents of the present volume edited by Dr. Jay Shankar Singh and Dr. Gamini Seneviratne entitled *Agro-Environmental Sustainability: Managing Crop Health (Volume I)*. The editors have done commendable research with publications related to sustainable agricultural development. This volume offers a lot of rational approaches that may help to improve and develop agricultural sustainability. I pass on good wishes to the editors and the subject expert contributors to this remarkable book.



Prof. Panjab Singh
National Academy of Agricultural Sciences (NAAS)
NASC Complex, DPS Marg, Pusa, New Delhi, India

Preface

Microbial signaling in ecosystems is a relatively novel subject being studied in the field of sustainable land use. New concepts have been put forward recently to explain the impetus of the signaling, which is responsible for equilibrated ecosystem functioning and sustained provision of ecosystem services, including crop production. The first chapter of this volume introduces the importance of microbial signaling for agricultural sustainability. Furthermore, it explains the current knowledge on the mechanisms of microbial signaling in plant-microbe interactions, technical advances in identifying signaling pathways between plants and soil, and avenues for future research in this field.

Subsequent chapters review the progress in microbial gene prospecting and its contemporary use in plant stress tolerance, and the role of fluorescent pseudomonads and biosynthesized antimicrobial secondary metabolites in rhizosphere functioning. Some chapters describe the potential use of the metabolites in more efficient control of plant diseases and postharvest applications, and in mycorrhizal induced resistance (MIR) in plant defense responses. Certain chapters deal with various N_2-fixing cyanobacterial systems in light of their use as biofertilizers, and reductions in fertilizer needs as high as 52 % by rice farmers in Vietnam. Furthermore, they discuss the potential of actinobacteria that produce antibiotics and active compounds as biofertilizers and biopesticides, thus helping reduce the use of harmful chemical inputs for restoration of soil fertility and bioremediation. One chapter reviews the recent progress in the improvement of soil fertility with cover crops via the addictive effects of symbiotic microbes. Micro-algal biomass production and its utilization is discussed in another chapter.

Scientists actively involved in a variety of research areas in soil science and agricultural microbiology from institutions worldwide have contributed to this book. This book is useful to students, teachers, and researchers in the disciplines of soil and agricultural microbiology, biochemistry, and biotechnology. We gratefully acknowledge the support and cooperation of all the contributing authors and note a

special thanks to Mr. Arun Kumar, Mr. Sashank Tiwari, Mr. Shobhit R. Vimal, and Mr. Sumit Kaushal, research scholars, Department of Environmental Microbiology, B.B. Ambedkar University, Lucknow, India, and Ms. J. S. Z. Ismail, Monash University, Australia, for editorial assistance in preparation of this volume.

Lucknow, Uttar Pradesh, India — Jay Shankar Singh
Kandy, Sri Lanka — Gamini Seneviratne

Contents

Contributors

V.S. Bharti Indian Council of Agricultural Research-Central Institute of Fisheries Education, Mumbai, Maharashtra, India

G.R. Bhuvaneswari Aquatic Environment and Health Management Division, Central Institute of Fisheries Education, Mumbai, Maharashtra, India

Kartikay Bisen Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Manoj Kumar Chitara Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Abu T.M.A. Choudhury Faculty of Agriculture and Environment, University of Sydney, Sydney, NSW, Australia

Rosalind J. Deaker Faculty of Agriculture and Environment, University of Sydney, Sydney, NSW, Australia

M.L. Dotaniya Indian Council of Agricultural Research-Indian Institute of Soil Science, Bhopal, Madhya Pradesh, India

Geetika Gambhir National Research Centre on Plant Biotechnology, New Delhi, Delhi, India

S. Gita Aquatic Environment and Health Management Division, Central Institute of Fisheries Education, Mumbai, Maharashtra, India

R. Gothalwal Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India

Supriya Gupta Department of Plant Pathology, College of Agriculture, G B Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

Ivan R. Kennedy Faculty of Agriculture and Environment, University of Sydney, Sydney, NSW, Australia

Chetan Keswani Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Rajesh Kumar Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (Central) University, Lucknow, Uttar Pradesh, India

D. Kumaresan Faculty of Natural and Agricultural Sciences, School of Earth and Environment, The University of Western Australia, Crawley, WA, Australia

Chandan Maharana Department of Plant Pathology, College of Agriculture, G B Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

Thanh Hien Nguyen Biofertilizer Action Research Center, Hanoi, Vietnam

Jasdeep Chatrath Padaria National Research Centre on Plant Biotechnology, New Delhi, Delhi, India

Thi Cong Phan Agriculture Green Future, Ho Chi Minh City, Vietnam

Amar Nath Rai Department of Biochemistry, North-Eastern Hill University, Shillong, Meghalaya, India

Anuradha Rai Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Pradeep Kumar Rai Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Pankaj Rautela Department of Plant Pathology, College of Agriculture, G B Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

Michael T. Rose NSW Department of Primary Industries, Wollongbar Primary Industries Institute, Wollongbar, NSW, Australia

Birinchi Kumar Sarma Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Tulasi Satyanarayana Department of Microbiology, University of Delhi South Campus, New Delhi, Delhi, India

G. Seneviratne Microbial Biotechnology Unit, National Institute of Fundamental Studies, Kandy, Sri Lanka

Prashant Kumar Sharma Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India

Beenu Shastri Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (Central) University, Lucknow, Uttar Pradesh, India

Shashi Shekhar National Research Centre on Plant Biotechnology, New Delhi, Delhi, India

L. Shivlata Department of Microbiology, University of Delhi South Campus, New Delhi, Delhi, India

S.P. Shukla Indian Council of Agricultural Research-Central Institute of Fisheries Education, Mumbai, Maharashtra, India

Arvind Kumar Singh Department of Biochemistry, North-Eastern Hill University, Shillong, Meghalaya, India

Harikesh Bahadur Singh Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi, Uttar Pradesh, India

K.P. Singh Department of Plant Pathology, College of Agriculture, G B Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India

Surendra Singh Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi, Uttar Pradesh, India

Mayashree B. Syiem Department of Biochemistry, North-Eastern Hill University, Shillong, Meghalaya, India

M.L.M.A.W. Weerasekara Faculty of Natural and Agricultural Sciences, School of Earth and Environment, The University of Western Australia, Crawley, WA, Australia

W.A.A.D.L. Wikramasinghe Aquatic Environment and Health Management Division, Central Institute of Fisheries Education, Mumbai, Maharashtra, India

V.K. Yadav Indian Council of Agricultural Research-Central Institute of Fisheries Education, Mumbai, Maharashtra, India

Qing Yao College of Horticulture, South China Agricultural University, Guangzhou, China

J.S. Zavahir Geocycle (SBF), Dandenong South, VIC, Australia

Yang Zhou College of Horticulture, South China Agricultural University, Guangzhou, China

Guangzhou Institute of Microbiology, Guangzhou, China

Honghui Zhu Guangzhou Institute of Microbiology, Guangzhou, China

Chapter 1
Microbial Signaling in Plant—Microbe Interactions and Its Role on Sustainability of Agroecosystems

G. Seneviratne, M.L.M.A.W. Weerasekara, D. Kumaresan, and J.S. Zavahir

Abstract Sustainability in agroecosystems is governed primarily by the functional balance between soil processes and plant productivity. Microorganisms are key drivers of important soil processes such as nutrient recycling, and their activity directly influences the functional stability and sustainability of the soil ecosystem. In nature, microbes tend to function as functional guilds or communities, thereby creating a complex network of microbial interactions. Therefore, microbial signalling processes play an important role in communication within a particular functional guild or among different guilds. Numerous chemical compounds acting as signalling molecules in the soil-plant system have been identified. However, the understanding of how these molecules contribute to soil ecosystem stability and sustainability through inter- and intra-species chemical signalling is incomplete. In particular, it is known that chemical inputs in agroecosystems can suppress some microbes (e.g. nitrogen fixers), which can also reduce the interactions between microbes due to destruction of the signalling networks, consequently breaking the delicate balance of the soil ecosystem. Understanding the impact of microbial signalling processes on soil ecosystem sustainability is imperative if we are to address this issue. This chapter reviews the current knowledge on the mechanisms of microbial signalling in plant–microbe interactions and technical advances in identifying signalling pathways between plants and soil and also proposes avenue for future research in this field.

Keywords Microbial signalling • Ecosystem sustainability • Plant–microbe interaction • Chemical fertilizers

G. Seneviratne (✉)
Microbial Biotechnology Unit, National Institute of Fundamental Studies, Hantana Road, Kandy, Sri Lanka
e-mail: gaminis@ifs.ac.lk

M.L.M.A.W. Weerasekara • D. Kumaresan
Faculty of Natural and Agricultural Sciences, School of Earth and Environment, The University of Western Australia, 35 Stirling Highway, Crawley 6009, WA, Australia

J.S. Zavahir
Geocycle (SBF), 92 Ordish Road, Dandenong South 3175, VIC, Australia

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1.1 Introduction

Soils are complex ecosystems, composed of both biotic and abiotic components with regular interactions between both these components to maintain ecosystem function. Bot and Benites (2005) defined a soil ecosystem as an interdependent life-support system, in which the abiotic components, air, water, minerals, and organic matter, function together and interact closely with their biotic components of macro and microorganisms. These biotic and abiotic components thus principally govern the sustainability of soil ecosystem by maintaining soil fertility, soil health, and plant productivity (van der Heijden et al. 1998; Tilak et al. 2005; Richardson and Simpson 2011; Ranjan et al. 2015).

Soil biotic components are key drivers of important soil processes such as decomposition of organic matter, nutrient recycling, detoxification of toxicants, and suppression of noxious and pathogenic organisms (Doran and Zeiss 2000; Singh 2015a, b). Such a wide variety of soil processes which are driven by biotic components define the composition and sustainability of the soil and environment (Bot and Benites 2005; Singh and Gupta 2016). The soil food web comprises of a community of organisms living all or part of their lives in soil with interdependency for sources of carbon and energy. The soil food web determines cycling processes of major elements across ecosystems and is also a better predictor of such processes than land use (de Vries et al. 2013).

The diversity and abundance of life that exists within the soil ecosystem is greater perhaps than in any other ecosystem. With 1g of soil holding up to ten billion microorganisms and thousands of different species (Knietsch et al. 2003), the impact of microbial, functional diversity cannot be underestimated. The extent of microbial diversity creates an intricate network of microbe–microbe and plant–microbe interactions with complex systems of intra- and inter-species communication (Lambers et al. 2009). Through their abundance, diversity, food-web trophic, and community interactions, soil organisms maintain the functional equilibrium of soil ecosystems (Doran and Zeiss 2000). As revealed by Lupatini et al. (2014), microbial species interactions in the soil food web could be more important to soil processes than just species richness and abundance. Such interactions have been reported to reduce inter-species competition and increase the number of coexisting species leading to an increase in biodiversity (Bastolla et al. 2009). In such biodiverse communities, an array of small hormone like molecules helps achieve both cell-to-cell signalling and communication between the microorganisms and their hosts (Hughes and Sperandio 2008). These chemical signaling compounds function as messengers for communicating among the diverse groups of organisms in the soil ecosystems for cooperative existence and are also attributable to the stability and balanced responses of a large number of individuals in the soil food web (Seneviratne 2015). Hence, understanding how these microorganisms maintain a correct balance between inter- and intra-species interactions is important for sustenance of soil ecosystems. There is at present an incomplete understanding of plant–microbial signaling compounds and the mechanisms underlying plant–microbe interaction in both symbiotic and

defence associations. In particular, the importance of chemical signaling in ecosystem sustainability is less documented and calls for further attention. Thus, this chapter highlights the fact that sustainability of soil ecosystem is an outcome of maintaining a robust signaling stability within soil microbes and also optimizing plant–microbe interactions.

1.2 Signalling Molecules in Plant–Microbe Interactions

Plant–microbe interaction is critical to maintain soil health and sustainability (Tak et al. 2013). Plant growth is influenced by microbial functional diversity through a variety of mechanisms, including biological nitrogen fixation by different classes of *Proteobacteria*, increased biotic and abiotic stress tolerance imparted by the presence of endophytic microbes, and direct and indirect advantages rendered by plant growth-promoting rhizobacteria (Barea et al. 2005). It has long been known that plants and microbes interact closely through the release of signaling compounds forming an array of symbiotic and defence associations.

To date, a plethora of such chemical compounds acting as signaling molecules in the soil-plant feedback system have been identified (Berg 2009; Ortíz-Castro et al. 2009; Mandal et al. 2010). Some of these signaling molecules are plant derived whilst some are from microorganisms and influence plant–microbe interactions, plant growth, and ecological balance (Lambrecht et al. 2000; Ortíz-Castro et al. 2009; Mabood et al. 2014; Ludwig-Müller 2015). The wide variety of signaling compounds produced by plants include both primary metabolites (carbohydrates, proteins, organic acids, etc.) and secondary metabolites (flavonoids, phenol, phytohormones, etc.) (Singh et al. 2016a, b). On the other hand, microorganisms in soil release small molecules or volatile compounds called phytohormones, which may act directly or indirectly to activate plant immunity or regulate plant growth and morphogenesis. The major classes of signals participating in the interactions that occur between plants and beneficial microorganisms are carried out by compounds which include phytohormones, *N*-acyl-L-homoserine lactones, volatile organic and secondary plant compounds, etc. (reviewed in Ortíz-Castro et al. 2009).

The signaling compounds play diverse roles in ensuring benefits to both parties of the plant–microbe interaction. Phytohormones, such as auxins and cytokinins, produced by either bacteria or fungi, can act as signaling molecules and affect cell proliferation or modify root system architecture by overproduction of lateral roots and root hairs with a subsequent increase of nutrient and water uptake (Ortíz-Castro et al. 2009). Of these two groups of compounds, the production of cytokinins by plant growth-promoting rhizobacteria has been well documented and correlated with an increased growth in plants (Nieto and Frankenberger 1990; Arkhipova et al. 2005; Ortíz-Castro et al. 2009). Cytokinins are also key regulators of intricate plant–microbe–insect interactions and contribute to plant growth-defence tasks of facing both mutualists and invaders (Giron et al. 2013). The synthesis of the other group of phytohormones namely auxins can take place via a diverse group of

bacteria during multiple pathways of their metabolism. These auxins include indole-3-acetic acid (IAA), the major naturally occurring auxin, indole-3-butyric acid (IBA), phenylacetic acid, 4-chlorindole-3-acetic acid, or their precursors (Spaepen et al. 2007). IAA in particular acts as a reciprocal signaling molecule in bacterial–plant interactions and some PGPR stimulate root proliferation by IAA biosynthesis (Lambrecht et al. 2000). As reviewed by Ludwig-Müller (2015), auxins also play a dual role in plant developmental processes; stimulation of cell division and cell elongation in healthy plants, and defence mechanisms by acting as defence molecules with antimicrobial activity. Many fungal species also produce auxins. Based on recent evidences, it has been suggested that fungi may use IAA and related compounds to interact with plants as part of its colonization strategy, which could primarily result in plant growth stimulation and modification of basal plant defence mechanisms (Ortíz-Castro et al. 2009). In a similar manner, bacterial volatiles such as acetoin and 2,3-butanediol produced by certain PGPR can be used as plant–bacteria communicators and as aforesaid plant growth promotion triggers (Ortíz-Castro et al. 2009). In addition to these compounds, some organic acids (e.g. citrate, oxalate, and malate) play a central role in aluminium tolerance mechanisms, such as the detoxification of aluminium in the plant rhizosphere by releasing organic acids that chelate aluminium (Ma et al. 2001).

Long-term close interactions between different biological species, such as symbiosis and pathogenesis, are common between plants and soil microorganisms. Among the astounding number of such mutualistic associations, the legume–rhizobia nitrogen-fixing symbiosis of plant–bacterial nature and that of mycorrhizae of plant–fungal nature are well documented. Such relationships rely largely on various signaling molecules to ensure their sustenance. For example, phenolic acids, the main polyphenols made by plants, carry out diverse tasks which include acting as signaling molecules in the initiation of legume–rhizobia symbioses, establishment of arbuscular mycorrhizal symbioses and acting as agents in plant defence mechanisms (Mandal et al. 2010). An assortment of secondary plant compounds such as flavonoids and strigolactones, the latter of which is excreted by roots, also carry importance as signaling molecules in several symbiotic and pathogenic plant–microbe interactions (Steinkellner et al. 2007). For example, plant roots release flavonoid compounds, which signal rhizobia to produce lipooligosaccharide, and details on rhizobia–legume communication and signal transduction pathways have been described by Garg and Chandel (2010). Also, in response to the secretion of signal molecules recognized to be plant hormones known as “strigolactones”, arbuscular mycorrhizal fungi penetrate and colonize plant roots (Haichar et al. 2014).

In nature, microbes tend to function as functional guilds or communities, sometimes comprising of billions densely packed cells. Biofilms, one such group of communities, are adherent cells embedded within a self-produced matrix of extra cellular polymeric substance (EPS). Coordination of metabolic interactions among such biofilms is known to occur predominantly through quorum sensing (Reading and Sperandio 2006). Quorum sensing is a form of cell-to-cell communication between bacteria mediated by small diffusible signaling molecules called autoinducers that increase concentration as a function of cell density; these generally vary

depending on the nature of bacteria as *N*-acyl-L-homoserine lactones (AHLs) for Gram-negative bacteria (Ortíz-Castro et al. 2009) and peptide-signaling molecules for Gram-positive bacteria (Walker et al. 2003). These bacteria also have a receptor that can specifically detect the autoinducer. When the microbial population grows the inducer reaches a threshold concentration activating the receptor which then causes specific genes to begin transcriptional activities at approximately the same time. These activities enable intercellular signals of a bacterial population to control the expression of genes in response to cell density. This coordinating behaviour of bacteria can be useful in a variety of situations. For example, quorum-sensing systems possessed by both Gram-negative and -positive bacteria, including important plant pathogenic bacteria such as *Erwinia* spp., *Pseudomonas* spp., and *Agrobacterium* spp., can control the expression of several genes required for pathogenicity as reviewed in Fray (2002).

AHLs play an important role in the quorum sensing of different species. For instance, it is used for regulating diverse behaviours in rhizosphere inhabiting bacteria where in some situations plants may produce their own metabolites which may interfere with quorum-sensing signaling (Ortíz-Castro et al. 2009). The responses to AHL also vary, where bacteria respond to AHLs via biofilm formation, production of virulence factors, and symbiosis with plants. On the other hand, the plant recognizes AHLs and responds by altering gene expression in roots and shoots, thereby modulating defence and cell metabolism, root architecture, hormone responses, protein processing, and cytoskeletal organization (Ortíz-Castro et al. 2009).

Apart from the customary cell-to-cell communication via quorum sensing, it has been reported recently that electrical signals like potassium ion-channelling can be used to coordinate metabolism and to communicate within the biofilm (Beagle and Lockless 2015; Masi et al. 2015; Prindle et al. 2015). Further, Turrà et al. (2015) reported that the catalytic activity of secreted class III peroxidases triggered directed growth of the soil-inhabiting plant pathogen *Fusarium oxysporum* towards the roots of the host plant tomato (*Solanum lycopersicum*). Thus, this wide array of signaling molecules and their specific functions within bacterial communities diversify the relationships in plant–microbe interactions, and their role within the soil food web can be further explored.

1.3 Microbial Coordination of Complex Network Interaction Within Soil Food Web and Plant–Microbe Interactions

It is a known fact that soil bacterial communities use species-specific quorum-sensing signals or auto-inducers to coordinate gene expression within them, according to the density of their local population. However, subsequent findings have identified non-species-specific auto-inducers that are capable of mediating intra- and inter-species communication among different bacteria (Galloway et al. 2012). The role of these autoinducers has been demonstrated by plant defence responses and root development (Bai et al. 2012). Some higher plants on the other hand were

shown to have interactions between them and soil bacteria by the production of bacterial auto-inducers or signal-mimic compounds (Teplitski et al. 2000). Berdy (2005) reported that endophytic microbes in higher plants are responsible for producing such mimic compounds and other metabolites. It is therefore evident that endophytes are bound tightly to biosynthetic pathways of secondary metabolites in the hosts. Plants can also detect molecules produced by potential pathogens and activate pathogen-response systems thus placing plant defence mechanism to be a common role of the secondary metabolites in plants. As such, we suggest that there could be a close communication between the plants and their endophytes for producing the secondary metabolites when the need arises, for example, in the case of a pest or pathogen attack. The flavonoid pathway in plants produces a diverse array of compounds with functions which include defence mechanisms against pathogens, signaling in symbiosis, auxin transport regulators, and roles as antioxidants and pigments (Hassan and Mathesius 2012). An example for such signaling functions is seen in alfalfa, where chemotaxis towards the host plant by symbiotic *Sinorhizobium meliloti* has been reported to mediate from the sensing of proline secreted by roots (Webb et al. 2014).

In the presence of host plant physiological stress, many eukaryotic signal molecules are released and detected by Gram-negative pathogenic bacteria which respond by adapting their physiology for virulence (Lesouhaitier et al. 2009). The two conflicting demands balanced by plant immune signaling networks in pathogenesis have been identified to be vigour against pathogenic perturbations and moderation of negative impacts of immune responses on plant fitness (Sato et al. 2010). Recent findings provide further evidence for the important role of microbial signaling, showing intriguing complex interactions mediated by signaling among plant–insect–microbe relationships. This can be seen in the aphid-mediated plant immunity against pathogen infection, where in particular the priming of defence responses against different pathogens through hormonal signaling has been found to help prepare the plant for subsequent pathogen attacks (Lee et al. 2012). Another exciting study reports that plants can exploit common mycorrhizal networks in the soil for herbivore-induced defence signal transfer and interplant defence communication to activate defence responses more rapidly and aggressively upon insect attack and to increase their insect resistance (Song et al. 2014). Thus, it is clear that it is the microbes living in association with plants and also in the soil that contribute to ecosystem balance through signaling in complex network interactions.

1.4 Regulation of Microbial Signalling Compounds by Biotic and Abiotic Factors

Organisms challenged by a change in the environment can respond to that by secreting common signaling compounds (Smith et al. 2015). The activation, alteration, diminution, or termination of some signaling components in organisms can be regulated by numerous biotic and abiotic factors. These factors include a complex

matrix of plant–microbe and microbe–microbe communications and various environmental changes, as shown in this section.

Altered temperature (Schwinghamer et al. 2015) and water stress (Prudent et al. 2015) are two main factors leading to release signalling compounds. The architecture of the root system, for example, undergoes modification by its endogenous auxin levels and by environmental stimuli such as the availability of water and mineral nutrients (López-Bucio et al. 2003; Pérez-Torres et al. 2008). Plant rootlets starved of soil nitrogen have been observed to secrete small peptides that are translocated to the shoot and received by specific receptors so that the signaling from the root to the shoot helps the plant adapt to fluctuations in local nutrient availability (Tabata et al. 2014). In this instance, the signaling may induce the action of endophytic nitrogen fixers for compensating the deficiency in supply of soil nitrogen to the plant (Seneviratne 2015).

Moreover, in response to high doses of UV-B radiation, an induction of signaling molecules such as abscisic acid (ABA), nitric oxide (NO), and calcium ions (Ca^{2+}) in plant and animal cells can occur to bring about stress tolerance (Tossi et al. 2012). It has also been reported by Flores et al. (1999) that an insecticidal defence response can be created by UV light penetrating soil layers leading to photo-activation of fluorescent β-carboline alkaloids secreted by oca roots. Another example of such a response has been reported by Ma et al. (2001) in maize and wheat, where the exudation occurs of some known signaling molecules such as citrate, malate, and related organic acids in response to high Al^{3+} concentrations. Further, significant physiological functions are implemented by NO that modulates the activities of cellular and extracellular proteins in various groups of organisms (Medinets et al. 2015). Nitric oxide can further play a signaling function to enhance microbial biofilm formation in the soil (Medinets et al. 2015), which renders numerous biochemical and physiological benefits to plant growth (Qurashi and Sabri 2012).

Beneficial soil bacteria confer immunity against a wide range of foliar diseases by activating plant defences, thereby reducing a plant's susceptibility to pathogen attack. This is clearly seen in the reporting of root secretions of L-malic acid which is induced by the foliar pathogen *Pseudomonas syringae* pv *tomato* and in the elevated levels of L-malic acid, which promote binding and biofilm formation of beneficial rhizobacterium *Bacillus subtilis* on Arabidopsis roots (Rudrappa et al. 2008). In addition, it has been observed that biofilm formation improves soil fertility through aggregate formation (Qurashi and Sabri 2012) and carbon storage (Seneviratne et al. 2011), which in turn govern the sustainability of the soil ecosystem.

1.5 Signalling Pathways in Soil Food Web Improve Ecosystem Functioning and Sustainability

Microbes play an important role in chemical signaling in plant–microbe interaction as discussed above. Thus, microbial signaling pathways in soil and plant–microbe interactions improve ecosystem functioning and sustainability (Fig. 1.1).

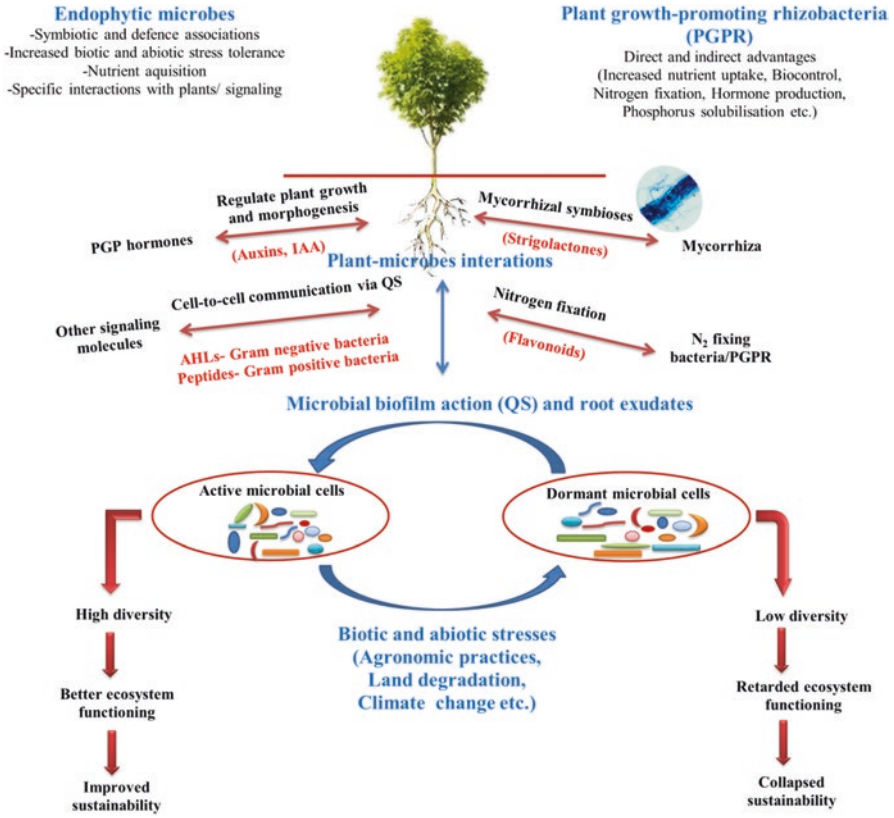

Fig. 1.1 Microbial signaling pathways in soil and plant–microbe interactions improve ecosystem functioning and sustainability in the long term

Beneficial microbes in plant and rhizosphere, such as endophytes and plant growth-promoting rhizobacteria (PGPR) stimulate plant growth though a wide range of mechanisms. Some direct and indirect advantages of PGPR and endophytes include: (1) increased nutrient uptake, (2) biocontrol, (3) nitrogen fixation, (4) hormone production, (5) phosphorus solubilization, (6) facilitation of symbiotic and defence associations, (7) increased biotic and abiotic stress tolerance, etc. (Smith et al. 2015).

On the other hand, root exudates of plant origin, which act as signaling molecules in plant–microbe interactions, are one of the key drivers of soil microbial community composition, diversity, and functional richness within the rhizosphere. Plant–microbe interactions in the rhizosphere create a highly structured and active microbial community and this ultimately leads to better ecosystem functioning and improved sustainability.

The knowledge that microbial signaling play as an integral part in plants and animals has also been confirmed (Seneviratne 2015). Microbes in macro-organisms provide their metabolic activity producing an amazing diversity of compounds and

signaling molecules for nutrition, protection, and development of the hosts (Selosse et al. 2014). However, the functional role of microbes-mediated chemical signaling in soil ecosystem sustainability is less documented. Also, the understanding of how these molecules contribute to soil ecosystem stability and sustainability through inter- and intra-species chemical signaling is limited. Seneviratne (2015) established an idea of the linkage between ecosystem sustainability and microbial chemical signaling, thereby introducing a new term edaphic ecosystem signal transduction (EST). The EST is defined as chemical signaling from signaling molecules to trigger a change in the activity or state at and within the edaphic ecosystem, considering the ecosystem to be a single unit like a cell. According to this concept, the EST is mediated by receptors of microbes in soil, plants, and animals of the ecosystem through a cascade in signal-receptor-process-response, thus leading to maintenance of a delicate balance among the interacting counterparts in the edaphic ecosystems. The resultant response could be a signal which again triggers receptors of other counterparts of the ecosystem, thereby instituting a signaling network.

However, when the signaling network is disrupted due to human impact, the sustenance of soil ecosystems collapses {e.g. chemical inputs in agricultural practices (Fox et al. 2007), tillage, and global change}. This can be seen in the example of nitrogen fixers who play a key role in the growth and persistence of effective microbial communities in the soil by supplying nitrogen through biological nitrogen fixation (Seneviratne et al. 2011). Here, a disruption of signaling networks occurs upon the use of nitrogenous fertilizers in agriculture and forestry which lead to a suppression of the action of microbes, particularly nitrogen fixers in agroecosystems. This tends to produce nitrogen-deficient, weak microbial communities with low biomass and activity, due to diminished nitrogen supply from the repressed nitrogen fixers, which paves the way to collapsed microbial diversity and ecosystem functioning. Under this circumstance, two negative impacts can be observed in the ecosystem, namely, (1) reduced soil fertility and organic matter build up (Scholes and Scholes 2013; Bi et al. 2015) leading to low soil moisture retention, and hence drought stress, and (2) yield decline (Kumar and Yadav 2001), possibly due to lack of rhizoremediation, resulting in phytotoxin accumulation (Dams et al. 2007), which are prevalent in collapsed sustainability. These effects can be minimized or restored by manipulating soil microbial diversity in the ecosystems for re-establishing communication through EST, but not solely from nutrients and water management, as suggested in conventional agriculture and forestry (Seneviratne 2015).

1.6 Technical Advances in Identifying Signalling Pathways in Soil-Plant System

The rhizosphere, the soil in the immediate vicinity of growing plant roots, is a complex, structured, and dynamic system with myriads of microorganisms. The relative abundance of particular microbial species that interact with plants can be shifted within different plant species and their genotypes, and also in response to both

abiotic and environmental factors. Therefore, assessment of relative abundance, taxonomic diversity, and functions of plant associated microorganisms are vital to reveal unknown signaling pathways between plant and soil microbes. This can be achieved successfully through an integrated approach of currently available molecular tools. For example, recent advances in multi-omics techniques, particularly in analytical capabilities (NMR, GC-MS), that aid detection of metabolites in environmental sample at low concentrations can be employed to improve our knowledge on microbial signaling. Whilst a detailed review of the techniques is out of scope for this chapter, we provide an overview of the techniques and its application to study plant–microbe interactions.

Majority of the microorganisms in the environment are recalcitrant to isolate and grow in the laboratory and thus cultivation-dependent techniques offer a very limited view of the microbial diversity and function. The advent of cultivation-independent techniques, i.e. nucleic acid-based evaluation of microbial diversity initiated a paradigm shift in our understanding of the microbial world. These cultivation-independent techniques rely on the extraction of nucleic acids directly from the environmental samples, such as soils, followed by either amplicon or shotgun sequencing to profile the microbial community. Traditionally, amplicon sequencing was performed on PCR-amplified marker genes using specific set of primers targeting either the 16S rRNA gene or metabolic genes, to infer phylogeny. Recent advances in sequencing chemistries has enabled researchers to use high-throughput sequencing (HTS), which provides an advantage over traditional Sanger sequencing and have resulted in a better understanding of microbial functional diversity in the environment, particularly plant–microbe interactions (Knief 2014). Amplicon sequencing is a cost-effective HTS technique to assess the taxonomic diversity of microbial community in an environment (Di Bella et al. 2013). HTS of marker genes has been used for characterization of the microbial community composition in phyllosphere and rhizosphere (Jiang et al. 2013; Bokulich et al. 2014; Bulgarelli et al. 2015) and to address key questions such as whether or not plant taxa select their microbial community composition (Delmotte et al. 2009; Bokulich et al. 2014) and how microbial community composition differs among different plant compartments (Bodenhausen et al. 2013; Ottesen et al. 2013).

Shotgun sequencing of the community DNA, i.e. metagenome, allows the researchers to not only identify the taxonomic diversity, but also to access the metabolic blueprint of the microbial community, i.e. genes and their functionalities (Di Bella et al. 2013). Moreover, shotgun metagenomes eliminate primer bias, i.e. sequencing without the need for targeting and amplifying a particular marker gene (Poretsky et al. 2014). Researchers have employed shotgun approaches to characterize phyllosphere microbial communities in rice, clover, soybean, and tomato (Delmotte et al. 2009; Atamna-Ismaeel et al. 2012; Knief et al. 2012; Ottesen et al. 2013). Using shotgun sequencing, Mendes et al. (2014) reported that rhizosphere microbiome in Soybean is a subset of the microorganisms observed in the bulk soil.

Global metagenome datasets are available in public repositories (JGI, MG-RAST, NCBI) and can be used to mine for genes that are identified to be involved in microbial signaling pathways. Whilst metagenomes can provide us information on the

genes which do not necessarily indicate activity. Transcriptomics allows the analysis of messenger RNA (mRNA) molecules, or gene transcripts, produced by an isolate or a specific gene (targeted transcriptomics) or a microbial population in an environment (meta-transcriptomics) at a specific developmental stage or physiological condition (Wang et al. 2009; Zhang et al. 2010). The mRNA represents the template for protein synthesis and the genes that respond the environmental stimuli are actively expressed and are reflected in the transcriptome (Horgan and Kenny 2011). Advances in the RNA sequencing techniques (RNA-seq) have significantly transformed the analysis of microbial transcriptomes (Croucher and Thomson 2010). RNA-seq can be used to determine gene expression levels and their dynamics across different microbial cells or induced environmental stimuli. Understanding microbial signaling pathways, in isolates or in co-culture experiments, will be aided by this technique by comparing up- or down-regulated genes in response to a specific treatment or stimuli and identification of specific groups of molecular processes, in this case genes that are involved in signaling pathways (Wit et al. 2012).

Proteins can also be used as biomarkers for biological functions as they represent the activity of metabolic reactions and provide more information on specific microbial processes (Keller and Hettich 2009). With recent advances in protein extraction techniques from environmental samples, proteomic techniques, and databases, it is now feasible to identify at least 50–70 % of a predicted bacterial proteome (Keller and Hettich 2009; Branca et al. 2014; Meyer et al. 2014; Yang et al. 2015). Whilst a strong correlation between mRNA expression levels and protein abundance can be assumed (Zhang et al. 2010), several studies have reported that it is not the case (Taniguchi et al. 2010; Vogel and Marcotte 2012). Therefore, it is essential to use transcriptomics and proteomics in tandem to obtain insights into microbial functions (Zhang et al. 2010). Whilst the metaproteome represents the composite of all proteins recovered from an environmental sample, metaproteomics has been applied to profile microbial community and function in various environmental samples, for example, soils (Bastida et al. 2014; Wang et al. 2010), freshwater (Habicht et al. 2011; Hanson et al. 2014), marine environment (Sowell et al. 2011; Stokke et al. 2012), and plants (Delmotte et al. 2009; Knief et al. 2012).

Further, metaproteomics can be used to study plant–microbe interactions, yet certain technical challenges remain in the separation of plant and microbial materials (Delmotte et al. 2009; Knief et al. 2012), as the microorganisms associated with the plants may not be well characterized (both isolates and the availability of isolate genomes). Therefore, protein identification depends on either the availability of whole genomes from related microbes or a metagenome from the same sample. Using metagenome in tandem to metaproteome significantly enhances the identification of peptides and its phylogenetic affinity (Delmotte et al. 2009; Knief et al. 2012). Thus, in the future, metaproteogenomic (combining metaproteomics and metagenomics) will be an integral part of researchers' tool kit intending to unravel the impact of microbial signaling on ecosystem health and productivity.

Environmental metabolomics is the application of metabolomics to analyse endogenous and exogenous low molecular mass metabolites in the environment to study organismal interactions as reviewed in Lankadurai et al. (2013).

Metabolomics is an excellent platform to detect metabolites, i.e. signaling molecules in the environment and understand the impact of environmental stimuli on microbial signaling. Researchers have used metabolomics to understand the response of microbes to external stress such as heavy metal or organic pollutants (Marles-Wright and Lewis 2007; Ye et al. 2012).

1.7 Conclusions and Future Perspectives

A better understanding of microbial signaling pathways is a key to success in manipulating beneficial plant–microbe association for better nutrient management (e.g. biofertilizers), suppression of pathogens (biopesticides), and crop stress alleviation which paves the way to environmental sustainability. Soil microbes maintain a complex interaction with other micro- and macro-organisms in the soil food web and plants via various signaling mechanisms. These communications are vital for nutrient assimilation, development, and activation of defence mechanisms in positive microbe–plant interactions. In addition, plant stress responses play an important role in the release of signaling compounds in the rhizosphere, and a better understanding of the relationship between environmental plant stresses and signaling could help in developing technologies that utilize plant signaling in crop stress alleviation (Barea 2015; Smith et al. 2015). Further, variable environmental factors may account for some of inconsistencies observed in field trials, and hence a more complete understanding of how plant–microbe communication is influenced by environmental factors would be beneficial (Smith et al. 2015). Recent advancements in molecular biology including the development of next generation sequencing approaches (e.g. such as metatranscriptomics, metagenomics, proteomics, metabolomics) means it is now possible to understand these microbial signaling mechanisms based on common genes, signalling pathways, and systems in a variety of ecosystems. Therefore, there is an increasing interest in the use of "multi-omics" approaches leading to improved mechanistic models of microbial community structure and function across soil ecosystem and plant–microbe interactions. This helps to upgrade our current knowledge in microbial signaling pathways and the factors that regulate the signalling in the soil ecosystem. This understanding can be used to manipulate the beneficial associations in disturbed ecosystems like croplands, particularly in agroecosystems where chemical inputs weaken the interactions through collapsing the signaling networks, consequently breaking the delicate balance of the ecosystem. Thus, bridging the knowledge gaps in microbial signaling in soil and plant–microbe interactions can no doubt lead to sustainable agricultural practices by developing more effective, low-cost, and eco-friendly agricultural practices.

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Chapter 2
Exploiting Beneficial Traits of Plant-Associated Fluorescent Pseudomonads for Plant Health

Anuradha Rai, Pradeep Kumar Rai, and Surendra Singh

Abstract Plants have recently been recognized as meta-organisms harboring distinct microbiome and reveling close symbiotic relationship with the associated microflora. Each plant has a unique niche and possesses species-specific microbes to a certain proportion and majority of the ubiquitous microbes that fulfill important host as well as ecosystem function. Currently, agricultural crops are facing challenges due to imbalance of micronutrients, deterioration of soil health, fluctuating environmental conditions, and increasing pest and pathogen attack. The rhizosphere region of the plants is the most extensively studied area due to its remarkable microbial diversity. Fluorescent pseudomonads are Gram-negative, motile, rod-shaped bacteria predominantly inhabiting the vicinity of rhizosphere and sometimes even the root interior. They effectively colonize the plant roots and rhizosphere soil because of their excellent ability to utilize a variety of organic substrates exuded by the plant roots. The study on the role of fluorescent pseudomonads in agriculture has been a matter of great interest attributable to their ability to control plant diseases, maintain soil health, and influence the plant growth directly or indirectly. They directly promote the plant growth by producing secondary metabolites such as siderophores and phosphatases that can chelate iron and solubilize phosphorus, respectively, from the soil and make them available to the plants. They also produce indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase that sequesters ACC, the precursor of ethylene. They also indirectly promote the plant growth mainly by suppressing the plant pathogens by producing an array of antibiotics and fungal cell wall degrading enzymes. Specific metabolites produced by fluorescent pseudomonads may elicit defense reactions and induce systemic resistance of the host plants. Introduction of such multifunctional rhizobacteria

A. Rai • P.K. Rai • S. Singh (✉)
Centre of Advanced Study in Botany, Banaras Hindu University,
Varanasi 221005, Uttar Pradesh, India
e-mail: surendrasingh.bhu@gmail.com

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to the plant roots can lead to increased plant growth and protection against phytopathogens. This chapter reviews the beneficial traits of the fluorescent pseudomonads and their relationship to the functioning in the rhizosphere.

Keywords PGPR • Micronutrient • Medicinal plants • Fluorescent *Pseudomonas* • Rhizosphere

2.1 Introduction

Photosynthetic plants play a key role in ecosystems functioning (Hartmann et al. 2009). In plant-based ecosystems, they contribute to the establishment of unique microbial ecological niches. Recently, plants have been recognized as meta-organisms harboring distinct microbial community and reveling close symbiotic relationship with the associated microflora. Hence, such a microbial community associated with plant roots can be referred to as a microbiome (Chaparro et al. 2013). Each plant has a unique niche which possesses species-specific microbes to a certain proportion, and majority being the ubiquitous microbes that fulfill important host as well as ecosystem function. Plant roots are colonized by an outstanding number of bacteria. These bacteria have profound effects on plant growth and development and are known as plant growth promoting rhizobacteria (PGPR) (Kloepper et al. 1980). PGPR are heterogeneous in nature comprising bacteria, fungi, and actinomycetes that survive in and around the rhizosphere (Singh 2013, 2014). They colonize the root surface as well as interior of the roots (Gray and Smith 2005). PGPR function in three different ways: by synthesizing particular compounds for the plants, facilitating the uptake of certain nutrients from the soil, and lessening or preventing the plants from diseases. Bacteria of diverse genera have been identified as PGPR, of which *Bacillus* and *Pseudomonas* spp. are predominant (Podile and Kishore 2006). It is a well-established fact that only 1–2 % of bacteria promote plant growth in the rhizosphere (Antoun and Kloepper 2001).

In recent years, fluorescent pseudomonads have drawn the attention worldwide owing to their ability to produce secondary metabolites such as antibiotics, volatile compounds, hydrogen cyanide (HCN), siderophores, cell wall degrading enzymes, and phytohormones (Bakker and Schippers 1987; O'Sullivan and O'Gara 1992; Nielsen et al. 2000). Plant-associated *Pseudomonas* can be categorized into beneficial, deleterious, and neutral groups on the basis of their effects on plant growth although they may colonize the same ecological niche (Dobbelaere et al. 2003). Beneficial fluorescent pseudomonads include *Pseudomonas putida*, *Pseudomonas chlororaphis*, *Pseudomonas aureofaciens*, *Pseudomonas aeruginosa*, and *Pseudomonas syringae*. Fluorescent pseudomonads are diverse group of aerobic, gram-negative, chemoheterotrophic, and rod-shaped bacteria. They are characterized by production of water soluble yellow-green fluorescent pigments pyoverdins or pseudobactins under low iron condition. All fluorescent pseudomonads fall into one of

the five rRNA groups. The G + C content of their DNA ranges from 58 to 68 mol% (Palleroni et al. 1973). They are heterogenous and ubiquitous in nature. They have simple nutritional requirements and are well adapted to numerous ecological niches (Stanier et al. 1966). Their universal distribution suggests a remarkable degree of their physiological and genetic adaptability (Spiers et al. 2005). Most species are saprophytic and commonly found in water and soil, and are used in biotechnological applications to improve plant growth and/or plant health and in the bioremediation of agricultural pollutants (Ramamoorthy et al. 2001). They are aggressive root colonizers of different crops and have a broad spectrum of antagonistic activity against a wide group of phytopathogens. Fluorescent pseudomonads are very common, diverse, and well-studied PGPR. *P. fluorescens* biovar III has been reported as the dominant group of bacteria among fluorescent pseudomonads associated with rhizosphere of rice (Sakthivel and Gnanamanickam 1987). Few members of this genus are associated with animal, plant and human diseases, and food spoilage. Fluorescent pseudomonads have been isolated from the rhizosphere of various crops and medicinal plants owing to their ability to utilize diverse organic substances and mobility. They have the ability to produce indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase that sequesters ACC, the precursor of ethylene. They reside in the rhizospheric region and at times in the root interior. This review focuses on the plant-beneficial fluorescent pseudomonads interaction and its effect on the plant growth promotion and biocontrol potential. Both the aspects confer this bacterial group as an alternative to agrochemicals, which fit environment-friendly strategies to be implemented in a modern sustainable agriculture framework.

2.2 Rhizosphere and Plant–Microbe Interaction

The term rhizosphere was first defined by Lorenz Hiltner as “the soil compartment influenced by the root” (Hiltner 1904). The rhizosphere is a hot spot for numerous organisms and is influenced by plant roots, and considered as one of the most complex ecosystems on Earth (Pierret et al. 2007; Jones and Hinsinger 2008; Raaijmakers et al. 2009; Singh et al. 2016a, b). From decades, it is known that an association exists between microbes and plants (Singh 2015a, b). All beneficial traits that fluorescent pseudomonads provide to plants would be useless if some fundamental requirements are not fulfilled (Singh et al. 2011a, b, c). They must be efficient root colonizers. They must persist, multiply, and compete with other microbiota. Plant roots synthesize, accumulate, and secrete a diverse array of compounds called root exudates (Walker et al. 2003). These compounds act as chemoattractants as well as repellents for a large number of heterogenous, diverse, and actively metabolizing soil microbes (Badri and Vivanco 2009). Root exudates are an immediate source of carbon, nitrogen, and energy. A wide range of chemical compounds of root exudates modifies physicochemical properties of the soil and regulates the composition of soil microbial community (Dakora and Phillips 2002). The chemical composition of

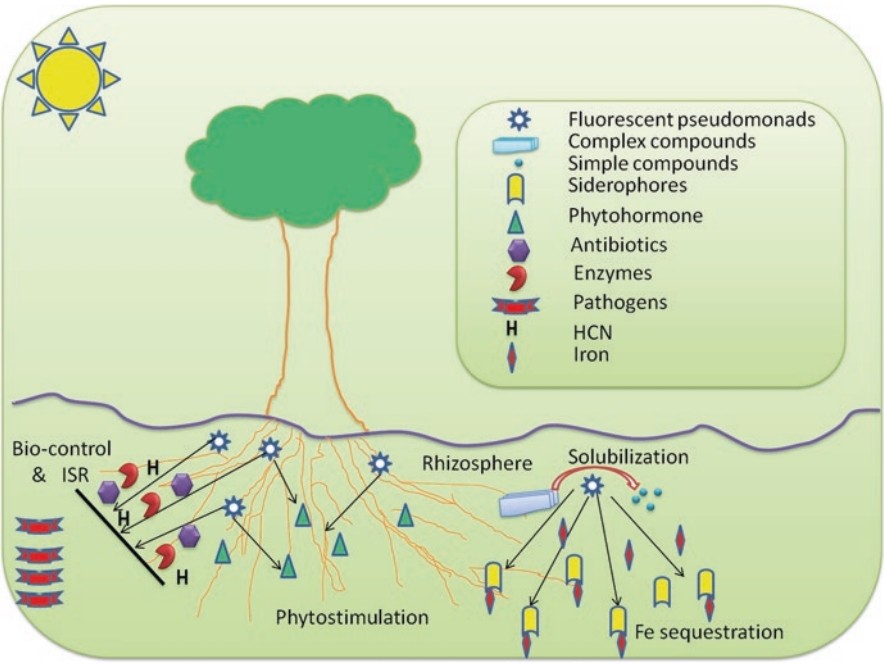

Fig. 2.1 Diagrammatic representation of different mechanisms of fluorescent pseudomonads

the root exudates also depends on plant species and microbes (Kang et al. 2010). Bacteria compete with each other and with other soil microorganisms for these carbon resources. In addition, severe competition between microorganisms in the rhizosphere involves specific communication between microorganisms, including quorum-sensing (QS) and complex mechanisms that modulate it (Faure et al. 2009). Plants can produce and secrete various compounds that mimic QS signals of bacteria and, thereby, alter bacterial activities in the rhizosphere (Bauer and Mathesius 2004).

In addition to chemotactic factors, bacterial lipopolysaccharides (LPS) and pilli can also contribute to root colonization in several PGPR. Fluorescent *Pseudomonas* chemotactically reaches root surfaces through flagella motility (De Weger et al. 1987; Turnbull et al. 2001a, b; De Weert et al. 2002). Different mechanisms of fluorescent pseudomonads in the rhizosphere have been diagrammatically represented in Fig. 2.1, and they are self-explanatory. Besides the flagella motility, O-antigenic side chain of LPS of *P. fluorescens* PCL1205 contributes to a major role in tomato root colonization (de Weger et al. 1989; Dekkers et al. 1998). *P. fluorescens* WCS365 strain isolated from potato (*Solanum tuberosum* L.) is a good colonizer of both potato (Brand et al. 1991) and tomato (*Lycopersicon esculentum* L.) (Simons et al. 1996) roots. The root colonization genes (*rhi*) associated with the colonization of *P. fluorescens* are specifically expressed by means of in vivo expression technology (IEVT) (Bloemberg and Lugtenberg 2001).

Microbes residing inside the plants go one step forward in their colonization ability compared to rhizospheric bacteria and almost all plants harbor endophytic bacteria (Singh and Singh 2013; Singh and Strong 2016; Singh and Gupta 2016; Singh et al. 2016a, b). Beneficial effects of *Pseudomonas* in combination with other endophytic bacterial genera have been reported in soybean (Kuklinsky-Sobral et al. 2004), rice (Adhikari et al. 2001), oilseed rape, tomato (Nejad and Johnson 2000), and hybrid spruce (Chanway et al. 2000).

2.3 Mechanisms of Plant Growth Promotion

PGPR enhance the plant growth and yield either directly or indirectly, without conferring pathogenicity (Hariprasad and Niranjana 2009). Indirect plant growth promotion includes the prevention of the deleterious effects of phytopathogenic organisms. This can be achieved by the production of siderophores, hydrogen cyanide (HCN), antibiotics, and fungal cell wall degrading enzymes, e.g., chitinase and ß-1, 3-glucanase. Direct plant growth promotion includes production of phytohormones and volatile compounds, nitrogen-fixation, and mineral nutrient solubilization that affect the plant signaling pathways. Whereas bacterial genera such as *Pseudomonas*, *Azospirillum*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Alcaligenes*, *Acinetobacter*, and *Flavobacterium* have been studied and used as PGPR inoculants, bacteria belonging to genera *Bacillus*, *Streptomyces*, *Pseudomonas*, *Burkholderia*, and *Agrobacterium* are, however, predominantly studied and increasingly marketed as biological control agents.

2.3.1 Phosphate Solubilization

Phosphorus (P), the second most important plant growth limiting mineral nutrient next to nitrogen, is present in the form of insoluble phosphates which cannot be utilized by the plants (Pradhan and Sukla 2006). Plants absorb P in two soluble forms, the monobasic [phosphoric acid (HPO_4^{2-})] and the dibasic [dihydrogen phosphate ($H_2PO_4^-$)] ions (Galleguillos et al. 2000). Indian soils are normally deficient in available phosphorus (Johri et al. 2003). Of the total P exists in a soluble form, only 0.1 % is available for plant uptake (Zhou et al. 2003) due to P fixation into an unavailable form. To overcome P deficiency in soils, available P level has to be maintained by adding chemical P fertilizers. Plants absorb only trace amounts of chemical P fertilizers whereas the rest is converted into insoluble complexes (Mckenzie and Roberts 1990). The regular application of phosphatic fertilizers poses adverse environmental impacts on soil health (Tilman et al. 2001), disturbing the microbial diversity. This has led to search for an ecologically safe and economically viable option for improving crop production in low P soils. Phosphate solubilizing microorganisms (PSMs) play an important role in supplying phosphate to

plants through various mechanisms of solubilization and mineralization. Among the different organic acids, gluconic acid production seems to be the most common mechanism of phosphate solubilization used by *P. fluorescens* (Di Simine et al. 1998). A considerable population of PSMs is present in the rhizosphere and secretion of organic acids and phosphatases is commonly involved in facilitating the conversion of insoluble forms of P to plant available forms. Bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Aereobacter*, *Flavobacterium*, and *Erwinia* have the ability to solubilize insoluble form of P to a form available to plants (Rodriguez and Fraga 1999). Among PSB, fluorescent pseudomonads spp. such as *P. chlororaphis*, *P. putida*, *P. aeruginosa*, *P. monteilii*, *P. plecoglossicida*, *P. fluorescens*, *P. fulva*, and *P. mosselii* have been identified as the most potent phosphate solubilizers (Gaur 1990; Cattelan et al. 1999; Bano and Musarrat 2003; Sunish Kumar et al. 2005; Ravindra Naik et al. 2008; Jha et al. 2009). *P. fluorescens* solubilizes $ZnPO_4$ in the presence of glucose as the carbon source (Di Simine et al. 1998). Das et al. (2003) has reported that cold-tolerant mutants of *P. fluorescens* were more efficient solubilizers of tricalcium phosphate than their respective wild-type counterparts at low temperatures. *P. fluorescens* strain Psd isolated from the rhizosphere of *Vigna mungo* also has a significant phosphate solubilization ability in addition to IAA production.

2.3.2 Phytohormones

Phytohormones are compounds that are produced by plants, and are involved in the developmental activities of plant like cell division, tissue differentiation, cell elongation, nutrients movements, apical dominance, ripening, and abscission. In addition to plants, fluorescent pseudomonads also produce various phytohormones such as auxins, gibberellins, cytokinins, and abscisic acid (ABA). Among these, IAA is involved in growth and development throughout the plant cell cycle, root initiation, apical dominance flowering, fruit ripening, senescence, and stimulation of plant growth (Xie et al. 1996). It has been found that about 80 % of the PGPR are involved in IAA production (Khalid et al. 2004; Patten and Glick 2002). Cytokinins produced by fluorescent pseudomonads (Vessey 2003) are involved in promoting the cell division, root development, and root hair formation (Frankenberger and Arshad 1995). Cytokinins are also involved in inhibition of auxin-induced apical dominance, prevention of senescence of plant parts mainly in leaves, chloroplast differentiation, and stimulation of opening of stomata (Crozier et al. 2001). Gibberellins synthesized by fluorescence pseudomonads induce cell elongation within the subapical meristem resulting in stem elongation (Dobbelaere et al. 2003), affecting seed germination, pollen tube growth, and development of reproductive part of various plants (Crozier et al. 2001). No reports are available regarding the ABA production by fluorescent pseudomonads; however, *Azospirillum* sp. and *Rhizobium* sp. are reported to produce this phytohormone (Dobbelaere et al. 2003). Interestingly, almost all species of bacteria synthesize ethylene (Primrose 1979). The synthesis of

Table 2.1 Hormones, siderophores, and enzymes produced by fluorescent pseudomonads

Hormones	Fluorescent pseudomonads	References
Auxins	*P. putida* GR12-2	Xie et al. (1996)
Cytokinins	*P. fluorescens*	Garcia de Salamone et al. (2001); Vessey (2003)
ACC deaminase	*P. fluorescens*	Wang et al. (2000)
Gibberellins	*Pseudomonas* spp.	Gutierrez-Manero et al. (2001)
Siderophores		
Pyoverdins	*P. fluorescens* WCS374	Mohammad et al. (2009)
Pyochelin	*P. aeruginosa*	Sun et al. (2006)
Pseudomonine	*P. fluorescens* WCS374	Mohammad et al. (2009)
Yersiniabactin	*P. syringae*	Jones et al. (2007); Petermann et al. (2008)
Quinolobactin	*P. fluorescens* 1740	Matthijs et al. (2007)
Achromobactin	*P. syringae* B728a	Berti and Thomas (2009)
Corrugatin	*P. fluorescens*	Matthijs et al. (2007)
Ornicorrugatin	*P. fluorescens* AF76	Matthijs et al. (2008)
Enzymes		
Chitinase	*P. stutzeri* YPL-1	Lim et al. (1991); Ayyadurai et al. (2007)
	P. aeruginosa P10	
β-1,3 glucanase	*P. cepacia*	Fridlender et al. (1993)
Laminarinase	*P. stutzeri* YPL-1	Lim et al. (1991)
Phosphatase	*P. mosselii* FP13	Jha et al. (2009)
Denitrifying enzymes	*P. aeruginosa* PUPa3	Sunish Kumar et al. (2005)
	P. aeruginosa FP10	Ayyadurai et al. (2006)
	P.aeruginosa FPB9, FPB15	Ravindra Naik et al. (2008)

ethylene is generally induced by wounding in plants and consequently it inhibits root growth development (Salisbury 1994). However, synthesis of ethylene induces ripening of fruits, senescence, development of adventitious root and root hair and breaks dormancy of seeds. Different strains of fluorescent *Pseudomonas* produce ACC deaminase, an enzyme that cleaves ACC, an immediate precursor of ethylene, resulting in the inhibition of ethylene production. The phytohormones produced by fluorescent pseudomonads are listed in Table 2.1.

2.3.3 Siderophores

Siderophores are water soluble, low molecular weight, organic compounds synthesized by many microorganisms under iron-deficient condition. These molecules show high affinity with ferric irons (Fe^{3+}) and form a stable chelate for transport into

the cell (Neilands 1981). Fe^{3+} is the most abundant form of iron in soil and an essential nutrient for the development of plants (Salisbury and Ross 1992). Fe^{3+} concentration along with ferric oxide hydrates is about 10^{-17} M in soil having neutral pH (Budzikiewicz 2010). However, rhizobacteria require iron concentrations higher than 10^{-6} M, and when its concentration declines below this level, they start producing siderophores (Miethke and Marahiel 2007). Various species of fluorescent pseudomonads produce fluorescent yellow siderophores such as pyoverdins (Budzikiewicz 1993, 1997), pseudomonine (Lewis et al. 2000; Mercado-Blanco et al. 2001), quinolobactin (Matthijs et al. 2007), pyochelin (Cox et al. 1981), and ornicorrugatin (Matthijs et al. 2008). These siderophores trap the limited iron in the rhizosphere and make it unavailable to deleterious fungi, resulting in the inhibition of fungal growth (Keel et al. 1992). Around 500 different siderophores with known structures have been reported (Boukhalfa and Crumbliss 2002) and many of them have been purified (Hider and Kong 2010). Siderophores produced by fluorescent pseudomonads are listed in Table 2.1.

2.3.4 Antibiotics

Fluorescent pseudomonads play an active role in the suppression of pathogenic microorganisms by secreting antibiotics. These antibiotics are low molecular weight organic compounds and are deleterious to the growth and metabolism of pathogenic microorganisms, even at low concentrations. Production of antibiotics by fluorescent pseudomonads is an important factor in the disease-suppressing ability of this group of bacteria (Thomashow et al. 1990). Antibiotics namely phenazines (Thomashow et al. 1990), pyoluteorin (Howell and Stipanovic 1980), 2,4-diacetylphloroglucinol (DAPG) (Shanahan et al. 1992), and pyrrolnitrin (Hammer et al. 1997) are produced by fluorescent pseudomonads. Some of the antibiotics have broad-spectrum activity and inhibit the growth of different groups of macro- and microorganisms. For example, DAPG produced by the fluorescent pseudomonads exhibits antibacterial, antifungal, and antihelminthic activities (Thomashow and Weller 1996). These antibiotics are grouped into nonvolatile and volatile antibiotics. Volatile antibiotics are alcohols, aldehydes, HCN, sulfides, and ketones. Nonvolatile antibiotics are DAPG, mupirocin, pyocyanin, phenazine-1-carboxylic acid, hydroxy phenazines (de Souza and Raaijmakers 2003), and phenylpyrrole (pyrrolnitrin) (Ahmad et al. 2008). Soilborne plant diseases are also suppressed by the introduction of antagonistic fluorescent pseudomonads into the rhizosphere. The extracellular secretion of antibiotics by pseudomonads also triggers the induced systemic resistance (ISR) in plants leading to protection from pathogens. Some important antibiotics produced by fluorescent pseudomonads are listed in Table 2.2.

Table 2.2 Some important antibiotics produced by fluorescent pseudomonads

Fluorescent pseudomonads	Antibiotics	Target pathogens	References
P. fluorescens F113	2,4-diacetylphloroglucinol	*Pythium* sp.	Shanahan et al. (1992)
P. fluorescens 2-79	Phenazines	*Gaeumannomyces graminis var. tritici*	Thomashow et al. (1990)
P. fluorescens	Pyoluteorin	*Py. ultimum*	Howell and Stipanovic (1980)
P. aureofaciens	Phenazine-1-caboxylate	*Sclerotinia homeocarpa*	Powell et al. (2000)
P. chlororaphis	Phenazine-1-carboxamide	*Fusarium oxysporum* f. sp. *radicis-lycopersici*	Chin-A-Woeng et al. (1998); Bolwerk et al. (2003)
P. fluorescens	Viscosinamide	*R. solani*	Nielsen et al. (2002)
P. fluorescens	Amphisin	*Py. ultimum* and *R. solani*	Andersen et al. (2003)
P. fluorescens	Aerugine	*Phytophthora, Colletotrichum orbiculare*	Lee et al. (2003)
P. fluorescens, P. cepacia	Pyrrolnitrin	*Rhizoctonia solani, F. sambucinum*	Hammer et al. (1997); Burkhead et al. (1994)
P. agglomerans EH318	Pantocin A, B	*Erwinia herbicola*	Wright et al. (2001)

2.3.5 Enzymes

Fluorescent *Pseudomonas* secretes several enzymes such as chitinases, proteases, pectinases, cellulases, xylanase, β-1, 3-glucanases, and some others, which can inhibit the growth and activities of pathogens. *P. fluorescens* CHA0 produces extracellular proteases and acts as an important biocontrol agent against the root-knot nematode disease caused by *Meloidogyne incognita* (Siddiqui and Shaukat 2005). *P. fluorescens* Pf-5 produces extracellular hydrolases and provides benefits to plant nutrition (Paulsen et al. 2005). Fluorescence pseudomonads strain, *Pseudomonas* GRC 2, produces chitinases (Gupta et al. 2001). This enzyme along with other secondary metabolites causes destruction and production of deformities of hypha, mycelia, and sclerotia of *Macrophomina phaseolina* and *Sclerotinia sclerotiorum. P. cepacia* produces β-1,3 glucanase which inhibits the growth and pathogenicity of *S. rolfsii*, *P. ultimum*, and *R. solani* (Fridlender et al. 1993). Rhizospheric fluorescent pseudomonads also produce cell wall degrading enzyme endochitinase which inhibits the pathogenicity of *R. solani* in sugar beet (Nielsen et al. 1998). In addition to enzyme production, *P. fluorescens* also induces the activity of laccase which is

involved in the pathogenicity of *R. solani* (Crowe and Olsson 2001). Enzymes produced by fluorescent pseudomonads are listed in Table 2.1. In addition to the above discussed enzymes, fluorescent pseudomonads also produce ACC deaminase, which helps in plant growth promotion by inhibiting the biosynthesis of ethylene in roots of plant, resulting in increase in the lengthening of root and root hairs. The production of ACC deaminase has also been reported from wild-type and genetically modified fluorescent pseudomonads (Ravindra Naik et al. 2008). ACC deaminase promotes the PGPR activity in *Arabidopsis thaliana* by reducing the ethylene concentration in root system (Desbrosses et al. 2009).

2.3.6 Hydrogen Cyanide

Hydrogen cyanide (HCN), a volatile compound which acts as an important biocontrol agent against plant pathogens, is produced by fluorescent pseudomonads (Rodriguez and Fraga 1999; Siddiqui 2006). HCN inhibits the cytochrome oxidase and metalloenzymes (Voisard et al. 1989) of pathogenic organisms and is highly toxic to all aerobic microorganisms at very low concentration. This helps fluorescent pseudomonads protect plants from soilborne diseases (Blumer and Haas 2000). *P. fluorescens* CHA0 produces HCN and suppresses the black root rot of tobacco caused by the fungus *Thielaviopsis basicola* and take-all disease of wheat caused by *G. graminis* var. *tritici* (Defago et al. 1990). HCN synthase is an important enzyme responsible for HCN production, and is encoded by three different genes *hcn* A, *hcn* B, and *hcn* C (Ramette et al. 2003). HCN causes death of the organisms by inhibiting electron transport resulting in loss of energy production inside the cell.

2.4 Induced Systemic Resistance

Development of a state of enhanced defensive capacity using external agents without modifying the genome of the plants is called induced systemic resistance (ISR) (Van Loon et al. 1998). These external agents may be a chemical or extracts of cells of living organisms or microorganisms (Romeiro 2000). The fluorescent pseudomonads and other PGPR have been reported to induce systemic resistance in the plants against bacterial, fungal, and viral diseases (Kloepper et al. 1996). ISR can be local or systemic and provides protection against a broad spectrum of phytopathogens (Jamsen 2000). Bacterial components such as LPS, flagella, siderophores, cyclic lipopeptides, 2, 4-diacetylphloroglucinol, homoserine lactones, and volatiles like acetoin and 2, 3-butanediol induce ISR in plants (Lugtenberg and Kamilova 2009).

The plant–microbe association involves molecular recognition between the two partners through a signaling network mediated by the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene. JA and ethylene have been described as

signal transduction molecules for ISR due to the effect of beneficial microbes, and the signal transduction pathway through SA accumulation is found in the systemic acquired resistance (SAR) induced by the attack of pathogens. The increased amount of SA, a putative resistance signal in leaves, is correlated with the root colonization of *P. fluorescens* CHA0 and its derivatives (Maurhofer et al. 1994). The application of PGPR results in several biochemical or physiological changes in plants. ISR-mediated enhanced resistance by PGPR is achieved by the accumulation of pathogenesis-related (PR) proteins and induction of defense compounds of the phenylpropanoid pathway. A correlation exists between the colonization of bean root by fluorescent bacteria and induction of PR proteins along with systemic resistance against *Botrytis cinerea* (Zdor and Anderson 1992). ISR triggered in some rhizobacterial strains depends on SA signaling in the plants. Induced resistance by *P. aeruginosa* 7NSK2 was found to be iron regulated and involved three siderophores, pyoverdin, pyochelin, and SA. SA is also a precursor in the production of SA-containing siderophores, such as pseudomonine in *P. fluorescens* WCS374 (Audenaert et al. 2002). *P. fluorescens* WCS417r-mediated ISR has been found effective against a wide range of pathogens, namely, *F. oxysporum* causing vascular wilts in *Arabidopsis* (Pieterse et al. 1996), *Alternaria brassicicola* and *Pseudomonas syringae* pv. tomato causing necrotic lesions in radish (Hoffland et al. 1996). *P. putida* 89 B–27 offered resistance against *Colletotrichum orbiculare* (Wei et al. 1991). Increased activity of penylalanine ammonia lyase (PAL) was observed in *P. fluorescens*-treated tomato and pepper plants in response to infection by *F. oxysporum* f. sp. *lycopersici* and *Colletotrichum capsici* (Ramamoorthy et al. 2001). PAL is the first enzyme involved in phenylpropanoid pathway and plays a key role in the biosynthesis of phenolics and phytoalexins.

2.5 Biological Control of Plant Pathogens

PGPR play a major role in the biocontrol of plant pathogens by suppressing a broad spectrum of bacterial, fungal, viral, and nematode diseases and also providing protection against viral diseases. Fluorescent pseudomonads are the most promising group of beneficial bacteria due to their multiple attributes for crop productivity and ability to suppress a wide variety of plant diseases. This specific group of bacteria could be used as prospective agents due to their ability to maintain soil health, promote plant growth, and suppress phytopathogens. Certain plant-associated fluorescent pseudomonads produced DAPG, a phenolic molecule that has antifungal, antibacterial, antihelminthic, and phytotoxic properties. *P. fluorescens* strain CHA0 suppressed black root rot of tobacco caused by *Thielaviopsis basicola* and take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* due to the production of DAPG (Keel et al. 1992). Several strains of fluorescent pseudomonads produce antifungal metabolites such as phenazines, a nitrogen-containing pigment having broad-spectrum antibiotic activity (Thomashow et al. 1997). Suppression of take-all disease of wheat by *P. fluorescens* strain 2-79 was mainly due to the production of antibiotic phenazine carboxylic acid (Thomashow et al. 1990).

Siderophores are low molecular weight molecules secreted by microorganisms with a high affinity for Fe^{3+}. Siderophores show antagonistic activity by sequestering iron from the environment and thereby limiting the iron availability for pathogens (Bakker et al. 1986; Loper and Buyer 1991). Lemanceau and Alabouvette (1992, 1993) have reported that the capacity to produce pseudobactin 358 by *P. putida* WCS358 is responsible for the suppression of *Fusarium* wilt of carnation. Apart from antibiotic production, fluorescent *Pseudomonas* produces several lytic enzymes that can hydrolyze a wide range of polymeric compounds and consequently suppress phytopathogenic fungi directly or indirectly (Martin and Loper 1999; Picard et al. 2000). Chitinase producing *Serratia plymuthica* C48 inhibited the spore germination and germ tube elongation in *B. cinerea* (Frankowski et al. 2001). HCN, a volatile compound produced by fluorescent pseudomonads, was found to have antagonistic activity against plant pathogen (Rodriguez and Fraga 1999; Siddiqui 2006). *P. fluorescens* CHA0 played an indispensable role in suppression of *Thielaviopsis basicola*, casual organism of black root rot of tobacco, mainly by producing HCN (Voisard et al. 1989). Fluorescent pseudomonads also produce an array of cyclic lipopeptides (CLPs) which are peptide antibiotics (Nielsen et al. 2002), which play a significant role in biocontrol. Tensin, a CLP, produced by *P. fluorescens*, exhibited potent antagonistic activity against *R. solani* infection in sugar beet (Nielsen et al. 2000). Fluorescent *Pseudomonas* also produce pyrrolnitrin and pyoluteorin of which pyoluteorin is a broad-spectrum antifungal metabolite inhibiting the growth of *Phytophthora capsici*, the pathogen on black pepper (Paul and Sarma 2006). Application of *P. fluorescens* 7-14 as a biocontrol agent controls the rice blast disease caused by *Magnaporthe grisea* (Gnanamanickam and Mew 1992; Chatterjee et al. 1996) (Table 2.3).

2.6 Fluorescent Pseudomonads in Agriculture and Plant Health

Pest and disease management is a vital part of sustainable agriculture and this includes the use of beneficial microorganisms for the effective and sustained production of agricultural and horticultural crops. PGPR, viz., *Pseudomonas*, *Bacillus*, and fungal antagonist *Trichoderma*, have been well exploited for the management of plant diseases. They may play a regulatory role in plant growth and development (Schippers et al. 1987; Fravel 2005). They also protect plant root surfaces from colonization of pathogenic microbes through direct competitive effects and production of antimicrobial agents. PGPR have been used as soil inoculants intended to improve the supply of various nutrients like phosphorus, nitrogen, and potassium to crop plants. PGPR can either increase plant health or protect them from diseases, and thus their commercial application depends on the type of PGPR. The extensive colonization and biocontrol ability of rhizospheric fluorescent pseudomonads have generated increased interest in their use as crop protectants (Schippers et al. 1987;

Table 2.3 Fluorescent pseudomonads mediated induced systemic resistance and biocontrol activity against phytopathogens

Fluorescent pseudomonads	Pathogens	Diseases	Plants	References
P. fluorescens S97	*P. syringae* pv. *phaseolicola*	Halo blight	Bean	Alstrom (1991)
P. aeruginosa 7NSK2	*Botrytis cinerea*	Grey mold	Bean	De Meyer and Hofte (1997)
P. aureofaciens 25-33	*Colletotrichum orbiculare*	Anthracnose	Cucumber	Wei et al. (1991)
P. corrugata 13	*Pythium aphanidermatum*	Crown rot	Cucumber	Chen et al. (2000)
P. fluorescens WCS374	*F. oxysporum raphani*	Vascular wilt	Radish	Leeman et al. (1995)
P. fluorescens WCS417	*Alternaria brassicicola*	Necrotic lesions	Radish	Ton et al. (2002)
P. fluorescens WCS417	*F. oxysporum* f. sp. *lycopersici*	Vascular wilt	Tomato	Duijff et al. (1998)
P. putida	*F. oxysporum*	Fusarium wilt	Radish	Scher and Baker (1982)
P. fluorescens Q8r1-96	*Gaeumannomyces gramini* var. *tritici*	Take-all	Wheat	Raaijmakers and Weller (1998)
P. aeruginosa	*Septoria tritici*	Foliar disease	Wheat	Baron et al. (1997); Flaishman et al. (1990)
P. fluorescens Pf-5	*Pythium ultimum, R. solani*	Seedling diseases of cotton	Cotton	Howell and Stipanovic (1979, 1980)
P. cepacia 5.5B	*R. solani*	Damping-off	Cotton	Cartwright et al. (1995)
P. fluorescens CHA0	*P. splendens*	Damping-off	Tomato	Buysens et al. (1994)
P. putida NIR	*P. ultimum*	Damping-off	Soybean	Paulitz (1991)
P. fluorescens Hv37a	*P. ultimum*	Damping-off	Barley	Gutterson et al. (1986)
P. fluorescens DR54	*R. solani*	Damping-off	Sugar beet	Nielsen et al. (1999)
P. fluorescens PfMDU	*R. solani*	Sheath blight	Rice	Nagrajkumar et al. (2005)
P. putida KKM1	*C. falcatum*	Red rot	Sugarcane	Malathi et al. (2002)
P. fluorescens PGS12	*F. oxysporum*	Damping-off	Corn	Georgakopoulos et al. (1994)
P. aeruginosa PNA1	*F. oxysporum*	Damping-off	Chickpea	Anjaiah et al. (1998, 2003)

Weller 1988; Lam and Gaffney 1993; Fravel 2005). Rice plants treated with bioformulation containing *P. fluorescens* strains Pf1 and AH1 and *Beauveria bassiana* isolate B2 showed a greater accumulation of defense enzymes, lipoxygenase and chitinase against leaf folder (Karthiba et al. 2010). Siderophore-mediated competition for iron between the two biocontrol agents *P. putida* WCS358 and *P. fluorescens* WCS374 decreased the colonization of radish roots by *P. fluorescens* WCS374 (Raaijmakers et al. 1995a, b). Combination of chitinase-producing *Streptomyces* spp., *Bacillus cereus*, and antibiotic-producing *P. fluorescens* and *Burkholderia cepacia* had a synergistic effect on the suppression of rice sheath blight (Sung and Chung 1997). Treatments of seed, soil, or root by *P. fluorescence* could control the foliar diseases and could also protect the leaves of cucumber (Wei et al. 1991).

The combined use of PGPR and specific contaminant-degrading bacteria can successfully remove complex contaminants (Huang et al. 2005). ACC deaminase containing plant growth promoting fluorescent pseudomonads could suppress accelerated endogenous ethylene synthesis and thus may facilitate root elongation, nodulation and improve growth and yield of plant (Zafar-ul-Hye 2008). Co-inoculation studies with PGPR and rhizobia have shown increased plant nodulation and N_2 fixation in leguminous plants such as soybean, pea, peanut, and alfalfa (Vessey and Buss 2002; Figueiredo et al. 2007). Co-inoculation of *P. fluorescens* and *Azospirillum* also stimulated root growth in spring wheat (Combes-Meynet et al. 2011).

2.7 Conclusions

Increased concern about the cleaner environment and excessive and deliberate use of chemicals in modern agriculture has necessitated the search for eco-friendly alternatives. PGPB offer an attractive alternative for sustainable agriculture and are gaining worldwide importance and acceptance in agriculture. Rhizospheres inhabiting fluorescent pseudomonads are a metabolically and functionally diverse group of bacteria which exhibit multiple mechanisms that mediate their ability to both suppress phytopathogens and promote crop growth and yield. Fluorescent pseudomonads provide benefit to the plants by various mechanisms which include competitive root colonization, phosphate solubilization, iron sequestration, production of plant growth regulators, enhancing nutrient uptake via mineral solubilization and synthesis of lytic enzymes along with the induction of ISR against phytopathogens. A better understanding of different mechanisms involved in the plant–microbe interaction is a prerequisite and necessarily required to develop new strategies for improving crop yields. These microorganisms can be used as model systems for providing novel genetic constituents and bioactive chemicals and can be used as a potential tool for sustainable agriculture.

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Chapter 3
N_2-Fixing Cyanobacterial Systems as Biofertilizer

Mayashree B. Syiem, Arvind Kumar Singh, and Amar Nath Rai

Abstract Soil and water surfaces, as well as plant surfaces and tissues are the known locations that harbor free-living phototrophic N_2-fixing cyanobacteria. These organisms are known to contribute substantial amounts of fixed nitrogen (20–30 kg N ha^{-1}annually). In continents where rice is the prime crop for majority of the population (amounting to over 40 % of world's population), these organisms assume great importance. Two third of the total of 180 million tons of fixed nitrogen that gets added to the earth's surface globally, comes from biological activities mainly contributed by these and other microbes. Rice field ecosystems are ideal for cyanobacterial growth as they provide optimum growth conditions. *Azolla-Anabaena* symbiotic association, another cyanobacterial system has been exploited as a biofertilizer in many Asian countries. This symbiosis is very important agronomically because its contribution has been estimated to be ~600 kg N ha^{-1}. With the adverse consequences of chemical agriculture, focus on nitrogen enrichment has shifted again to biological nitrogen fixation, especially towards both free-living and symbiotic cyanobacteria. During past few decades, research studies have yielded a large quantity of information on cyanobacterial nitrogen fixation from isolation, molecular understanding and manipulations to large-scale production for agriculture. Substantial research studies have also been devoted towards creating and understanding the artificial associations of cyanobacteria with crop plants. In this chapter, various N_2-fixing cyanobacterial systems in light of their use as biofertilizers are reviewed.

Keywords *Azolla* • Agriculture • Cyanobacteria • Nitrogen fixation • Rhizobia

M.B. Syiem • A.K. Singh • A.N. Rai (✉)
Department of Biochemistry, North-Eastern Hill University, Shillong, Meghalaya, India
e-mail: raiamarnath@gmail.com

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3.1 Introduction

Nitrogen is an indispensable nutrient required for all plant growth and although 79 % of the total air composition is elemental nitrogen, living organisms barring a few prokaryotes are unable to use this vast source of nitrogen directly as nutrient (Singh et al. 2011a, b, c). These prokaryotes include rhizobia as well as cyanobacteria (Singh 2013, 2014). Cultivation of rice in many countries of the world is fundamental as majority of the calorie requirement of ~40 % of the world's population depends on rice (Singh et al. 2016a, b) In rice cultivation, the availability of fixed nitrogen in the soil is the most crucial limiting factor. With increasing population, use of refined and highly developed technologies in the agricultural sector has resulted in increased crop productivity. However, quest for higher yield has also seen the indiscriminate use of chemical fertilizers, herbicides, and pesticides whose continued presence in excess has led to deterioration of soil texture, quality, and fertility as well as ground water quality. On the global scale, two third of the total amount of usable nitrogen that gets added to the soil (amounting to ~180 million tons) comes from microbial activities, mainly nitrogen fixation, performed by the above mentioned prokaryotes (Kaushik 2014). De (1939) had attributed soil fertility of rice fields due to the cyanobacterial nitrogen fixation (Roger et al. 1987). They reported presence of 10–10^7 counts of cyanobacteria in 396 soil samples from rice fields across ten countries. The flooded rice fields represent unique ecosystems characterized by aerobic and anaerobic zones. There are complex levels of microbial presence that perform diverse forms of biological nitrogen fixation (Singh and Strong 2016). The flood water and soil surface as well as the rhizospere form the aerobic zone, while the plow layer forms the anaerobic zones (Venkataraman 1993). In the aerobic zone, the free-living cyanobacteria reside, which perform nitrogen fixation independently (Roger and Kulasooriya 1980; Vaishampayan 1996; Whitton 2000; Vaishampayan et al. 2001) and this is also the preferred site for luxuriant growth of *Azolla-Anabaena* N_2-fixing complex that fix atmospheric nitrogen symbiotically (Singh 1979; Venkataraman 1988; Vaishampayan 1994; Vaishampayan et al. 2000). Heterotrophic N_2-fixation occurs in the rhizosphere and the anaerobic zone (Matsuguchi 1978; Hegde et al. 1999).

A diverse range of free-living cyanobacteria have been identified with biofertilizer potential. Additionally, *Azolla-Anabaena* N_2-fixing complex has been in use as an established and naturally competent biofertilizer in many rice-cultivating countries (Singh and Gupta 2016). Apart from being a biofertilizer in terms of enriching soil nitrogen content, cyanobacteria are known to have a positive influence on the physicochemical properties of soil such as pH, electrical conductivity, availability of phosphorus, and grain quality in terms of protein content (Vaishampayan et al. 2001; Kaushik 2014). Over the years, the cyanobacteria belonging to the genera *Nostoc* and *Anabaena* have emerged as microbes of choice with high biofertilizer potential and also having potential to form symbiotic association with other organisms (Singh 2013; Singh and Singh 2013; Singh 2014). Many of these microbes produce signaling molecules that can alter gene expression in the host plants

resulting in qualitative and quantitative alterations in the composition of soil microflora in the vicinity of the rhizophere (Whitton et al. 1988; Misra and Kaushik 1989).

In this chapter, we would look into free-living cyanobacteria, *Azolla-Anabaena* N_2-fixing complex and the artificial associations comprehensively to assess performance of these N_2-fixing cyanobacterial systems as biofertilizer.

3.2 Distribution of Cyanobacteria

Cyanobacteria are an ancient group of microbes that probably evolved in the Precambrian era (Schopf 1970) and are thought to be responsible for bringing about oxygenation to earth from an anoxygenic environment. They are unique as they combine oxygenic photosynthesis with biological nitrogen fixation where the key enzyme of the process is highly sensitive to the presence of oxygen. Simple nutrient requirements along with carbon and nitrogen independence have allowed these organisms to populate nutritionally poor habitats. Additionally, they have slowly, but surely adapted to enormously diverse environments ranging from most favorable locations like rice fields to extreme environmental conditions in terms of temperature, pH, light intensity, desiccation, flooding, salinity, and pollution (Whitton 2000; Vaishampayan et al. 2001).

3.3 Free-Living Cyanobacteria

Free-living cyanobacteria are ubiquitously present in all types of environments, and they enjoy nutritional freedom due to their carbon and nitrogen-fixing abilities. Most free-living cyanobacteria can fix both atmospheric carbon and nitrogen and flourish in rice fields as they provide optimum growth conditions for cyanobacteria in terms of light, temperature, and water requirements. With their nitrogen-fixing capabilities cyanobacteria are thought to be beneficial to rice fields in sustaining nitrogen status of the soil. Cyanobacterial abundance in rice fields was first reported by Fritsch in 1907. There is a significant amount of information relating to cyanobacterial presence in paddy field ecosystem from countries such as Australia, Egypt, India, Indonesia, Iraq, Japan, Morocco, Philippines, Senegal, and Southeast Asia (Desikachary 1959; De and Sulaiman 1950; Singh 1961; Pandey 1965; Gupta 1966; Stewart et al. 1979; Roger and Kulasooriya 1980; Watanabe and Brotonegoro 1981; Al-Mousawi and Whitton 1983; Selvi Thamizh and Sivakumar 2012). Some of the common rice field cyanobacteria are *Aphanothece, Gloeocapsa, Microcystis, Chroococcus* (Unicellular forms); *Oscillaroria, Plectonema, Lyngbya* and *Phormidium* (filamentous non-heterocystous forms) and *Anabaena, Aulosira, Calorhrix, Cylindrospermum, Camptylonema, Fiscerella, Gloeotrichia, Mastigocladus, Nostoc, Nodularia, Nostochopsis, Rivularia, Scyronema, Westiella,*

Westiellopsis (filamentous heterocystous forms) (Venkataraman 1993; Sinha and Hader 1996; Vaishampayan et al. 2001; Fernandez-Valiente and Quesada 2004). *Nostoc* sp. and *Anabaena* spare the most common N_2-fixing cyanobacteria in rice fields occurring frequently as free-floating blooms and forming microbial mats. Characteristically, about 50 % of the cyanobacterial genera are heterocystous (Vaishampayan et al. 2001). The frequency of N_2-fixing cyanobacteria was recorded to be high in tropical and subtropical regions, and species like *Tolypothrix tenuis* were found to be the active nitrogen fixers (Watanabe and Brotonegoro 1981).

Although rice plants have the dominant presence in rice fields, cyanobacteria are the prominent photosynthetic aquatic biomass along with various algae and vascular macrophytes that grow during the different phases of rice growth. All forms of benthic, planktonic, and epiphytic cyanobacteria are widespread in rice fields. Studies on cyanobacterial successions have been performed in different rice fields all over the world (Gupta 1966; Roger and Reynaud 1976; Grant et al. 1986). Fernandez-Valiente and Quesada (2004) reported that phytoplankton; mainly chlorophyceans develop early in the cultivation cycle until the tillering phase. The highest values of photosynthetic aquatic biomass are seen up to the initiation of panicle. During this phase, most dominant are the non-N_2-fixing cyanobacteria even though abundance of N_2-fixing cyanobacteria is seen in some places. From panicle initiation to harvest N_2-fixing cyanobacteria become dominant. Watanabe (1951a, b) have reported an input of 20 kg/ha nitrogen by *Tolypothrix tenuis* in rice fields of Japan. MacRae and Castro (1967) also reported a similar value of 10–15 kg/ha cyanobacterial nitrogen contribution in rice fields. (Henriksson 1971) estimated an annual nitrogen fixation of 15–51 kg/ha in agriculture with *Nostoc* as dominant cyanobacteria. A value as high as 90 kg/ha had been reported by Metting (1981) for cyanobacterial nitrogen input in rice fields. These varied estimates of cyanobacterial nitrogen contribution may reflect the dependence of cyanobacterial growth and nitrogen fixation on the climatic and biotic constituents as well as on the physicochemical properties of the test sites and samples. In a review, (Kaushik 2014) summarized various reports of the quantitative assessments of cyanobacterial N contribution for different countries: 15–53 kg/ha for India (De and Biswas 1952; Singh 1961; Venkataraman 1979); 11–23 kg/ha for Japan (Okuda and Yamaguchi 1955; Singh 1980), 18–33 kg/ha for Philippines (Alimagno and Yoshida 1977; Watanabe and Lee 1975), 0–30 kg/ha for Senegal (Reynaud and Roger 1978), and 50–80 kg/ha in Mali (Traore et al. 1978). These values indicate how rice cultivations have been supported over the centuries without the application of fertilizers (Watanabe et al. 1987). Addition of phosphorus-based fertilizers is shown to have favorable consequences on the establishment, growth, and long-term maintenance of nitrogen-fixing cyanobacteria in rice fields (Jha et al. 1965). As early as 1979, Rodger and Reynaud reported that nutrients fixed by microbes are available to soil by either exudation from live cells or after disintegration of microbial cells after death. This process gradually builds up the soil organic matter adding fertility to the soil. Wilson et al. (1980) reported that 39 % of N was transferred from ^{15}N-labeled *Aulosira* sp. to the rice crop. Fernandez-Valiente et al. (2002) stated that key source of N for rice was soil N and they estimated only 8–10 % of the total N taken up by rice plants was derived from

N-based fertilizers. However, based on ^{15}N-labeled cyanobacteria they further showed that the amount of nitrogen recovered by the soil-rice plant from cyanobacteria was higher than that from the chemical fertilizers. The most determining factor in soil fertility is the organic carbon. Algae and cyanobacteria that are photosynthetic in nature immensely contribute to the total organic carbon in rice fields (De and Sulaiman 1950; Nekrasova and Aleksandrova 1982; Roger et al. 1987).

Apart from carbon and nitrogen contribution, many researchers have also reported production and release of various growth-promoting substances like cytokinins, auxins, gibberellins, abscisic acids, and vitamins by cyanobacteria that have beneficial effects on seed germination, grain quality, yield, and nutritional value (Watanabe 1951a, b; Gupta et al. 1967; Ahmad and Winter 1968; Venkataraman and Goyal 1969; Singh and Trehan 1973; Grieco and Desrochers 1978; Kaushik and Venkataraman 1979; Roger and Reynaud 1979; Marsalek et al. 1992; Kaushik 2014). The presence of cyanobacteria has also been credited to many fold increase in bacteria, fungi, and actinomycetes consortia in the rhizospere of crop plant (Rao and Burns 1990). The development of diverse microbial conglomerate in the soil increases soil fertility due to their varied activities. Cyanobacteria are also known to increase phosphorus availability in the dissolved organic form. Cyanobacterial presence helps improve physical properties of soil as they excrete a number of compounds such as polysaccharides, peptides, and lipids during their growth that bind soil particles into micro- and eventually macroparticles along with organic carbon added via their biomass (Roychoudhury et al. 1979; Rogers et al. 1991; Rogers and Burns 1994). The extracellular polysaccharide of cyanobacterial origin along with the release of oxygen due to their photosynthetic activities have been found to influence the form in which essential mineral such as Fe, Mn, and Zn occur in soil (Das et al. 1991). A decrease in the readily available Fe may help in reducing Zn deficiency in rice. Lange in 1976 reported the chelation of Fe, Cu, Mo, Zn, Co, and Mn in the gelatinous sheath of many cyanobacteria. The sheath may influence the availability of these minerals to other organisms (Belnap and Harper 1995). Whitton (2002a, b) examined that a cyanobacterial sheath may also aid in adsorbing cations and reduce soil erosion.

3.4 *Azolla-Anabaena* Symbiotic System

Another important biofertilizer of cyanobacterial origin that benefits rice cultivation is the *Azolla-Anabaena* symbiotic system. *Azolla* is found in both tropical and temperate climates and grows luxuriantly in ponds, paddy fields, and ditches (Dommergues et al. 1986). The use of *Azolla* in increasing rice productivity has been known for centuries (Fogg et al. 1973; Watanabe and Liu 1992; Yadav et al. 2014). Chinese have records of *Azolla* use dated 2000 years back (Chu 1979; Shi and Hall 1988). It is widely cultivated in many rice growing Asian countries where it is either integrated into the soil before rice transplantation or grown along with rice as dual crop. *Azolla* is classified under Phylum—Pteridophyta; Class—Filicopsida;

Order—Salvoniales; Family—Azollaceae; Genus—Azolla. Studies conducted at morphological and molecular level using randomly amplified polymorphic DNA (RAPD) showed grouping of various *Azolla* species in two distinct clusters. Cluster I (*A. pinnata* and *A. nilotica*) and cluster II (*A. microphylla, A. filiculoides, A. caroliniana,* and *A. mexicana*). *A. rubra* somehow stood independently that clustered differently depending on the method used (Pereira et al. 2011).

The free-floating heterosporous pteridophyte *Azolla* is always found with an obligate cyanobacterial associate as its endosymbiont. The obligate endosymbiont described earlier as *Anabaena azollae* taxonomically has been placed under Phylum—Cyanophyta; Order—Nostocales; Family—Nostocaceae. Different researchers have identified the cyanobiont as *Nostoc* (Meeks et al. 1988; Plazinski and Gresshof 1990; Kim et al. 1997) or as *Anabaena azollae* or as *Trichormus azollae* (Bergman et al. 1992; Grilli Caiola et al. 1993). Others have reported the presence of more than one strain of *Anabaena* within a single species of *Azolla*. There are other contrasting reports of the presence of different *Anabaena* sp. in different *Azolla* species (Ladha and Watanabe 1982; Gebhardt and Nierzwicki-Bauer 1991). The third key partner of the association have been indicated to be an eubacterium from *Arthrobacter* species that comprises almost 90 % of the total bacterial population regardless of the *Azolla* species used as inoculum (Braun-Howland and Nierzwicki-Bauer 1990). Rest of the bacterial population includes members of *Pseudomonas* species, *Alkaligenes faecalis, Caulobacter fusiformis,* and *Azotobacter* species (Plazinski et al. 1990; Malliga and Subramanian 1995).

Morphologically, *Anabaena azollae* is composed of unbranched trichomes. The vegetative cells are 6–8 μm long and 10–12 μm wide and are highly pigmented (Singh 1979; Van Hove 1989). Heterocysts exist in the range of 15–30 % and are bigger than the vegetative cells with light pigmentation and thick walls (Becking 1976; Singh 1977a). Akinetes have thick walls and are resting spores and are not commonly found (Lumpkin and Plucknett 1980; Kannaiyan 1994). *Azolla* requires all macro- and micronutrients and are sensitive to excess or deficiency of suitable nutrient concentrations that are essential during its growth. Phosphorus is the most common limiting macronutrient for the growth of *Azolla*. Phosphorus concentration of ~0.5 μM has been reported to be ideal for optimum growth of *Azolla* (Sah 1989). Due to the presence of symbiotic cyanobacteria *Azolla* can grow independent of external nitrogen sources. The presence of different nitrogen sources significantly affects nitrogen fixation (Manna and Singh 1991). However, it has been indicated that this symbiotic association shows noticeable adjustment to N-deficiency and N-supplementation mediated largely via the GS-GOGAT pathway of the cyanobiont (Pabby et al. 2000).

This *Anabaena azollae* present in the leaf cavities of the *Azolla* is capable of higher nitrogen fixation as it becomes free of the responsibility of fixing carbohydrate that gets provided by the host plant *Azolla*. The agronomic importance of *Azolla* comes from the fact that it can effectively grow in nitrogen poor soil and under waterlogged conditions. These characteristics have been spotted early on and *Azolla* have been successfully used as biofertilizer in rice cultivation. Many other benefits have been also realized from *Azolla*. Its growth improves water quality by

removing excess quantities of nitrate and phosphorus; it is used as fodder and feed for fish, ducks, rabbits, etc. and functions as a controlling agent for weed and mosquitoes (Wagner 1997). The biggest attribute of *Azolla* that promotes it as biofertilizer is its high multiplication rate which allows it to completely cover the entire water surface within 2–3 days of application (Pabby et al. 2003). Since it floats, it does not compete with rice plant for light and space. The shallow, water-logged conditions of rice field and the shade offered by the rice leaf canopy provide the most ideal growth conditions for *Azolla*. Nitrogen fixed by the *Azolla-Anabaena* symbiosis is immediately available to the rice plants and has been reported to be most readily assimilable source of organic nitrogen for rice plant whenever this system has been employed as biofertilizer in rice cultivation (Kannaiyan 1993). The estimated organic nitrogen supplemented by *Azolla* ranges between 40 and 60 kg/ha/crop (Plazinski et al. 1990). *Azolla* starts to die as rice plants approaches maturity due to depleted nutrient conditions of the soil and disintegrates releasing nutrients to the immediate environment. This becomes available to rice for grain development (Dey 1999). Productivity of rice plants is influenced by the availability of fixed nitrogen. Since conditions for rice cultivation and optimal growth of *Azolla* are similar, *Azolla* can be co-cultivated as biofertilizer to maximize rice production. Field experiments using high yielding varieties of rice conducted by Central Rice Research Institute, Cuttack, India showed that rice production with application of 30 kg N ha^{-1} is similar to that with application of 10 ton/ha of fresh *Azolla* (Singh 1977b; Singh 1978).

Watanabe (1982) pointed out that when using *Azolla* as nitrogen fertilizer, the crucial factor is the inoculum density and time of inoculation for best growth and biomass production. If inoculum density used is too high, then growth rate and nitrogen fixation rates are inhibited. If the inoculum density used is too low, then *Azolla* could be outgrown by weeds and algae (Ashton 1974). *Azolla*-derived N incorporation has been reported to increase the rice grain yield up to 38–45 % (Reynaud 1982; Tung and Shen 1985). Experiments conducted in Davis, California, showed 112 % increase in rice yield when *Azolla* was used as intercrop and 216 % increase compared to control when *Azolla* was applied both as mono crop and intercrop (Peters 1975). *Azolla* was found to compensate for urea between 30 and 60 kg N ha^{-1} (Vlek et al. 1995; Lakshmanan et al. 1997). Numerous studies have indicated that growing *Azolla* before and after rice plantation is equivalent to application of 30–40 kg N ha^{-1} which of course differ from species to species of *Azolla* used (Singh 1977a, b; Rains and Talleys 1979; Watanabe 1982; Lakshmanan et al. 1997). Further studies indicated that 45–50 % of the total *Azolla* N used by rice plants is incorporated into rice straw, 10–20 % in the roots, and 30–45 % in the grain (Ito and Watanabe 1985). *Azolla* N has also been reported to improve the grain quality by increasing protein content (Singh 1977b; Liu 1979).

Kannaiyan (1993) had reported that combined application of *Azolla* and free-living cyanobacterial biofertilizers on rice variety CO-41 showed remarkable increase in rice grain yield compared to when *Azolla* or free-living cyanobacteria were applied independently. Application of *Azolla* seems to be far more gainful than using free-living cyanobacteria in rice cultivation (Vaishampayan et al. 1998).

Because of its high protein, amino acids, and lipid content, *Azolla* is also used in China and many south Asian countries as fodder for chicken, duck, fish, and pigs. Nierzwicki-Bauer (1990) had reported that the dry *Azolla* powder contains about 27 % assimilable protein that can support a considerable increase in carotene content and egg production in poultry. Despite its high efficiency *Azolla* mass cultivation is restricted to certain coastal areas as this symbiotic association requires high humidity and is highly thermo-sensitive. The ideal temperature for *Azolla* cultivation is around 20–30 °C. This limits its application in regions with higher temperature. Also *Azolla* requires high phosphate input for its growth and metabolic activities. Another drawback is that its spores do not germinate into sporophytes readily (Sinha et al. 1999). To overcome these limitations and to make use of *Azolla* throughout the year many researchers have tried introducing somaclonal mutations. Vaishampayan and Awasthi (1997) have pointed out that constitutive production of some key enzymes such as phosphofructokinase, glucose 6-phosphate dehydrogenase, alcohol dehydrogenase, and superoxide dismutase offer considerable thermostability to various metabolic processes like chlorophyll and amino acid synthesis and photosynthesis. These approaches have been applied to bring about temperature tolerance in *Azolla* by manipulation at the genetic and/or enzymatic level. Additionally, introduction of a thermo-insensitive gene from naturally occurring *Azolla* from a warmer region (e.g., *A. pinnata* from Africa) was also tried into more common *Azolla* species of a temperate region (e.g., *A. pinnata* var. *pinnata* in India). The mutant strain of *Azolla* was found to grow well up to a temperature of 40 °C. Somaclonal mutation for reduced phosphate requirement has been achieved by treatment with *N*-methyl-N′-nitro-N-nitroguanidin (MNNG) in *A. pinnata* using shoot or frond meristem as starting material that retained the germplasm for the cyanobiont *Anabaena azollae* (Vaishampayan et al. 2000).

The considerable rise in rice grain yield on application of *Azolla* as biofertilizer may be attributed to the enhanced biomass buildup that on mineralization adds significant amount of organic matter and nitrogen to the soil increasing in turn the C:N ratio of the soil. The profuse growth of *Azolla* also restricts the growth of other phototrophs as it reduces light penetration. This in turn reduces photo-dependent CO_2 uptake by these organisms.

3.5 Artificial Cyanobacterial-Plant Association

In nature, cyanobacteria have evolved to exist in association with several eucaryotic hosts with varying degrees of intimacy. However, these associations do not involve crop plants and, therefore, are of little significance. Details of cyanobacterial associations with various hosts comprising fungi, algae, bryophytes, pteridophytes, gymnosperms, and angiosperms are available elsewhere (Rai et al. 2000; Rai et al. 2002; Dworkin et al. 2006; Meeks 2006; Adams et al. 2013). In natural associations, cyanobacteria are severely modified and release a significant quantity of N_2-derived ammonia which is partly stimulated by host-mediated decrease in cyanobacterial

glutamine synthetase activity. The diazotrophic partner thus enables the non-diazotrophic host to grow in nitrogen poor soil. These facts have focused the attention of researchers to characterize various physicochemical parameters for artificial association between N_2-fixing cyanobacteria and crop plants (Gusev and Korzhenevskaya 1990; Svircev et al. 1997; Gusev et al. 2002; Nilsson et al. 2002, 2005, 2006; Akoijam et al. 2012). Several laboratory studies have evaluated the capacities of diazotrophic cyanobacteria to associate with and support the growth of wheat and rice plants (Gantar et al. 1991a; Akoijam et al. 2012). In one such coculture experiment, two types of associations were recognized. Filaments of *Anabaena* growing between root hairs formed loose association while certain *Nostoc* strains developed tight associations with the wheat root surface (Gantar et al. 1991a). It has also been shown that *Nostoc* strains intimately associated with root surfaces maintained a substantial level of nitrogenase activity (Gantar et al. 1995a). The attachment of cyanobacteria to root surface is facilitated by extracellular polysaccharide. The intimacy of such association is partly determined by the protein component in the exopolysaccharide layer of filaments. These associations in some cases develop to such a strong extent that it becomes difficult to disassociate the symbiotic partners without damaging the epidermis (Gantar et al. 1995b).

Our laboratory and collaborators have characterized the kinetics of adsorption of cyanobacteria to rice roots. The association of cyanobacteria to rice plant exhibits a biphasic pattern. The rapid first phase last for 60–120 min. This is followed by a slower second phase after a lag period (Nilsson et al. 2002; Akoijam et al. 2012). Many cyanobacteria have been tested successfully to colonize rice root as well (Nilsson et al. 2002). The cyanobacteria associated with rice root surfaces maintained high level of N_2-fixation compared to their free-living control culture (Nilsson et al. 2002). Experiments have been carried out also to test the influence of strains in mixed population on the colonization ability of other strains. A 16S rRNA gene fragment-based DGGE analysis of rice root colonization pattern by mixed cyanobacterial inoculum exhibited that certain strains suppressed the colonization of other strains while retaining their own colonization ability. These results indicate that all strains are not equally competent to colonize rice root (Akoijam et al. 2012).

3.6 Molecular Signaling Mechanism

The mechanistic details of association process have been analyzed to a certain degree. Cyanobacteria in symbiosis with plants have low photosynthetic activity and are supplemented by sugar from plants. It has been reported that *Nostoc punctiforme* defective in glucose permease (GlcP) was unable to infect hornwort *Anthoceros punctatus* (Ekman et al. 2013) thus suggesting the involvement of GlcP for establishing symbiosis. Sugars are known to act also as chemo-attractants (Nilsson et al. 2006) and repressors of hormogonia formation after symbiosis has been established (Khamar et al. 2010). During initial stages of symbiosis between *Athoceros punctatus* and *Nostoc* strain, the bryophyte releases a low molecular

weight and heat-labile compound that stimulates hormogonia formation (Campbell and Meeks 1989). The hormogonia-inducing factor (HIF) acts by antagonizing the effect of auto-inhibitor of hormogonia differentiation in aseriate filaments (Gantar et al. 1993). The functioning of HIF is effectively suppressed by $NaNO_3$, NaCl, and KNO_3 (Gantar et al. 1993). The nature of the class of these distinct biochemical inducers still remains to be established. The HIF-like compounds have been observed in wheat root exudate (Gantar et al. 1993), *Gunnera* stem glands mucilage (Rasmussen et al. 1994), and *Blasia* exudates (Adams and Duggan 2008). The chemotaxis of symbiotic cyanobacteria (*Nostoc* strains 8964:3 and PCC 73102) towards extract from natural hosts *Gunnera manicata*, *Cycas revoluta*, and *Blasia pusilla*, and the non-host plants *Trifolium repens*, *Arabidopsis thaliana*, and *Oryza sativa* has been observed (Nilsson et al. 2006) which is directed by chemo-attractants in plant exudates (Knight and Adams 1996; Watts et al. 1999). In spite of a widespread presence of chemo-attractants in host and non-host plants, cyanobacterial symbioses are limited only to certain plant groups. It will be an interesting proposition, therefore, to examine the factors responsible for this. The motility of hormogonia towards host plant is probably regulated by chemotaxis-related signal transduction system (Duggan et al. 2013). One such communication system has been characterized in *N. punctiforme* ATCC 29133 (Duggan et al. 2013). The genome of *N. punctiforme* ATCC 29133 contains five *che* loci (*che1-che5*) exhibiting sequence similarity to chemotaxis genes from other bacterium. The *che5* locus comprises seven *che*-like ORFs (NpR0244-NpR0250) containing genes encoding a response regulator receiver modulated CheB methylesterase (NpR0244) and a CheR type methyl accepting chemotaxis protein (MCP) methyltransferase (NpR0248). The CheB and CheR modulate the methylation status of MCPs leading to adaptive response of cyanobacteria in the background of chemo-attractants (Duggan et al. 2013). Disruption of *che-R* (NpR0248) gene in *N. punctiforme* ATCC29133 resulted in the loss of motility and decrease in symbiotic competency with liverwort *Blasia pusilla*. A dissection of molecular communication during early stages of *Gunnera-Nostoc* symbiosis has shown that a protein compound was responsible for inducing hormogonia differentiation (Rasmussen et al. 1994). Although hormogonia production is an inevitable step for successful association, all hormogonia-producing strains do not associate with roots (Enderlin and Meeks 1983; Gantar et al. 1991a; Johansson and Bergman 1994; Rasmussen et al. 1994) implicating roles for additional factors in symbiotic competence development. A role for pili-like appendages in symbiosis development was reported. Pili are expressed on the surface of hormogonia and disruption of these structures by inactivating *pilD* and *pilT* components in *N. punctiforme* specifically altered surface piliation and reduced symbiotic competency (Duggan et al. 2007). Compounds like synthetic auxin (2, 4-D) treatment enhance colonization of wheat roots by cyanobacteria (Gantar and Elhai 1999). Indeed, a subsequent study has supported the positive impact of auxin on the development of artificial associations by demonstrating better association capacity of high auxin producing cyanobacterial strains (Ahmed et al. 2010). Ultrastructural analyses of colonized roots have revealed penetration of cyanobacteria into root epidermis and cortex (Gantar et al. 1991b; Ahmed et al. 2010). In addition to root surface, competent strains adsorb on the leaves and stems of wheat plant. Penetration

of associated strain into internal tissue occurs through cuts in epidermis. The activity of hydrolytic enzymes in wheat root has been found to increase when associated with cyanobacteria. The action of these cell-wall hydrolyzing enzyme may partly mediate entry of cyanobacteria in to the subepidermal layers (Babu et al. 2015). This observation was supported by showing a strong positive correlation between associative N_2-fixation and wheat root endoglucanase activity.

3.7 Selection of Competent Biofertilizer Strains

The N_2-fixing cyanobacterial strains (such as *Nostoc*) that associate with rice and wheat roots can be used as in situ biofertilizers providing host plant with fixed nitrogen. The observation that only a limited number of cyanobacteria associate with plant host necessitate the development of methods for the selection of competent strains and their performance in the field conditions (Nilsson et al. 2002; Akoijam et al. 2012). Under laboratory conditions, association of cyanobacteria with rice roots has been followed by measurement of chl *a* content (Nilsson et al. 2002). Such measurements reflect association capacity of individual strains. On the other hand, in natural conditions many species are likely to compete for colonizing the host plants. Detection and identification of competent strains with biofertilizer potential by classic method is not a feasible option because a major fraction of microbial community defies their cultivation. Therefore, the inevitable consequence of the cultivation-based approach is the gross underestimation of microbial diversity and community composition (Amann et al. 1995). Excellent molecular techniques for direct analysis of microbial diversity in environmental samples have been described (Theron and Cloete 2000; Handelsman 2004). These methods are 16S rRNA gene clone analysis, denaturing gradient gel electrophoresis (DGGE), qPCR, and metagenomics. Denaturing condition-based molecular methods which distinguish closely related strain by producing strain-specific unique bands (Becker et al. 2004; Nilsson et al. 2005) has become a powerful and quick method for the assessment of relative ability of different cyanobacterial strains to associate with the host plants (Figs. 3.1 and 3.2) (Nilsson et al. 2005; Akoijam et al. 2012). In this process, DNA of cyanobacteria associated on root surface is extracted which is followed by PCR amplification of a fragment of 16S rRNA gene or a functional gene (het R) using GC clamped primers. The resolution of PCR amplicons on DGGE gel provides information about the number and identity of organisms attached on rice root. In the case, when a mix culture of unknown composition is used for colonization assay, sequencing of individual bands can reveal the identity of various root-associated strains. As described above, DGGE has been successfully used for assessment of cyanobacterial colonization of rice roots in mixed population (Nilsson et al. 2005; Akoijam et al. 2012). The abundance of individual strains can be determined by qPCR analysis targeting the organisms producing strong DGGE signal. qPCR has limited application as it provides quantitative abundance of only few organisms. Extremely powerful new generation techniques like metagenomics are more suitable for the analysis of environmental samples that provide direct information on rank

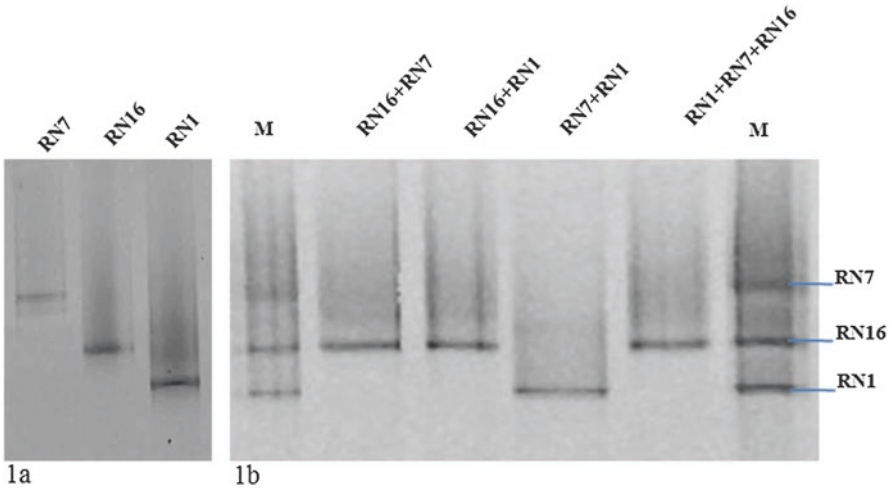

Fig. 3.1 DGGE based profiling of *Nostoc* genotypes using 16S rDNA amplicons generated with PCR primer pair CYA106FGC (5 ′-CGCCCGCCGC GCCCCGCGCCGGTCCCGCCGCC CCCGCCC GCGGACGGGTGAGTAACG CGTGA-3′) and CYA781R (CYA781Ra 5′-GACTACT GGGGTATCTAATCCCATT-3′ and CYA781Rb 5′-GACT ACAGGGGTATCTAATCCCTTT-3′) over a denaturing gradient of 20–60 %. (**a**) 16S rDNA profile of individual *Nostoc* strains RN7, RN16, and RN1 (**b**) 16S rDNA profile-based identification of *Nostoc* genotypes exposed to rice roots. Captions on top of lanes represent cyanobacterial mix used in competition experiments. Lane "M" represents mixture of 16S rDNA amplicons. Figure is reproduced from Akoijam et al. (2012) with permission

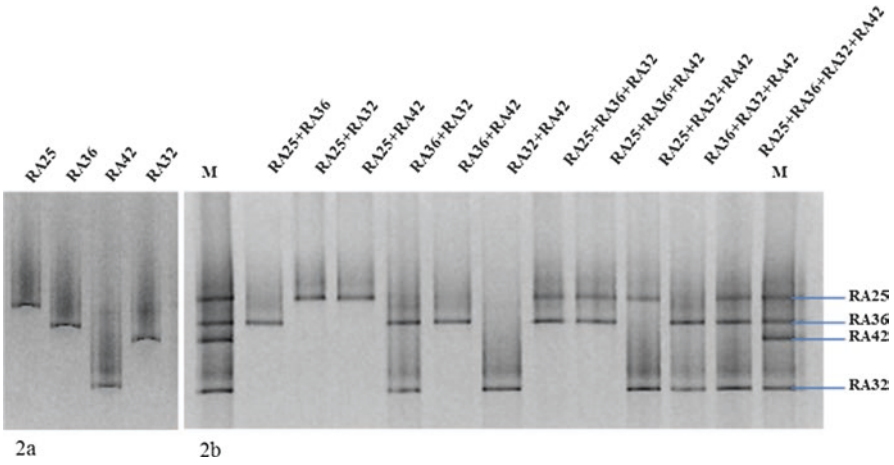

Fig. 3.2 DGGE based profiling of *Anabaena* genotypes using 16S rDNA amplicons generated with PCR primer CYA106FGC (5′-CGCCCGCCGCGCCCCG CGCCGGTCCCGCCGCCCCC GCCCGCGGACGGGTGAGTAACG CGTGA-3′) and CYA781R (CYA781Ra 5′-GACTACT GGGGTATCTAATCCCATT-3′ and CYA781Rb 5′-GACT ACAGGGGTATCTAATCCCTTT-3′) over a denaturing gradient of 20–60 %. (**a**) 16S rDNA profile of individual *Anabaena* strains RA25, RA36, RA42, and RA32 (**b**) 16S rDNA profile-based identification of *Anabaena* genotypes exposed to rice roots. Captions on top of lanes represent cyanobacterial mix used in competition experiments. Lane "M" represents mixture of 16S rDNA amplicons. Figure is reproduced from Akoijam et al. (2012) with permission

abundance of various operational taxonomic units (OUT). Such OUT can also be generated by preparing 16S rRNA gene clone library. Combined application of these methods can be extremely useful for the identification and selection of cyanobacterial biofertilizer strains able to associate with crop plants in field conditions.

3.8 Conclusions

Nitrogen-fixing cyanobacterial systems play a vital role in rice plantations by enhancing the soil ecosystems physicochemical properties. A diverse range of free-living cyanobacteria have been identified with biofertilizer potential. Their contribution is in the form of carbon and nitrogen nutrition as well as production of various growth-promoting substances. A plethora of molecular signal mechanisms of symbiotic cyanobacteria have been studied opening the gateways to an increased potential to further develop their activity as successful biofertilizers.

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Chapter 4
Exploring the Role of Secondary Metabolites of *Trichoderma* in Tripartite Interaction with Plant and Pathogens

Chetan Keswani, Kartikay Bisen, Manoj Kumar Chitara, Birinchi Kumar Sarma, and Harikesh Bahadur Singh

Abstract Exploitation of agriculturally important microorganisms in plant growth promotion and antagonistic potential is a well-investigated area. *Trichoderma* spp. are widely acknowledged for their potential to parasitize plant pathogenic fungi and have been efficiently utilized for biocontrol of wide range of seed and soil-borne phytopathogens. The antagonistic activity of *Trichoderma* spp. is largely credited to production of various antimicrobial secondary metabolites and has also been reported for plant growth promotion, management of the phytopathogens, and induction of systemic resistance in plants. Secondary metabolites-based formulation may have an additional benefit of longer shelf-life and immediate effect in comparison to spore-based formulations. Hence, this chapter will focus on the role of biosynthesized antimicrobial secondary metabolites of *Trichoderma* in tripartite interactions.

Keywords Biocontrol • Agriculturally important microorganisms • Secondary metabolites • Bioformulation • Systemic resistance

4.1 Introduction

Phytopathogens including fungi, bacteria, nematodes, viruses, and molicutes impose a serious threat to the global food production. Plant diseases alone cause about 27–42 % crop loss globally (Singh 2014a). Chemical control utilizing synthetic pesticides contribute to a major share in plant disease management all over the world. However, indiscriminate use of chemicals in agriculture has posed detrimental effects such as pesticide residues in agricultural, environmental pollution,

C. Keswani • K. Bisen • M.K. Chitara • B.K. Sarma • H.B. Singh (✉)
Department of Mycology and Plant Pathology, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi 221005, Uttar Pradesh, India
e-mail: hbs1@rediffmail.com

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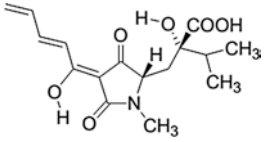
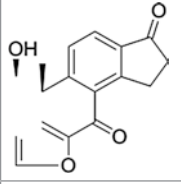
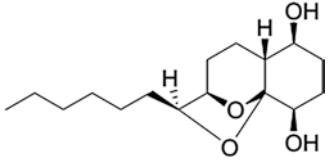
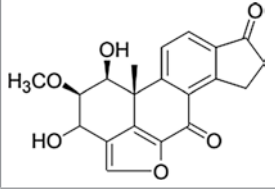
and resurgence of minor pests and pathogens. A worldwide rising of alarms against synthetic pesticides has driven the human interest towards safer alternatives for plant disease management including use of resistant varieties, GM crops, and biological control. Developing resistance varieties with plant breeding is a time-taking process and resistance against all diseases may not be possible. Likewise, GM crops have not been allowed for cultivation by many countries. Biological control includes use of antagonistic microorganisms and botanicals for eco-friendly and effective management of plant disease.

Various biocontrol agents (BCAs) have been studied for their biocontrol potential including *Trichoderma* spp., *Bacillus subtilis*, *Pseudomonas fluorescence, Agrobacterium radiobacter, Beauvaria bassiana,* atoxigenic *Aspergillus niger, Ampelomyces quisqualis,* nonpathogenic *Fusarium*, *Coniothyrium,* etc. (Singh 2006; Keswani et al. 2014; Keswani 2015a, b; Mishra et al. 2015; Bisen et al. 2015). The use of beneficial microorganisms for plant disease management is an effective approach. Application of beneficial microorganism result in various advantages including: (1) fortification of plant rhizospheric soil with beneficial microorganism; (2) suppression of phytopathogens; (3) Plant growth promotion and improvement of plant health; (4) increased nutrient availability and use efficiency in plant; and (5) induced resistance in plants to biotic and abiotic stresses (Sarma et al. 2015; Singh et al. 2014; Harman 2000; Harman et al. 2004; Vinale et al. 2008).

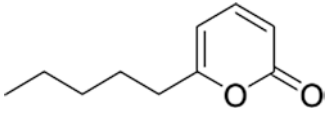
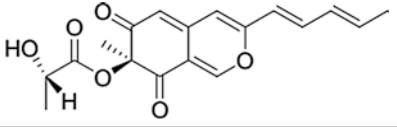
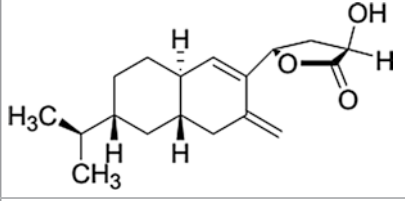
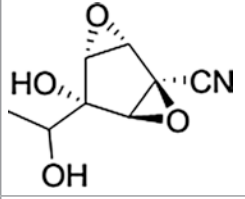
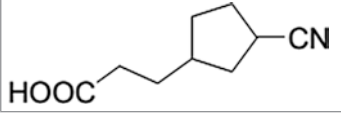
Among various fungal BCAs, *Trichoderma* spp. are most investigated and are currently being employed in biopesticide industries globally (Whipps and Lumsden 2001; Singh et al. 2012; Keswani et al. 2013). Filamentous *Trichoderma* (teleomorph *Hypocrea*) is a saprophytic fungus commonly found in the rhizospheric region of plant. *Trichoderma* spp. is antagonists to a wide range of soil-borne phytopathogenic fungi. Currently, *Trichoderma* spp. are the most frequently investigated fungal biocontrol agents and more than 60 % of the commercially available registered biofungicides worldwide are based on different formulations of *Trichoderma* (Keswani et al. 2013). About 250 products are commercially available for field applications in India alone (Singh et al. 2012). The antagonistic ability of *Trichoderma* spp. against a wide range of pathogens has been one of the most entrancing and fascinating subjects of research in plant defense. Genome sequencing of seven spp. including *T. virens*, *T. atroviride,* and *T. reesei* has offered new insights in understanding the biological control mechanisms using metabolomics and proteomics approaches (Kubicek et al. 2011). Biocontrol mechanism of *Trichoderma* spp. include: (1) mycoparasitism—direct interaction with fungal pathogens, cell wall degradation, and penetration through the secretion of various hydrolytic enzymes (Woo and Lorito 2007); (2) antibiosis, secretion of antimicrobial secondary metabolites (Table 4.1) and antibiotics around its vicinity (Sivasithamparam and Ghisalberti 1998); (3) competition—quenching of micronutrients in soil through efficient mobilization and uptake (Benitez and Rincon 2004; Verma et al. 2007). Plethora of cell wall degrading enzymes and secondary metabolites (SMs) with antimicrobial activity are secreted by *Trichoderma* spp. during the mycoparasitism which effectively hydrolyze the host fungal cell wall (Kubicek et al. 2011; Woo et al. 2006). *Trichoderma* spp. biosynthesize wide range secondary

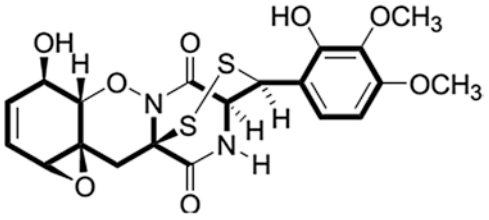
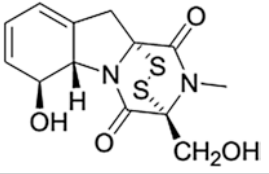
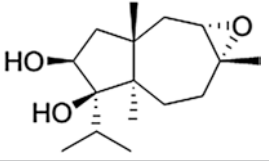
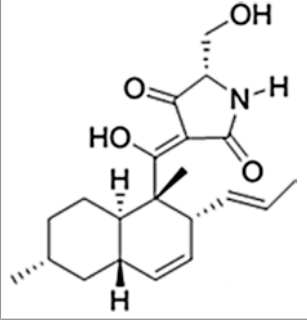
Table 4.1 Secondary metabolites secreted by various *Trichoderma* spp.

Secondary metabolites	Group	Structure	Application	Isolated from *Trichoderma* spp.	References
Harzianic acid	Nitrogen heterocyclic compounds		Biological control, plant growth regulation	*T. harzianum*	Oh et al. (2000), Stoppacher et al. (2010), Fujiwara et al. (1982), Dickinson et al. (1989), Almassi et al. (1991)
Viridin	Viridins		Biological control, antineoplastic, antiatherosclerosis	*T. viride*, *T. koningii*, *T. virens*	Ghisalberti et al. (1992), Ghisalberti and Rowland (1993), Lee et al. (1997), Parker et al. (1997), Marfori et al. (2002), Vinale et al. (2009a, b)
Koninginin A	Koninginins		Biological control, plant growth regulation	*T. koningii, T. harzianum*	Tamura et al. (1975), Itoh et al. (1980), Simon et al. (1988), Cutler et al. (1989), Dunlop et al. (1989), Cutler and Jacyno (1991)
Viridiol	Viridins		Weedicidal, anti-aging	*T. viride*, *T. hamatum*	Cutler et al. (1991a, b), Parker et al. (1995a, b), Huang et al. (1995a, b), Mukhopadhyay et al. (1996)

(continued)

Table 4.1 (continued)

Secondary metabolites	Group	Structure	Application	Isolated from *Trichoderma* spp.	References
6-pentyl-2H-pyran-2-one (6-PP)	Pyrones		Biological control, plant growth promotion, coconut aroma	*T. harzianum*, *T. viride*, *T. koningii*, *T. atroviride*	Watts et al. (1988), Brueckner et al. (1984)
T22-azaphilone	Azaphilones		Biological control	*T. harzianum* T22	Sperry et al. (1998), Endo et al. (1985), Nakano et al. (1990), Vicente et al. (2001)
Cerinolactone	Hydroxy-lactones		Plant growth promotion	*T. cerinum*	Blight and Grove (1986), Brian and McGowan (1945), Stipanovic and Howell (1982), Garo et al. (2003)
Isonitrile trichoviridin	Isocyano		Antibiotic	*T. viride* and *T. koningii*	Tamura et al. (1975); Fujiwara et al. (1982); Coats et al. (1971); Nobuhara et al. (1976)
Dermadin	Isocyano		Antibiotic	*T. hamatum*, *T. viride* and *T. koningii*	Fujiwara et al. (1982), Meyer (1966), Tamura et al. (1975)

Gliovirin	Diketopiperazines		Biocontrol	*T. longibrachiatum*, *T. virens*	Singh et al. (2005), Macias et al. (2000), Kubicek et al. (2011)
Gliotoxin	Diketopiperazines		Antimalarial, immune system suppressor	*T. hamatum*, *T. viride*	Endo et al. (1985), Sivasithamparam and Ghisalberti (1998), Luo et al. (2010)
Trichocaranes A	Daucanes		Plant growth regulation	*T. viride*	Watts et al. (1988), Astudillo et al. (2000), Jaworski et al. (1999), Harris et al. (1993), Fujita et al. (1994)
Trichosetin	Setin-like metabolites		Antibiotic	*T. harzianum*	Sivasithamparam and Ghisalberti (1998), Evidente et al. (2003), Degenkolb et al. (2006), Krause et al. (2006), Ghisalberti (2002)

metabolites which contribute significantly in signaling, biocontrol, and communication with other organisms in various ways. Secondary metabolites are relatively low-molecular-weight chemical compounds (in most cases <3 kDa) mainly produced by microorganisms. These compounds are biosynthesized by fungal primary metabolites in particular pathways (i.e., mevalonate pathways or polyketides derived from amino acids or Acetyl Coenzyme A) (Vinale et al. 2012a, b). SMs confirm various biological functions related to survival of the microorganism including interactions with other micro- and macroorganisms, metal transport, and symbiosis. Typically, in fungi the biosynthesis of SMs has been frequently linked to the growth phase and associated to the stages of morphological differentiation (Keller et al. 2005; Chiang et al. 2009).

Secondary metabolites isolated and purified from *Trichoderma* spp. show various biological functions and engage in regulation of interactions between organisms (Hanson 2003). These secondary metabolites include mycotoxins that are toxic to humans and animals; phytotoxins, that are toxic to plants; antibiotics, natural products that inhibit or kill other microorganisms and pigments, colored compounds with antioxidant activity (Keller et al. 2005; Demain and Fang 2000; Chiang et al. 2009). However, biological activities are not essentially restricted to one particular metabolite or single group (Hanson 2003; Hanson 2008). Some secondary metabolites inhibit specific biological processes such as spore production and hyphal growth, subsequently leading to the death of competing fungi, while others appear to alter the metabolic rate and growth patterns of plants (Keller et al. 2005). *Trichoderma* secondary metabolites play a crucial role in various biological activities and have considerable impact on crop production.

4.2 *Trichoderma* Secondary Metabolites in *Trichoderma*–Pathogen Interaction

Fungal secondary metabolites have been largely used in pharmaceutical industries and are sources of important drugs including antibiotics such as penicillin, streptomycin, cephalosporins, and cyclosporine (Fleming 1929; Ruegger et al. 1976; Endo et al. 1976; Endo and Monacolin 1979; Alberts 1980). Biosynthesis of antibiotics in *Trichoderma* spp. is a well-studied phenomenon and is generally linked to the biocontrol potential of the microorganism (Howell 1998; Sivasithamparam and Ghisalberti 1998). The role of toxic secondary metabolites from *Trichoderma* spp. in interaction with fungal pathogens has been decisively established by various studies (Fravel 1988; Keswani et al. 2014). *Trichoderma* spp. is the most widely used biocontrol agent worldwide owing to their capacity to secrete chemically diverse secondary metabolites that are toxic to a broad range of phytopathogens (Fig. 4.1).

Complex pyranes secondary metabolites Koninginins have been isolated and purified from *T. koningii*, *T. harzianum,* and *T. aureoviride* (Ghisalberti 2002). Koninginins A, B, D, E, and G exhibit potential antagonistic effects against *Gaeumannomyces graminis* var. *tritici* causing take-all disease in wheat (Almassi

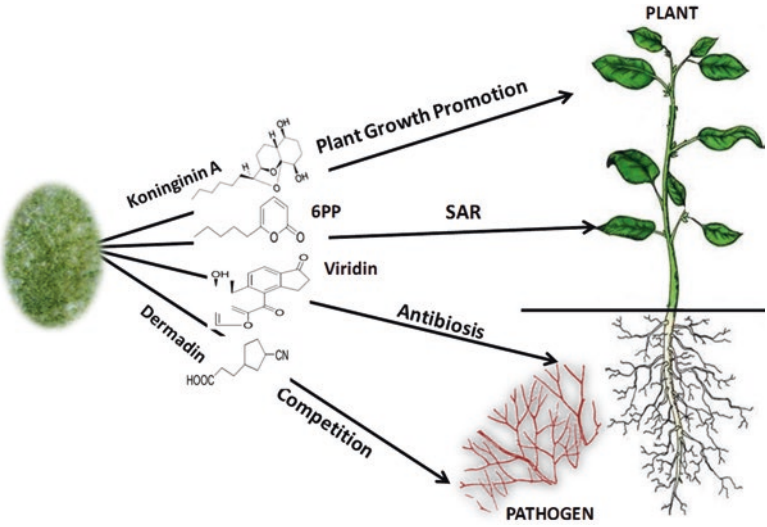


Fig. 4.1 Role of secondary metabolites in *Trichoderma*–plant–pathogen interaction

et al. 1991; Ghisalberti and Rowland 1993). Similarly, Koninginin D has been reported to act against other important soil-borne plant pathogens, including *Phytophthora cinnamomi*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Pythiummiddletonii,* and *Bipolaris sorokiniana* (Dunlop et al. 1989). Koningic acid showed high biocontrol activity against *Bacteroides fragiles* in vitro (Itoh et al. 1980).

Viridin is an antifungal secondary metabolite and mostly produced by from diverse *Trichoderma* spp. including *T. viride*, *T. koningii*, and *T. virens* (Golder and Watson 1980; Singh et al. 2005). Viridin has been reported to prevent spore germination of several other fungi including *Colletotrichum lini*, *Botrytis allii*, *Fusarium caeruleum*, *Aspergillus niger*, *Penicillium expansum,* and *Stachybotrysatra* (Reino et al. 2008; Brian and McGowan 1945). A similar antifungal compound viridiol, produced by *T. viride*, *T. hamatum,* and *Gliocladium* spp. showed biocontrol activity against various pathogens in vivo including *Botrytis cinerea, R. solani,and S. sclerotiorum.*

The pyrone 6-pentyl-2H-pyran-2-one (6PP) commonly isolated from *T. harzianum*, *T. viride*, *T. koningii*, and *T. atroviride* produces coconut aroma in axenically developed colonies. 6PP has shown biocontrol activity both in vitro and in vivo against several phytopathogenic fungi including *Fusarium oxysporum* f. sp. *lycopersici* and *Rhizoctonia solani* (Scarselletti and Faull 1994; Worasatit et al. 1994). Secondary metabolites azaphilones has been isolated from *T. harzianum* T22 showed significant inhibition of various pathogens including *P. ultimum*, *R. solani,* and *G. graminis* var. *tritici* (Vinale et al. 2006). A nitrogen heterocyclic compound named harzianopyridone, produced by *T. harzianum*was found to be very active against *R. solani*, *Botrytiscinerea* (Dickinson et al. 1989), *Pythiumultimum,* and *G. graminis* var. *tritici* (Vinale et al. 2006). Harzianic acid has been isolated and purified from a *T. harzianum* which demonstrated potential antagonistic activity against *Sclerotinia sclerotiorum*, *Pythiumirregulare,* and *R. solani* in vitro (Vinale et al. 2009a).

Butenolides including harzianolide and its derivatives, deydroharzianolide and T39butenolide, have been isolated and purified from different strains of *T. harzianum* (Claydon et al. 1999; Ordentlich et al. 1992). Antifungal activity of these against various phytopathogens has been reported (Almassi et al. 1991; Vinale et al. 2006). A new hydroxy-lactone derivative, cerinolactone, has been isolated from *T. cerinum*, showing antifungal activity against *R. solani*, *B. cinerea,* and *P. ultimum* in vitro (Vinale et al. 2012a, b).

Characteristic 5-membered ring isocyano metabolites are produced by various *Trichoderma* spp. which are very difficult to isolate due to their poor stability (Reino et al. 2008). Isonitrile trichoviridin secondary metabolite biosynthesized by *T. viride* and *T. koningii* (Tamura et al. 1975; Fujiwara et al. 1982; Coats et al. 1971; Nobuhara et al. 1976) exhibited high antifungal activities in vitro (Yamano et al. 1970). Dermadin isolated from *T. hamatum* (Fujiwara et al. 1982), *T. viride* (Meyer 1966), and *T. koningii* (Tamura et al. 1975) was patented in 1971 (Coats et al. 1971). Cyclopentenes isocyano metabolites such as homothallins isolated from *T. koningii* have degrading effect on the morphology of *Phytophthora* spp. (Reino et al. 2008).

Gliovirin and gliotoxin are the two most important secondary metabolites from *Trichoderma* spp. that belong to class diketopiperazines. Gliotoxin was the first metabolite described from *Trichoderma* earlier recognized as *Trichoderma lignorum*, later as *Gliocladium virens*, and currently assigned to *Hypocrea virens* (Brian 1944; Brian and Hemming 1945). Gliotoxin is fungistatic in nature and was already utilized as an antagonist against *Rhizoctonia*in the 1930s (Weindling 1934; Weindling and Emerson 1936). Q group strains of *Gliocladium virens* produce gliotoxin which showed significant antifungal activity against to *R. solani*, but was less active against *P. ultimum*. Strains of the P group produce gliovirin, which acted successfully against *P. ultimum*, but was not active against *R. solani* (Howell 1999). Peptaibols metabolites secondary metabolites, alamethicin from *T. viride* showed inhibitory action against host enzyme α-glucan synthase activity, while interacting with α-glucanases of *T. harzianum* and prevented the cell wall synthesis.

Trichoderma spp. produce various organic acids viz. citric acid, gluconic acid, and fumaric acid which help them in mobilization and uptake of soil minerals. These acids regulate the soil pH that allow the solubilization and uptake of micronutrients and minerals like iron, magnesium, and manganese (Vinale et al. 2008). Competition between various soil microorganisms for nitrogen, carbon, and iron is an important factor during the interactions between *Trichoderma* and phytopathogemic fungi and is one of the biocontrol mechanisms applied by nonpathogenic *Fusarium* and *Trichoderma* spp. (Vinale et al. 2008).

4.3 *Trichoderma* Secondary Metabolites in *Trichoderma*–Plant Interactions

In addition to the direct antagonism against various phytopathogens, *Trichoderma* spp. are also potential root colonizers. They are able to substantially alter the plant metabolism (Harman et al. 2004). It is a decisively established fact that some strains

of *Trichoderma* promote plant growth, enhance disease resistance in plants, increase nutrient availability and use efficiency, and improve crop production (Harman et al. 2004).

4.4 Secondary Metabolites Mediated Induction of Defense Response in Plants

Trichoderma spp. produce various metabolites which induce systemic resistance in plant, such as proteins, i.e., xylanase, low-molecular-weight compounds produced either from plant or fungal cell walls (Harman et al. 2004; Woo et al. 2006) and a virulence-like gene products (Harman et al. 2004). Various low-molecular-weight products from fungal cell walls have been characterized (Woo et al. 2006). They have been reported for elicitation of defense reaction in the host plant when applied to roots or leaves.

Peptaibols are plant defense elicitors produced by *Trichoderma* spp. Alamethicin released by *T. viride*, elicited strong defense responses in *Arabidopsis thaliana* and *Phaseolus lunatus* (Engelberth et al. 2000; Chen et al. 2003). However, knockdown of the non-ribosomal peptide synthetases (NRPS) gene in *T. virens* inhibited the production of peptaibols resulting in considerable reduction in defenseability of plant (Viterbo et al. 2007). Mutation in polyketide synthase/non-ribosomal peptide synthetases (PKS/NRPS) genes resulted in the reduced induction of resistance gene pal (phenylalanine ammonia lyase) by *T. virens,* confirming the role of coupled secondary metabolites derived from polyketide pathway (Mukherjee et al. 2012).

Canola and tomato seedlings specifically treated with 6PP and inoculated with *Leptosphaeria maculanshas* and *B. cinerea*, respectively, have been demonstrated to have reduced disease symptoms (Vinale et al. 2012a, b). 6PP when applied as a soil drench before inoculation with pathogen *Fusarium moniliforme* showed substantial reduction of disease seedling blight and significant plant growth promotion (El-Hasan and Buchenauer 2009). In maize seedlings, 6PP markedly enhanced the activities of polyphenoloxidase, peroxidase, and α-1, 3-glucanase in plant tissues suggesting their role in inducing resistance in maize (El-Hasan and Buchenauer 2009). Foliar application of trichokonin on *Nicotiana tabacum* activated defense responses against tobacco mosaic virus infection. Spray of trichokonin at concentration of 100 nM resulted in 54 % and 30 % of lesion inhibition and reduction in lesion size, respectively (Luo et al. 2010).

In recent study, Malmierca et al. (2015) demonstrated that the trichodiene production by *T. harzianum* erg1-silenced strain demonstrated the importance of the sterol biosynthetic pathway in expression of gene related to the plant defense. The terpene trichodiene (TD) induces the expression of defense and virulence genes. Trichodiene itself is capable of inducing the expression of *Botrytis* genes which leads to the synthesis of bortydial and also induces gene expression in *Trichoderma* related to the terpene biosynthesis. In addition to structural components of the fungal cell membranes, terpene ergosterol also acts as a defense elicitor in plants (Malmierca et al. 2015).

4.5 Secondary Metabolites Mediated Growth Regulation in Plants

Trichoderma produce various chemically diverse secondary metabolites capable of plant growth promotion and inhibition (Fig. 4.1) (Vinale et al. 2008, 2009a, b; Luo et al. 2010). Some world-wide used strains have been reported to stimulate plant development by the production of indole-3-acetic acid (IAA) or auxin analogues via auxin-dependent mechanism (Contreras-Cornejo et al. 2009).

The beneficial effect of several *Trichoderma* secondary metabolites on plant growth and development have been reported (Vinale et al. 2012a, b). 6PP, koninginins, trichocaranes A-D, cyclonerodiol, harzianopyridone, harzianic acid, and harzianolide are some examples of secondary metabolites affecting plant growth (Ghisalberti and Rowland 1993; Cutler et al. 1989; Parker et al. 1997). Harzianolide when applied to *Solanum lycopersicum* and *Brassica napus* at concentration of 1 mg/L resulted in enhanced growth (Vinale et al. 2008). Cerinolactone metabolite isolated from *T. cerinum* when applied to the tomato seedling showed positive results for growth (Vinale et al. 2012a, b).

The inhibitory effects of some secondary metabolites have also been reported. Application of harzianic acid at concentration of 100 and 10 µg/seed resulted in 45 % and 33 % inhibition in canola stem, respectively. Furthermore, application of harzianic acid at a rate of 100, 10, and 1 ng/seed resulted in 42 %, 44 %, and 52 % increased stem length, respectively (Vinale et al. 2009a, b). Application of trichocaranes A and B at a concentration of 10^{-4} M have shown 40 % inhibitory effect in wheat coleoptiles and trichocarane C at concentration of 10^{-3} M resulted in up to 86 % inhibition in growth (Macias et al. 2000). Likewise, koninginins B and C also have shown negative impact on growth of wheat coleoptiles at 10^{-3} M concentration (Parker et al. 1995a) and application of koninginins E and G at concentration of 10^{-3} M resulted in 65 % inhibition of wheat coleoptiles (Cutler et al. 1989; Parker et al. 1995b). It is assumed that *Trichoderma* secondary metabolites act as auxin-like compounds and their optimum activity is recorded between at $10^{-5}$and 10^{-6} M. However, at higher concentrations such metabolites showed an inhibitory effect (Thimann 1937; Cleland 1972; Brenner 1981).

Trichosetin has shown an 87 % inhibitory effect in root growth of *Oryza sativa*, 67 % in *Vigna radiate*, and 91 % in *Lycopersicum esculentum* (Marfori et al. 2002). The harzianopyridone caused complete inhibition of wheat coleoptiles at 10^{-3} M concentration and also caused necrosis in tobacco, bean, and corn at higher concentration (Cutler and Jacyno 1991). 6PP treated seedling showed growth promotion at lower concentrations and inhibitory effects at higher concentrations in wheat seedlings. Interestingly, foliar application of same compound on tomato at 0.166 mg/L showed more prominent growth (Vinale et al. 2008).

The application of secondary metabolites produced and secreted by biocontrol agents for management of weed control is an innovative measure for managing troublesome weeds. In an interesting study, compounds such as viridiols, (3H)-benzoxazolinone, and 2, 4-dihydroxy-1, 4-(2H) benzoxazine-3-one were

identified as principal compounds during composting of chicken manure and showed herbicidal activity against leaf and grass weeds namely *Setaria viridis* and *Amaranthus retroflexus* (Heraux et al. 2005; Javaid and Ali 2011). Herbicidal activity of four *Trichoderma* spp. viz. *T. pseudokoningii* Rifai, *T. harzianum* Rifai, *T. viride* Pers., and *T. reesei* Simmons against *Rumex dentatus*, a noxious weed of wheat was reported when applied as foliar application (Javaid and Ali 2011). Though the weedicidal effect of *Trichoderma* secondary metabolites is at initial stage, the results have been promising at least in green house condition.

4.6 Biosynthesized Secondary Metabolites-Based Bioformulation

Commercial success of biopesticides is largely based on the survival and proliferation of microorganisms in the field condition, prolonged shelf-life, high biocontrol potential, low production cost, and easy mode of application. Microbial pesticides are now gaining popularity among users worldwide. Progress has been achieved in the production of biopesticides with prolonged shelf-life of microorganism in various formulations. Biopesticides are now available in different formulations according to their application such as for seed treatment, foliar spray, drenching, and dusting. However, their contribution in commercial markets is much lower and chemical pesticides still occupy more than 95 % of the total market share. Various factors are responsible for the inconsistency of microbial pesticides in field condition, such as poor shelf-life of microorganism, instability of microorganism in formulation, competition with other microorganisms in soil, and detrimental effect of various abiotic stresses on microorganism in field.

The help of fundamental and applied researches conducted with new ideas and approaches may allow us in the near future to overcome these obstacles associated with application of microbial pesticides. Success can be achieved by the introduction of next generation biosynthesized secondary metabolites-based biopesticides in order to reduce the risks associated with the introduction of microorganisms into the environment and maximize the desired effect on plants. Secondary metabolites could be used in the field as inducers of systemic resistance and stimulators of plant growth promotion. The application of *Trichoderma* secondary metabolites-based bioformulation for crop protection and plant growth promotion may become a reality in the near future. Shelf-life of biosynthesized secondary metabolites-based bioformulations will be prolonged in comparison to whole organism-based formulations. Biosynthesized secondary metabolites-based bioformulations will be easy to handle and can be applied as seed treatment, spray drench easily. Despite varied environmental conditions, it performs better in field conditions as its effects will not be hampered by abiotic stress. Performance of secondary metabolites will not be checked by other fungal biomass in soil as in the case of whole organism-based pesticides. In comparison to the whole organism-based biopesticides, secondary metabolites would perform better in low concentrations, thus being more cost effective.

4.7 Constrains in Commercialization of Secondary Metabolites-Based Bioformulation

Despite being a novel and comparatively beneficial approach than currently available microbial biopesticides and synthetic chemicals, the production of secondary metabolites-based bioformulation has not commenced due to various reasons. Biosafety concerns are the major constrains for secondary metabolites-based bioformulation. Nontarget effects of these bioformulation have been under rigorous review by different committees who chalk out various possible problems with beneficial microorganisms, phytotoxicity, and toxicity to animals and human (Singh 2014a). Opportunistic human pathogenic species of *Trichoderma* have been reported, and this toxicity is directly related to the production of certain secondary metabolites (Keswani et al. 2014). Peptaibols, particularly α-aminoisobutyric acid (Aib) richantibiotics, paracelsin, alamethicins, and trichobrachins showed high toxicity to mammalian model *Daphnia magna*, *Crassostrea gigas,* and *Artemia salina* (Favilla et al. 2006; Poiriera et al. 2007). Another issue related to the secondary metabolite-based product is the persistence of these biosynthesized secondary metabolites in soil, water, and plants.

4.8 Conclusions

Secondary metabolites produced by different classes of fungi have been isolated and characterized. *Trichoderma* spp. is widely recognized for secretion of antimicrobial secondary metabolites and their role in biocontrol of wide range of phytopathogens. In addition to antagonistic effect against phytopathogens, secondary metabolites from various *Trichoderma* spp. including *T. harzianum*, *T.* virens, and *T. atroviride* showed enhanced systemic and localized resistance in plants. Furthermore, considerable influence of secondary metabolites from *Trichoderma* spp. has been reported on plant growth regulation (Harman et al. 2004). *Trichoderma* metabolites are capable of acting on specific pathways of plants including resistance to biotic and abiotic stresses, nutrient uptake, and hormones synthesis, thus altering the plant metabolome and proteome (Vinale et al. 2012a, b).

Although biosynthesized secondary metabolites from antagonistic fungi exhibited greater response in vitro, they have not been commercialized yet despite the possibility of next-generation biosynthesized antimicrobial secondary metabolites-based bioformulations for management of phytopathogens could offer an edge over chemically synthesized pesticides and whole organism formulations. Current research on fungal secondary metabolites has led to new insights in the development of secondary metabolites-based novel biopesticides formulations, which would perform much better in field conditions as compared to whole organisms (Keswani 2015a).

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Chapter 5
Managing Soil Fertility Through Microbes: Prospects, Challenges and Future Strategies

V.S. Bharti, M.L. Dotaniya, S.P. Shukla, and V.K. Yadav

Abstract Soil fertility is the inherent capacity of a soil to provide the essential plant nutrients in adequate amounts and proper proportions for plant growth. There is an immense possibility to enhance soil fertility through microbes, as microbes are "built-in" soil regulators and catalysts contributing to recycling of nutrients into available inorganic forms and provide early warning of land degradation. The focus of this chapter is on the prospect of using microbes as decomposers (cellulose, protein and lignin), formers (humus, nitrate and nitrite), nitrogen fixers, ammonifiers, oxidizers (iron, hydrogen and sulfur), phosphorus solubilizers and denitrifiers. In this context, the factors viz. environmental contaminants and climate change that limit the enhancement of soil fertility through microbes are also discussed. In the latter part of the chapter, the strategies like practising organic farming, zero-tillage, mixed cropping, nano-biofertilizer, biopesticides and soil carbon sequestration for management of soil fertility through microbes are highlighted.

Keywords Carbon sequestration • Biofertilizer • Microbes • Organic farming • Plant nutrients

5.1 Introduction

Soil fertility is a key component which governs the functioning of an agricultural ecosystem. Soil is a complex system comprising of texture, organic matter, soil microbial biomass, air, water and others. Soil functioning is an indicator of soil microbial diversity and crop productivity potential (Singh et al. 2011a, b, c). In general, soil fertility is the ability of a soil to provide plant nutrient to crop, ease of

V.S. Bharti (✉) • S.P. Shukla • V.K. Yadav
Indian Council of Agricultural Research-Central Institute of Fisheries Education, Mumbai 400061, Maharashtra, India
e-mail: bhartivs@rediffmail.com

M.L. Dotaniya
Indian Council of Agricultural Research-Indian Institute of Soil Science, Bhopal 462038, Madhya Pradesh, India

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tillage, penetration for seed germination and support to plant growth (Dotaniya and Kushwah 2013). The fertility of soil enhances the microbial diversity and population, or in other words soil microbial population enhances the fertility levels in agricultural systems (Singh 2013; Singh and Singh 2013; Singh and Pandey 2013; Singh 2014). From eras ago man has beaked the surface soil and sown the seed of the crop, thus practising the art of ancient agriculture. At present, most agricultural practices are rudimentary in nature and primitive in cumulative output. In the early ages slashing and burning was practised to raise food crop to mitigate the requirement of a hungry mouth, with the population increasing in quantum pace, and the higher requirement of food in the mid-60s, there came a change in agricultural technologies in terms of green revolution. As time passed, people understood the role of soil fertility in relation to crop productivity across the globe. Initially, researchers and policymakers concentrated mostly on water and inorganic fertilizers. The nitrogen (N) fertilizers have revolutionized the agricultural production system and food availability in developed and developing countries (Meena et al. 2013). This phase removed the food scarcity from many countries including India. The maximum exploration of arable lands and excessive use of chemical fertilizers showed crop stagnation and decline of soil health, which is a major concern in developing and developed countries (Singh 2015a, b). The intensive cropping system exhausted plant nutrients from the soil and showed nutrient deficiency symptoms on plant parts and also reduced the crop yield and quality. The current farmers' practices relating to food grain production reduced the recycling of organics and other waste generated from the farm, leading to a decline in soil organic matter in soils (Shukla et al. 2013). Thus the world scientific community rethinks the age-old practices, use of natural resources, crop residues, microorganisms and organic amendments (Singh and Strong 2016). In this phase, the uses of microorganism are increasing at a tremendous rate, and the agricultural crop productivity has boosted from a stagnation level. This revolution increased the importance of soil microbial diversity and population in agricultural crop production. Soil microorganisms are mainly responsible for the nutrient release dynamics from any source in soil. It is the integral part of the plant nutrient cycle, soil properties, organic matter decomposition, and organic and inorganic degradation in soil. Microorganisms in soil alter the toxic effect of heavy metals, pesticides, herbicides and other chemicals. They degrade the compounds into smaller fractions which are less toxic. Apart from their role as a natural scavenger, they enrich the soil fertility levels, i.e. fixation of atmospheric nitrogen, mobilization of in situ fixed phosphorus (P) and potassium (K), secreting siderophores. The atmospheric nitrogen fixation by soil microorganism in root nodules of legume crop accounted for more than half of the crop requirement (Singh et al. 2016). Across the globe contribution of biological nitrogen fixation is estimated at 105 Tg $year^{-1}$ (Rao 2014). In the improvement of soil health and crop productivity, the soil microbial biomass plays a vital role and ensures the agricultural and environmental sustainability (Singh et al. 2010; Singh and Singh 2012). In modern agriculture, the non-return or less return of crop residue into the field as a source of organic matter reduced the food for soil microorganism and soil health (Dotaniya 2013). According to Rao (2013), more than 90 % of the earth's

biodiversity is presented in soils and it affects the plant nutrient cycles i.e. nitrogen cycle, phosphorus cycle and carbon cycle (Singh 2015c). The great species diversity in soil is one of the important parameters to measure the soil biodiversity and soil fertility level (Rao 2013). In this communication, a comprehensive report on the role of microorganisms in management of soil fertility has been discussed.

5.2 Organisms in Soil

The soil is teeming with millions of living organisms, which make soil a live and dynamic system. The soil organisms are classified into two groups: (1) soil flora, mainly belonging to the plant kingdom, and (2) soil fauna, thus belonging to the animal kingdom (Fig. 5.1). These soil organisms not only help in the soil developmental process/soil formation, but are also responsible for a number of transformations, and also facilitate the availability of plant nutrients to the crop. The incorporated organic materials are decomposed with the help of soil biota and converted it into an assimilated form of plant nutrient. The activities of the organisms are affected by the soil environment, soil fertility status and the available substrate in soil. The soil organisms are further divided into subgroups: macroorganisms, those that are big enough to be seen with a naked eye, and microorganisms, organism small in size and cannot be seen by the naked eye, requiring magnification (e.g. microscope). However, these organisms are also divided into various groups on the basis of food materials used, or basis of temperature, and molecular oxygen taken for respiration.

On the basis of oxygen taken during the respiration, organisms that cannot survive without oxygen are known as obligate aerobes. Whereas, facultative anaerobes are organisms which are adapted to grow in the presence of oxidized substances i.e.

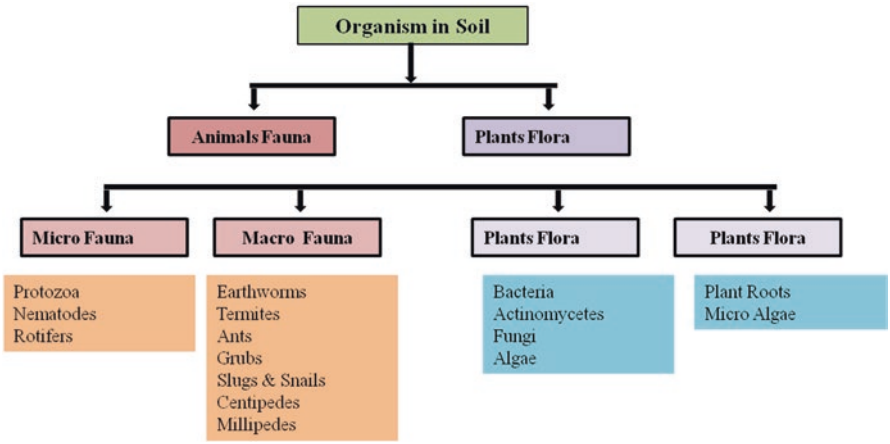

Fig. 5.1 Soil organisms present in soil

SO_4, NO_3 and CO_2. These substances are used as terminal electron acceptors in place of O_2 during respiration. Some organisms grow in the absence of molecular oxygen and are called obligate anaerobes, such as *Clostridia* and *Methanobacteria*. On the basis of tolerance of temperature, organisms are categorized into three groups. They are:

1. Psychrophiles: Organisms capable of growth and reproduction in below 10 °C. Such types of temperatures are found in the few pockets of salted water surrounded by the sea ice.
2. Mesophiles: Organisms having an optimum growth temperature between 20 and 35 °C. Such types of organisms are found in neither too hot nor too cold environments. This group is highly dominated and numerous in most of the agricultural soils of Indian conditions.
3. Thermopiles: Organisms that thrive in the higher temperatures and require optimum temperatures of more than 45 °C for growth. Such organisms are found in hot regions of the earth and in compost pits. The abovesaid particular temperature-sensitive organisms require a particular temperature for optimum growth. Any change in temperature condition reduces the population and growth of the organisms, sometime leading to their death.

Based on the mode of nutrition, organisms are categorized into two groups: autotrophs and heterotrophs. The autotrophs organisms derive their C from CO_2 for growth and cell synthesis, whereas heterotrophs derive energy by the oxidation of organic and inorganic substances. The group of autotrophs is further divided into two groups on the basis of their source of energy. Chemoautotrophs get energy from the oxidation of organic compounds, whereas photoautotrophs derive energy from sunlight for their growth. Chemoautotrophs generally comprise several groups: halophiles, sulfur oxidizers and reducers, nitrifiers, anammox bacteria, methanogens and thermoacidophiles. These organisms derive their energy during the interconversion of inorganic species or organic substances decomposition.

5.3 Macroorganisms in Soil

Soil is a diverse entity, having varieties in fauna and flora (Table 5.1). The macroorganisms play a main role in soil ploughing for better crop production. The macroorganisms are highly mobile, and cannot be easily determined in the population and biomass estimation. It is not uniformly distributed in soil systems; an increasing soil depth reduced the biomass of soil organisms.

These organisms have sizes of more than 2 mm, and are very useful in soils as:

1. They eat plant materials and decompose soil by mixing, churning and fragmentation activities.
2. They form burrows and fine tunnels, which enhance the soil drainage and soil aeration and act as natural ploughers.

Table 5.1 Relative number and biomass of fauna and flora commonly found in the surface 15 cm of soil[a] Brady (1995)

Organisms	Number		Biomass[b]	
	Per m^2	Per gram	kg/ha	g/m^2
Microflora				
Bacteria	10^{13}–10^{14}	10^{8}–10^{9}	400–5000	40–500
Actinomycetes	10^{12}–10^{13}	10^{7}–10^{8}	400–5000	40–500
Fungi	10^{10}–10^{11}	10^{5}–10^{6}	1000–15,000	100–1500
Algae	10^{9}–10^{10}	10^{4}–10^{5}	10–500	1–50
Fauna				
Protozoa	10^{9}–10^{10}	10^{4}–10^{5}	20–200	2–20
Nematodes	10^{6}–10^{7}	10–10^{2}	10–150	1–15
Mites	10^{3}–10^{6}	1–10	5–150	0.5–1.5
Collembola	10^{3}–10^{6}	1–10	5–150	0.5–1.5
Earthworms	10–10^{3}		100–1500	10–150
Other fauna	10^{2}–10^{4}		10–100	1–10

[a]A greater depth is used for earthworm
[b]Biomass value on the basis of liveweight

3. Some macroorganisms eat the soil organic matter and convert it into more accessible plant nutrients.
4. Some of the macroorganisms act as a protector in crop production, and they eat the plant pathogens i.e. mites and termites.

5.3.1 Earthworms

Earthworms are called farmers' friends because they eat the organic material present in soil and convert it into easily digested material called worm casts also known as mull humus. It is a sign of good soil health or indicator of organic soils. It passes the organic matter into the body and grinds it with the help of tiny stones in their gizzard. After the gut digestion, the resulting material is the richest and finest forms of all humus. Earthworm belongs to the phylum Annelida and the class Oligochaeta, and a wide range of genera are found in various types of soils. Across the world, 36 families are identified; these families consist of aquatic or semiaquatic or terrestrials in nature. The earthworms are elongated, cylindrical, segmented, ranging in length from a few millimetres to 1.4 m, such as the giant Australian *Megascolides australis* (Lavelle 1996). In general, uncultivated grasslands have a higher number of earthworms as compared to the cultivated, due to higher amount of plant litter. The total biomass in soil ranged 100–1500 kg/ha in 15 cm surface soil. The earthworm population decreased with cultivation, decreasing the soil organic matter in soil. Earthworm cast is a source of plant nutrients mostly N, P and Ca, and contains more bacteria and organic matter. Some of the earthworm species, i.e. *Geophagus* ingests

the material per day, which is 5–36 times more than their body mass. The earthworms play a vital role in organic matter dynamics and nutrient cycling in soil. On the horizon O and A, the epigeic species are more dominated, and the soil organic matter feeding and casting activities and more microbial decomposition rate were observed. The burrows activity by endogeic earthworm enhanced the soil aeration and water infiltration rate, microbial activities, and plant nutrient mineralization of organic matter. The worm casts provide a platform for microbial activities, secretes various types of organic acids, augment the soil aggregation and improve the soil health.

5.3.2 Termites

In the list of macroorganisms towards the role in soil fertility or soil health, termites find a valuable place. It has cellulose decomposition microbes in the gut, and digests the resistant parts of soil organic matter. It helps to change the soil texture due to formation of mounds and movement of the clay fraction of soil from subsoil. It also modifies the nature and distribution of organic matter. Its excreta have less organic matter content than earthworm casts. The forest soils have many termite mounds which also help in soil drainage and soil moisture retention.

5.3.3 Plant Roots

A plant root constitutes the major part of the plant body in terms of mass and functions. More than 80 % of photosynthates are released into soil through plant roots. It acts as a source of food materials for soil organisms, improves the soil physical, chemical and biological properties and enhances the crop productivity and quality. It has an immense role in soil formation, fertility and productivity and can also be considered as one of the soil macroorganisms. The influence of root is mainly described by the rhizosphere zone activities and its environment. Ranges of root produce are as follows:

1. Exudates—chemical compounds leaking from the roots
2. Secretions—chemical compounds released from root through plant metabolism
3. Mucilages—complex compounds produced by roots or through bacterial degradation
4. Mucigels—gelatinous layers composed of a mixture of mucilages and soil particles
5. Lysates—compounds released from root cells through bacterial degradation

5.4 Microorganisms in Soil

Soil microorganisms play a crucial role in soil ecology and plant nutrient transformation across the global soils. It mediates the soil environment and affects the soil health. In general, fauna and flora having size less than 0.1 mm are known as microorganisms. They are actively involved in soil nutrient transformation and plant nutrient cycles in the soil plant continuum. The population and diversity of soil microorganisms vary in a wide range, and are affected by the fertility level, organic matter, the presence of toxic substances and climatic factors. They are minute in size and cannot be seen with the naked eyes. In a soil having approximately 10^9 soil bacteria, with the help of a microscope, can see only 1 % of the total population.

5.4.1 Bacteria

In view of soil fertility management, bacteria play an immense role to remove the agricultural crop yield stagnation and also manage soil health. It is a single cell organism, also known as the simplest and smallest form of life on earth. It is a light of research to develop biofertilizers and reduce the fertilizer dependency on chemical fertilizers. Its multiplication rate is very high and has a quick response to changes in environmental conditions. The shape and size of bacteria vary for example as round, spiral and rod-like (Coyne 1999). In soil, rod shaped bacteria is more dominant. The role of bacteria in soil forming processes, fertility management, plant nutrient cycles, decomposition and maintaining soil health is well addressed at the global level. Scientific communities long ago screened the role of particular soil bacteria in global nutrient cycling and the advancement of science it kept in the new horizons.

5.4.2 Actinomycetes

During rainy season, the smell emitted from soil is due to geosmins produced by actinomycetes. In general, they compose 10–50 % (10^5–10^8 propagules per gram) of total microbial population in soil, and are mostly found in soil, compost and sediment. Its population varies as pastures > cultivated land > fallow soil. They are prokaryotes and look like fungus structures and are sometimes called ray fungi. It grows in filamentous mycelia and produces spores during the growth period. The optimum temperature for the better growth of actinomycetes is between 28 and 37 °C, but few compost actinomytes can grow up to 65 °C. The actinomycetes do not tolerate waterlogged, drought or aridic conditions with less moisture, but the

spores can tolerate desiccation and can recover 30–90 % microbial growth. At lower temperature the growth of actinomycetes is affected, and below 5 °C the growth is very less, thus it is mostly isolated from hot climatic soils than colder soils. Apart from temperature, several of actinomycetes populations are also affected by the pH and EC of the soils. It is tolerant to high pH or alkaline conditions and can be 95 % isolated from such type of harsh conditions. Few of the unique identities of actinomycetes which distinguish them from fungi are:

- It is prokaryotic, having no cell nucleus.
- It has smaller hyphae (0.5–1.0 μm in diameter) than fungi (3–8 μm in diameter).
- In common, actinomytes are aerobic, whereas bacteria are aerobic and anaerobic in nature.

Frankia, one of the actinomycetes, which is mostly known for the nitrogen fixation in trees and shrubs, can fix nitrogen in inhospitable environmental conditions such as mine locations, degraded and reclaimed lands, and the range of N_2 fixation is 2–300 kg N $ha^{-1}year^{-1}$. It forms nodulation in 25 plant genera, and the population can vary 0–4600 infectious units per gram of soils. The Frankia mainly fixes the nitrogen in Betulaceae, Casuarinaceae, Coriariaceae, Datiscaceae, Elaeagnaceae, Myricaceae, Rhamnaceae families, etc. (Table 5.2).

5.4.3 Fungi

It belongs to the eukaryotic group that also includes yeasts and moulds. It has a nucleus and other cell organelles and is covered with membranes. One of the major differences of fungi from plant, animals and bacteria is having a chitin constituent in the cell wall. The kingdom of fungus has enormous diversity in ecologies and duration of the life cycle. Their morphologies vary from unicellular aquatic chytrids to the bigger mushrooms. It mostly has a filamentous structure with larger cell wall width than actinomycetes. The individual filamentous structures are called hyphae and the network is known as mycelium. In common, hyphae are divided by a cross wall called septa mycelium, whereas without septa they are called coenocytic, producing spore for multiplication (Chhonkar and Pareek 2002). Majorities of fungus are aerobic in nature and prefer optimum soil moisture for favourable growth. The fungi are heterotrophs and derive their nutrition from living organisms and dead tissues, and sometime act as a plant pathogen. The decomposition of organic matter or residue by saprophytic fungi is released to the plant nutrition for microbial action or in soil solution. At the plant nutrition point of view, fungi play a vital role as follows:

- It enhances the plant nutrient availability in soil solution by solubilizing insoluble forms present in the soil, especially P.
- The fungus hypha increases the nutrient absorption from soils and it also modifies root morphology.

Table 5.2 Common actinomycete genera in soil

Genus	Environment
Actinomadura	Wide distribution in cultivated soils
Actinomyces	Human and animal tissue
Actinoplanes	Worldwide distribution
Agromyces	Wide range of soil type
Amycolata	Forest soil and the rhizoplane
Amycolatopsis	Forest and cultivated soils
Arthrobacter	Numerous and widely distributed
Aureobacterium	Several different soil species isolated
Catellatospora	Woodland soils
Cellulomonas	Mostly soil isolates
Corynebacterium	Farmland soils and manure
Dactylosporangium	Sediment, water and plant litter
Frankia	Root nodules of N-fixing shrubs and trees
Geodermatophilus	Desert soils
Glycomyces	Global distribution in soils
Gordona	Isolated from soil and sediments
Kibdellosporangium	Desert and tropical soils
Microbispora	In cultivated soils
Micromonospora	Common in most soil and sediments
Microtetraspora	Cultivated soils
Mycobacterium	Saprophytes in many soils and animal pathogen
Nocardia	Widely distributed in soils
Nocardiodes	Clay soils, Savana grassland and plant litter
Oerskovia	Organic rich soils
Pilimelia	Wide range of soil types
Planobispora	Widely distributed in diverse soils
Pseudonocardia	Compost, manure, cultivated soils
Rhodococcus	Pastures, marine sediments and farmland
Saccharomonospora	Manure, compost and peat
Saccharothrix	Common in many soils
Streptomyces	Widespread in numerous soils
Streptosporangium	Widely distributed in cultivated soil and plant litter
Thermonospora	Compost, bagasse, manures and soils

Developed from Wellington and Toth (1994)

- It increases the plant nutrient mobility due to faster intracellular mobility; the fungus root absorbs the nutrient and traverses the mycorrhizal hyphae.
- Sometime it absorbs the toxic metals from the soil and the significant amount of it is transferred to the crop plant as well.
- The faster growth of fragmented hyphae enhances the absorption rate of nutrients.

Few fungi in nature form a symbiotic association with roots of higher plants and motivate the absorption of plant nutrients mainly less labile in soil. This symbiotic association is popular by the name of "Mycorrhizal association" across the globe. On the basis of fungus development, it can be broadly categorized into two groups.

(a) *Ectotrophic mycorrhizae*: In which fungus form a mantle or sheath around the surface of the root (known as Hartig net) and mycelium develops intercellularly. This type of association is mainly found in the species of *Boletus*, *Amenita*, etc.
(b) *Endotrophic mycorrhizae*: The developmental of fungus hyphae is in the intracellular site of the root without forming a Hartig net. By this association, the penetration of root cells is characterized by the formation of terminal spherical structures well-known as vesicles; it contains P and oil droplets. These associations are called "Vesicular Arbuscular Mycorrhizae" (VAM), and are mainly found in P-deficient agricultural soil. The stored P in vesicles diffuses out into the cytoplasm and is taken by the crop plants. Such types of symbiotic association are found in the genera *Glomus, Endogene,* etc.

Increasing the level of nutrient availability to mycorrhizae, enhanced the absorption surface area by the fine filamentous hyphae of fungus. In infested roots the absorption surface area is enhanced more than 10 times than in uninfested roots. The fine roots reactive fungus hyphae extended upto 8 cm in surrounding roots into the soil, thereby absorption of Cu, Zn and P plant nutrients are enhanced, which do not diffuse readily to the roots. The mycorrhizal infestation enhances the plant stress tolerance ability more than uninfested plants. Its infestation is affected by the climatic condition like drought, water logging, temperature, etc. It also transfers the P from one plant to nearby species mainly in grasslands. A number of researchers find out the effect of P on the uptake behaviour of plant nutrient in agricultural crops (Table 5.3).

5.4.4 Algae

From the inception of earth, it plays a crucial role in modification of earth materials by geochemical processes. Organisms having chlorophylls and other related photosynthetic pigments spread across the world and are mainly found in aquatic ecosystems, but also in all terrestrial ecosystems. The major groups of algae are blue green (Cyanophyta), green algae (Chlorophyta), golden brown (Bacillariophyta/Diatoms)

Table 5.3 Effect of mycorrhiza inoculation with phosphorus fertilizer on plant nutrients in corn

	Without P		P (25 mg/kg)	
	No mycorrhiza	Mycorrhiza	No mycorrhiza	Mycorrhiza
Elements	mg/plant		mg/plant	
P	750	1340	2970	5910
K	6000	9700	17,500	19,900
Ca	1200	1600	2700	3500
Mg	430	630	990	1750
Zn	28	95	48	169
Cu	7	14	12	30
Mn	72	101	159	238
Fe	80	147	161	277

By curtsey of Lambert et al. (1979)

and yellow green algae (Xanthophyta). The blue green algae are also known as cyanobacteria. It is unicellular, long, filamentous, looks like string or beads and has sexual reproduction by fusion and asexual reproduction by fission, fragmentation or zoospores. It is important for the formation of primary productivity through photosynthesis, organic compounds and improved soil fertility status. It helps to remove CO_2 from the atmosphere by formation of $CaCO_3$ and also the synthesis of organic compounds during photosynthesis processes. From the soil health management perspective chlamydomonas is the best algae; in light soil it is used for soil erosion control, and in clay soil it helps in soil aggregation. It enhances the infiltration rate by producing the extracellular carbohydrates during the growth period (Metting et al. 1988). The algae also have symbiotic association with fungi and are known as lichens. Here algae provide carbohydrate synthesis through photosynthesis, the fungi and fungal mass supply mineral nutrition and also regulate water supply. It helps during bioweathering due to excretion of organic acids. It is a source of food materials for earthworms, protozoa, nematodes and mites. Increasing the use of insecticide and pesticides reduces the chances of predatory insects and stimulates the algal growth. The temperature affects the algal growth; it is less active in winter , although few cold-loving algae can grow at less than 2 °C. The boom of algal growth widely seen after the rainfall has high K^+ and also helps to get optimum boon due to maintenance of osmotic balance in high salt soils. The role of algae in the agricultural production system is well known and Dr. R. N. Singh in early 1950s showed the significance of blue green algae in nitrogen fixation in a rice field. In general, blue green algae fix 20 kg N/ha atmospheric N into soil. The important major genera dominant in rice fields are *Nostoc, Anabaena* and *Tolypothrix*. The *Anabaena azollae*, a nitrogen fixing algae, form a symbiotic association with fresh water fern, *Azollae*, and fix atmospheric nitrogen. The *Azolla* is also used as a biofertilizer, and it contributes N as well as organic matter.

5.4.5 Protozoa

They are unicellular animals larger than any microorganisms present in soil. They are saprophytic in nature and feed off inorganic and organic materials dissolved in soil or water. They differ from fungi due to lack of chitin in their cell wall and also filamentous growth. Across the globe, more than 30,000 species are found and size ranges from 10 to 100 μm. The major groups of protozoa are Mastigophora, Sarcodina, Ciliophora and Sporozoa. The genera Mastigophora are mainly dominant in soil ecosystems and enhanced their population by means of sexual (fusion) or asexual (fission). The Euglena is a photosynthetic protozoan under the class of Mastigophora. The life cycle of protozoa consists of active and resting phases (cyst). In the optimum growth conditions active phase is dominant and multiply fast, but in the adverse climatic condition like drought, high temperature its vegetative cell cover it with a thick coating to combat unfavourable soil conditions, it is called as a cyst. Its body has short hairs known as cilia and a long whip structure called flagella, and also pseudopodia or false foot formed by internal protoplasmic movement. Protozoa have a limited role in soil fertility and mainly affect the soil pH, soil structure and organic cycles. In a fertile soil, number of protozoa may be up to one million per gram. Some protozoa also feed bacteria from soil, so that sometimes it reduces the soil fertility level by the detrimental effect on beneficial bacteria. On other side, it reduced the soil pathogenic bacteria and turnover of available plant nutrient in soil and reduces the immobilization of nutrients.

5.4.6 Nematodes

In the line of microfauna, the nematodes are next to protozoa in abundance. It is commonly called eelworms or threadworms, due to the narrow long bodies. It is microscopic in size, and cannot be seen by the naked eyes. It is mainly saprozoic in nature or fed as a parasitic on living crop plants. It does not affect the soil organic matter decomposition much. In most of the cases in agricultural fields it acts as a parasite mainly in vegetable crops. Across the globe, 10,000 nematode species are identified and among them only 1000 are found in soils. On an average, 90 % of nematodes are present in surface soil (15 cm depth). It does not directly affect the soil fertility, but indirectly affect it as regulator of microorganism population in soils. The free-living nematodes are parasites on other nematodes, soil microorganism and consumed as a part of food materials.

5.4.7 Viruses

It is also parasitic in nature and does not take part in plant nutrient transformation in soil; they are ultramicroscopic organisms and smaller than soil bacteria. It cannot be seen by the naked eyes and ordinary light microscope. They are mostly parasitic on

plants, animal and microorganisms. The viruses parasitizing bacteria are called bacteriophages. Sometime in legume crops, failure of nodulation or poor nodulation was observed due to the presence of bacteriophages. It may indirectly affect the plant nutrient cycles by affecting the microbial population in rhizosphere zone.

5.5 Role of Organism in Soil Fertility Enhancement

The soil organic matter is a key component of soil plant nutrient uptake pattern. Organic materials are intrinsic and essential parts of all soils. The organic matter is what makes the soil a living, dynamic system that supports all life on the beautiful earth planet.

5.5.1 N Transformations

Nitrogen is a chemical element present in the ecosystem having with the symbol N and atomic number 7. It is one of the important primary plant nutrients. The N fertilizers have revolutionized the global agricultural production and availability of food material at each corner of the world. It is absorbed by plant roots in the form of NO_3^- and in case of rice as NH_4^+ from the soil solution. It is present in the atmosphere, lithosphere, hydrosphere and biosphere, but more than 98 % present in the lithosphere comprises soils, sediments, silicate minerals, rocks and fossils. With the plant sufficiency condition the concentration of N varies 1–5 % much similar to the potassium concentration (Table 5.4). This is one of the most often deficient plant nutrients in soil, showing clear deficiency symptoms and having serious nutrition problems. It is a constituent of amino acids and protein in crop plants, which enhances the food value of products. Apart from this, they play a major role in cell and cell wall part, and contribute more than 5 % N. In combination of other plant nutrients like C, H, O, P and S, N concentration in cell cytoplasm and cell organelles vary. The long back Haber–Bosch process of ammonia manufacturing brought the tremendous changes in our survival and it was the most important invention in the past millennium (Smil 1999).

The applications of N in crop production have the following pathway in soil: (1) uptake by crop, (2) adsorbed by soil colloids, (3) volatilization, (4) leaching into ground water. The rate of application and the uptake by crop plant are affected by the N losses during the crop uptake and uptake potential of crop species. The N requirement varies from crop to crop or even species to species. It also varies with soil type, management practices and climate. The N is a mobile nutrient in the plant soil system and shows deficiency symptoms in lower leaves of the plant. It is a major constituent of plant cell and cell wall, (Brady 1995). It plays a crucial role in the synthesis of amino acids, nucleic acids, enzymes and coenzymes in plants. It improves the live of protein and quality of fodder and leafy vegetables and helps nutrient mobility in plants. Application of crop residues in soil for improving plant

Table 5.4 Approximate concentration of plant nutrients in mature leaf tissue of agricultural crop plants

Nutrients	Deficient	Sufficient normal	Toxic
N (%)	–	1–5	–
P (%)	–	0.1–0.4	–
K (%)	–	1–5	–
Ca (%)	–	0.2–1	–
Mg (%)	–	0.1–0.4	–
S (%)	–	0.1–0.4	–
Fe (mg/kg)	<50	100–500	>500
Mn (mg/kg)	15–25	20–300	300–500
Zn (mg/kg)	10–20	27–150	100–400
Cu (mg/kg)	2–5	5–30	200–100
B (mg/kg)	5–30	10–20	50–200
Mo (mg/kg)	0.03–0.15	0.1–2.0	>100
Cl (mg/kg)	<100	100–500	500–1000
Ni (mg/kg)	<0.1		

Modified from Jones (1991) and Tisdale et al. (1997)

Table 5.5 Nodulation, nitrogenase activity and N uptake by *Sesbania cannabina* at various growth stages (average daily values plant^{-1})

Growth stages (days)	Nodules	Nodule mass (mg)	Nitrogenase activity[a]	Shoot biomass (g)	N (%)	N uptake (mg)
15–21	13	148	24.0	0.27	2.59	7.1
22–27	19	272	28.0	0.60	2.97	17.6
29–34	30	520	34.7	1.80	3.00	53.2
36–41	55	1287	36.3	3.84	3.09	120.7
43–47	81	1459	23.9	9.73	3.32	322.6
51–54	84	1378	11.2	13.21	2.80	366.7
57–60	83	1609	8.0	22.97	2.83	655.0

Developed from Rao and Ghai (1995)
[a]μmoles of acetylene reduced g^{-1} nodule

nutrient concentration and soil properties enhanced the crop quality parameters and crop yield positively. The N fixation during the crop growth period of 15–45 days could almost meet the N requirement of plants. Therefore, growing of any green manure crops for N enrichment in soil beyond 45 days is not useful (Table 5.5), as they take the N from soil (Rao and Ghai 1995).

Applied N may be taken up by crop plants or losses occurring through various ways can also affect the nitrogen use efficiency. In general N use efficiency can be estimated with the help of following formulas:

(a) Apparent nitrogen recovery (%) =

$$\frac{\left[\text{N uptake in the fertilizer plot}\left(kg/ha\right)\right] - \left[\text{N uptake in the control plot}\left(kg/ha\right)\right]}{\text{Fertilizer N Applied}\left(kg/ha\right)} \text{X100}$$

(b) Agronomic efficiency (kg grain per kg N applied) =

$$\frac{\left[\text{Grain yield in the fertilizer plot}\left(kg/ha\right)\right] - \left[\text{Grain yield in the control plot}\left(kg/ha\right)\right]}{\text{Fertilizer N Applied}\left(kg/ha\right)} \text{X100}$$

(c) Production efficiency (kg grain per kg N absorbed) =

$$\frac{\left[\text{Grain yield in the fertilizer plot}\left(kg/ha\right)\right] - \left[\text{Grain yield in the control plot}\left(kg/ha\right)\right]}{\left[\text{Nitrogen uptake in the fertilized plot}\left(kg/ha\right)\right] - \left[\text{Nitrogen uptake in the fertilized plot}\left(kg/ha\right)\right]}$$

5.5.2 Phosphorus Cycle

It is one of the major plant nutrients, an essential component of energy adenosine triphosphate (ATP) and adenosine diphosphate (ADP), and is known as energy currency. It plays a vital role in the metabolic process, photosynthesis, crop maturation, nitrogen fixation, crop quality and straw strength of cereal crop and reduced the lodging. Inorganic P is largely absorbed by plants from soil as dihydrogen orthophosphate ion ($H_2PO_4^-$), but in high pH soils it is also taken up as monohydrogen orthophosphate ion (HPO_4^-). Apart from these inorganic forms of P, it is also taken up in organic forms like nucleic acids and phytins, but the amount is lower than inorganic uptake (Rattan and Goswami 2002). It is a mobile nutrient in plant and showing the dark green deficiency symptoms on lower leaves, due to accumulation of carbohydrates. The common, P-deficient plants are thin, erect, and have restricted foliage; poor development of later buds, narrow leaves, or in extreme deficiency older leaves become bronzed or show reddish purple tips or leaf margins. To maintain the highest production of food grain level, phosphatic fertilizers play a key role. The deposits of rock phosphate are located only in few countries, i.e. African countries, China, Russia, Brazil, and Australia (Dotaniya et al. 2013b, 2014g). Other countries of the world also have deposits of rock phosphate, but have low grade rock phosphate like India. As per the international standard, those rock phosphates having P concentration lower than 30 % P_2O_5 are called as low grade RP. The low grade RP cannot be used in P fertilizer manufacturing due to higher cost of production.

Inorganic P fertilizers are applied in soil during crop production; they are converted into unavailable form of P to plants. The phosphorus use efficiency (PUE) has varied 15–20 %. So it is a big challenge to the researcher and policymaker to enhance the PUE and economic growth. At present, the use of low grade RP in acidic soil is a common practice, and it reduces the use of commercial fertilizers. Application of crop residue during the crop production also helped to improve the P concentration in soil solution, but the direct supply of P from crop residue is very little. It improves the soil fertility parameters and indirectly enhances the P level in soil or PUE. The P supply from the soil solution to plant root is governed by the potential buffering capacity (PBC) *i.e.* $\Delta Q/\Delta I$, here, ΔQ means a change in the quantity (Q) factor, and ΔI is change in intensity (I) factor. The use of microorganisms for enhancing the in situ P availability during crop production is also a P management strategy. The use of phosphorus solubilizing microorganisms produced various types of organic acids and increased the P concentration in soil solution at various plant growth stages (Dotaniya et al. 2014f). Some of the microorganisms producing gluconic acid are *Pseudomonas sp., Erwinia herbicola, Pseudomonas cepacia,* and *Burkholderia cepacia*, whereas *Rhizobium leguminosarum, Rhizobium meliloti,* and *Bacillus firmus* produce 2-ketogluconic acid, which has the solubility potential of in situ P in soil. Dotaniya et al. (2014b) conducted the field experiment and showed that addition of sugarcane industries byproduct bagasse and press mud enhanced the P uptake by 77 % in wheat compared to control. The addition of sugarcane organic residue, having a concentration of sugar, enhanced the soil microbial population and diversity (Dotaniya et al. 2014e; Dotaniya and Datta 2014). The plant roots secreted the oxalic acid more in P stress condition, and enhanced the conversion of immobilized P to an available form in the soil solution (Dotaniya et al. 2013d). The availability of applied P depends on soil properties, concentration of P, level of N applied, and management practices. The high yielding dwarf wheat varieties gave a 35% higher yield response to P than tall varieties. The applied P efficiency is also affected by the presence of available N concentration in soil. The use of soil amendment is used to enhance the pH or reduce the pH, and it also improved the P level in soil (Dotaniya et al. 2013c).

5.5.3 Sulfur Cycle

Crop plants are dependent on the soil to supply the sulfur that they need for the synthesis of proteins and a number of essential vitamins and cofactors. In agricultural soils, most of the soil sulfur (>95 %) is present as sulphate esters or as carbon-bonded sulfur (sulphonates or amino acid sulfur), rather than inorganic sulphate. Plant sulfur nutrition depends primarily on the uptake of inorganic sulphate. The sulphate ester and sulphonate-pools of soil sulfur are also plant-bio-available, probably due to interconversion of carbon-bonded sulfur and sulphate ester sulfur to inorganic sulphate by soil microbes. In addition to this mineralization of bound

forms of sulfur, soil microbes are also responsible for the rapid immobilization of sulphate, first to sulphate esters and subsequently to carbon-bound sulfur. The rate of sulfur cycling depends on the microbial community present, and on its metabolic activity, though it is not yet known if specific microbial species or genera control this process. The genes present in rhizosphere bacterium, *P. putida*, are involved in the mobilization of sulphonate- and sulphate ester sulfur. Mutants of *P. putida* species are unable to transform sulphate esters and show reduced survival in the soil, indicating that sulphate esters are important for bacterial S nutrition in this environment. From the plant's perspective, the most important form of sulfur is inorganic sulphate since this is the starting point for cysteine biosynthesis. Though it forms only a very small part of the soil sulfur but the symptoms of sulfur deficiency are now frequently encountered in crop plants (Schnug and Haneklaus 1998). However, although inorganic sulphate generally makes up less than 5 % of the sulfur present in agricultural soils, this does not mean that these soils contain limiting amounts of total sulfur. The organic sulfur is present as a heterogeneous mixture of forms, partly included in microbial biomass and partly in the soil organic matter, and very little is known about the chemical identity of the specific sulfur-containing molecules. Traditionally, the types of sulfur species have been differentiated by their reactivity to reducing agents allowing the organosulfur pool to be divided up into three groups: (1) HI-reducible sulfur (thought to be primarily sulphate esters); (2) Raney-nickel-reducible sulfur (mainly amino acids; Freney et al. 1975); and (3) residual carbon-bonded sulfur (thought to be largely sulphonates and heterocyclic sulfur). In the rhizosphere, it is clear that microbes play a critical role as a link in allowing plants to access soil organosulfur. The two critical processes in sulfur cycling in soils, immobilization of inorganic sulfur and mobilization of organically bound sulfur, are both thought to be microbially mediated (Ghani et al. 1992), Probably the clearest finding regarding organosulfur transformations in soils is that the proportions of sulphate ester sulfur and C-bonded sulfur in a given soil, and the rates in which they are interconverted and mineralized, depend critically on the cropping of the soil concerned. The role of the plant in controlling sulfur transformations in the soil is thought to derive primarily from the increased microbial biomass present in the rhizosphere compared with the bulk soil (Castellano and Dick 1991). Sulfur metabolism in the soils can be divided into two pathways: 'biological' pathways catalysed by microorganisms and 'biochemical' pathways depending on free soil enzymes. McGill and Cole (1981) relied heavily on the idea of arylsulphatase as an enzyme secreted by bacteria into the external environment as a response to sulfur limitation. Sulfur can decrease soil pH by oxidation and proton production, at least in rhizosphere and can increase the availability of insoluble micronutrients in soil (Modaihsh et al. 1989). The sulfur microbial oxidation process in soil can take place by various groups of microorganisms. Some photolithotrophs, chemolithotrophs and chemoorganotrophs can accomplish this process. Among them, chemolithotrophs are efficient and their most important genus is *Thiobacillus*. But their population in calcareous soils is usually low. In this process, proton production is dependent on oxidizing microorganism's population in soil. Arthrobacters,

Micrococcus, Mycobacterium, Pseudomonas, and some actinomycetes and chemo-organotrophic fungi are able to oxidize sulphides to sulphates too. The populations of these microorganisms in soil are usually higher than chemolithotrophs and photolithotrophs (Aliasgharzadeh et al. 1998). Beneficial effects of sulfur application in soil, such as crop yield enhancement, pH reduction and increasing the availability of micronutrients, have been demonstrated by several studies (Modaihsh et al. 1989; Kalbasi et al. 1986). S addition increased NUE mainly by increasing the N recovery from the soil.

5.5.4 Other Mineral Elements

Micronutrients are those nutrients required in extremely small quantities (less than 100 ppm in plant dry weight). Plants as well as microorganisms require traces of iron, manganese, copper, zinc, molybdenum, calcium, boron, cobalt, etc. Iron is always abundant in terrestrial habitats, often in an unavailable form for utilization by plants, which leads to the serious deficiency in plants. Soil microorganisms play an important role in the transformations of iron, under anaerobic conditions. The sulphides formed from sulphate and organic sulfur compounds remove the iron from solution as ferrous sulphide. Microbes liberate organic acids and other carbonaceous products of metabolism resulting in the formation of soluble organic iron complex. Thus, iron may be precipitated in nature and immobilized by iron oxidizing bacteria under alkaline soil reaction and on the other hand solubilization of iron may occur through acid formation. Some bacteria are capable of reducing ferric iron to ferrous which lowers the oxidation-reduction potential of the environment (i.e. *Bacillus, Clostridium, Klebsiella*). However, some chemoautotrophic iron and sulfur bacteria such as *Thiobacillus ferrooxidans* and *Ferrobacillus ferrooxidans* can oxidize ferrous iron to ferric hydroxide which accumulates around the cells.

Most of the aerobic microorganisms live in an environment where iron exists in the oxidized, insoluble ferric hydroxide form. They produce iron-binding compounds in order to take up ferric iron. The iron-binding or chelating compounds/ligands produced by microorganisms are called “siderophores”. Bacterial siderophores may act as virulence factors in pathogenic bacteria and thus, bacteria that secrete siderophores are more virulent than non-siderophores producers. Therefore, siderophore producing bacteria can be used as biocontrol agents e.g. fluorescent pseudomonads used to control *Pythium,* causing damping-off diseases in seedlings. Recently VAM has been reported to increase uptake of iron. Microbial genera such as *Bacillus* sp., *Pseudomonas* sp. and *Aspergillus* sp. have zinc solubilizing potential. These organisms are able to perform solubilization with production of organic acids. *Thiobacillus thiooxidans*, *T. ferrooxidans* and facultative thermophilic iron oxidizers solubilized zinc from sulphide ore (sphalerite).

5.6 Environmental Contaminants and Climate Change

5.6.1 Potentially Toxic Element

Fast industrialization enhanced the potential toxic elements (PTEs) concentration in soil. The anthropogenic activities have increased the level of PTEs in the soil-plant-atmosphere system. The toxic elements can reach the soil by means of air borne or degradation of native rock materials. These trace metals are highly toxic to plants, animals and humans. They have a vital potential to impair the biological process of any living organism. The trace metals interact with organic and inorganic materials in the soil and become available to humans through plant consumption. The higher concentration of heavy metal in agricultural field reduced the crop yield and quality.

5.6.2 Chromium (Cr)

It is toxic to plant and soil microorganisms (Dotaniya et al. 2016a). It is divided mainly into Cr (III), less toxic, and Cr (VI) more toxic to plants (Fig. 5.2). The plants do not accumulate higher amounts of Cr, even when it's present in higher amounts its affect on the soil biological function and poor microbial population or diversity was observed. In the soil Cr (VI) is highly toxic to plant and shows adverse effects (poor germination, chlorosis, restricted root and stem developments and black colour of the roots).

Fig. 5.2 Chromium toxicity in spinach

5.6.3 Cadmium (Cd)

It is one of the major toxic elements in the list of trace metals. Accumulation of Cd concentration in the plant varies with species. It accumulates more in leafy vegetables than cereals and fruit crops. The vegetable production in peri-urban areas of megacities with sewage or industrial effluents has greater chances of Cd toxicity. The long-term use of Cd-contaminated wastewater reduces the fertility status of soil and also crop quality. The high concentration of Cd in water bodies or soil caused itai-itai disease in human due to consumption of high Cd-content of rice in Japan. Intake of higher concentration of Cd reduced the concentration of calcium and vitamin D in the human body, and adversely affected the liver and kidney.

5.6.4 Lead (Pb)

It is less toxic than cadmium, but higher concentration in the soil is necessary to produce a toxic response. It is soft, malleable and categorized into heavy post-transition metal. The main sources of lead are petrol, battery industries, pesticides and insecticides. The increase of the mining activities is also increasing the Pb concentration in soil and atmosphere. The accumulated Pb concentration in soil reduced the microbial population and soil organic carbon decomposition and nutrient cycles. It reduced the plant growth, and plants look like stunted growth and produce poor yield. It is fixed into soil by hydrolysis and polymerization. The soil pH, status of organic matter, and P-content affected the Pb uptake pattern of the crop plants.

5.6.5 Mercury (Hg)

Mercury is one of the most toxic heavy metals in nature. The major source of Hg is volcanoes and evaporation from natural water bodies and degassing of the earth's crust. Mercury is mainly used in batteries, thermometers and lamps industries. Apart from this, use of fungicides i.e. Ceresan in crop production is one of the sources of Hg for crop plants. Most of the countries dispose Hg thermometers without the prior treatments. It contaminates the soil and water systems and affects the soil health adversely. In plant tissue of uncontaminated soil systems, the concentration of Hg seldom exceeds 500 ppb, whereas the concentration in natural Hg-bearing rock is 3500 ppb. The concentration of Hg in plant parts is very low, but increasing concentration affects the enzyme and polynucleotides and adversely affects the plant nutrient uptake system. The toxicity of Hg in humans causes a number of diseases like diarrhoea, vision loss, loss of hair, vomiting, stomach problems, etc. The higher concentration in water bodies accumulated in fishes and reaches human bodies through food; this bioaccumulation is known as Minamata disease.

5.6.6 Arsenic (As)

Arsenic builds up in soil environments due to weathering of As-bearing rocks or use of contaminated water for irrigation or other uses. It is a highly toxic heavy metal and key additive in the rat killing poison. The inorganic form of As is found in meat, insecticides, and weedicides. The high rate of fungicides or insecticides application during crop production, leads to buildup of pesticides in soil and later transfer to plants through roots to shoot. It also negatively affects the chemical, physical and biological health of soils. Arsenic problems in soil and water are well known, as an example is West Bengal of India.

5.6.7 Selenium (Se)

This metal is mainly found in association with sulphide rocks in nature. Its derivatives are generated in copper and sulphide industries during commercial purification of metals. It is essential for animals, but not for plants. In the wastewater and sludge, Se presented in elemental Se and selenites bound to hydrous iron oxides and trimethyl selenonium salts. These forms of Se are not easily available to plants. Thus, the contamination chances of Se through contaminated water and sludge application are very limited. Some part of seleniferous soils have been identified in the northwestern part of India i.e. Hoshiarpur and Nawanshahr of Punjab (Dhillon and Dhillon 1997).

5.6.8 Radionuclides

Such type of elements emits radiations like alpha, beta, gamma rays. In simple words a nuclide that is showing radioactive behaviour. When it interacts with humans or plants, it causes modification in cells or even death of cells. The high radiation level creates diarrhoea, nausea, leukaemia, vomiting, etc. In general, the relative order of magnitude of plant uptake of fission products has been found as follows: $^{89\text{-}90}Sr \geq {}^{131}I > {}^{140}Ba > {}^{137}Cs$. The relative magnitude of radionuclides depends on management practices such as cultivation, fertilization and organic matter application.

5.7 Pesticides/Insecticides/Fungicides

Increase in the population requires the food production in higher horizons, and the rates of insecticide and pesticide are increasing 2–5 % annually. In long run the chemicals which are applied for crop protection get accumulated in soil and become

toxic to plants. It potential toxicity depends upon the degradation rate or half-life of the product. Some pesticides are having a too short half-life, whereas some have a half-life of years. The chemical structure of the product also affects the rate of degradation and persistence in the soil plant system. They affect the soil microbial population, modify soil organic matter and plant nutrients; and also area of non pest population in the agricultural fields.

5.8 Effluents

Industrial growth of any country generates an ample number of employments on one side, but on the other side, generates huge volumes of industrial effluent or waste. The uses of industrial effluents are more in developing nations like India. Some of the major industries effluents have adverse affects on soil fertility as mentioned below:

5.8.1 Paper and Distillery effluents

Enormous industrial growth was taken across the world and generated huge volumes of industrial effluents. In India, 75 % of fresh water supplied with paper and pulp industries is emerging as wastewater (Kumar et al. 2014). This wastewater is widely used for agricultural crop production or discharged into water bodies. It has a considerable amount of plant nutrients N, P, K, S and Ca. It has a high pH, Chemical Oxygen Demand (COD), Suspended Solids (SS), Biochemical Oxygen Demand (BOD) and higher amount of Adsorbable Organic Halides. During the paper manufacturing process a number of chlorinated acids is released, i.e. chlorinated resin acids, lignosulphonic acids, chlorinated phenols and derivatives of hydrocarbons in the paper effluents. In addition to this, it also has heavy metals like Cr, Pb, Ni and Hg. The continuous use of these effluents creating loss of soil biota reduced the rate of nutrient transformation, and reduced root exudates in soil.

5.8.2 Tannery Effluents

Tannery industries are one of the oldest industries in India, generating lot of revenue by exporting leather goods across the globe. It is both an organized and unorganized sector and dominated by the presence of family units. As per the production turnover in economics it is classified into small, medium and large units. The major tannery industries are located in Tamil Nadu, West Bengal, Uttar Pradesh, Maharashtra, Punjab, Karnataka, Andhra Pradesh, Haryana and Delhi (Dotaniya et al. 2016b). The tannery effluents generating from the units are huge, and farmers

use them knowingly and unknowingly for crop production. This effluent contains a high concentration of chromium (Cr) and various salts. The long-term use of tannery effluents accumulated the Cr 28–30 times more than fresh water irrigated fields (Dotaniya et al. 2014d). The high salt concentration changes the chemical properties of soil and reduces the soil microorganism, which has directly and indirectly affected the soil organic matter decomposition rate, nitrification rate, plant nutrient transformation and other soil properties (Wang et al. 2006). The accumulated concentration of Cr adversely affects the soil enzymatic activities and soil biomass (Sahu et al. 2007). Dotaniya et al. (2014a) reported that increasing the level of Cr concentration reduced the germination percent, root and shoot growth of wheat and pigeon pea crop (Dotaniya et al. 2014c).

5.9 Effect of Climate Change on Microbe-Mediated Soil Fertility

Increasing CO_2 concentration by the burning of fossil fuel and anthropogenic activities in the atmosphere enhanced the global climate change. The short wave coming from the sun reaches the soil and converts into long wave and reflects back to the atmosphere, but due to elevated concentrations of CO_2, reduced the long wave reflection rate back to the atmosphere. It enhanced the soil and atmospheric temperature. In the CO_2 scarcity regions, it improved the crop growth, but in higher concentration regions it adversely affected the biochemical cycles of crop plants. The higher growth of the vegetative part enhanced the root exudates more in soil and also microbial population and diversity (Kushwah et al. 2014). The rhizospheric population increases tremendously in higher vegetative growth phase due to more availability of food materials for microorganisms. Sometimes increasing the level of root exudates enhances the plant pathogen interaction and affects the plant health adversely (Siciliano et al. 1998). It helps in the nitrogen fixation process by providing the food materials for the bacteria, i.e. *Azospirillum* spp. and *Azotobacter paspali*. The elevated global climate change also promotes the plants to secrete toxic substances like glycosides and hydrocyanic acid, which may reduce the growth of pathogenic microorganisms (Dotaniya 2015; Kundu et al. 2013). The secreted root exudates increase the plant nutrient concentration in soil solution by improving soil pH and altering microclimate of rhizosphere (Dotaniya and Meena 2013; Dotaniya et al. 2013a). The soil temperature modifies the location of AMF hyphal network, the more vesicles in lower temperature soil to warmer soils (Hawkes et al. 2008). The climate change also affects the soil forming process, which is mediated by soil microorganisms. Sardans et al. (2008) showed that in Mediterranean shrubs land warming increased the urease activity by 10 % in soil during the study period (1999–2005). Increasing climate change disturbance also affected the rainfall intensity and pattern, and due to this, some area receive more rainfall and some face prolonged drought. It enhances the soil fertility by means of runoff and soil degradation and poor microbial reaction in soil in drought conditions.

5.10 Different Agronomic Practices for Soil Fertility Enhancement Through Microbes

5.10.1 Tillage and Cultivation

Tillage system with minimum disturbance of soil with the maintenance of some residue on the surface will reduce the erosion risk and maintain the organic matter, microbes and subsequently productivity in long run. Three well-recognized conservation tillage systems are: (1) mulch tillage, (2) ridge tillage, and (3) no tillage. The conservation tillage soils have shown significant ($p < 0.05$) increase in soil respiration (81.1 %), soil microbial biomass carbon (SMBC) (104 %) and soil dehydrogenase (DH) (59.2 %) compared to the conventional tillage soil. The maximum increase in soil microbial activities is found when sole organic source (50 % Farm Yard Manure + 25 % biofertilizer + 25 % Green Manure) has been used in combination with the conservation tillage and the optimum water supply. Microbial activity could be regulated by tillage, water and nitrogen management in the soil in a sustainable manner. Sharma et al. (2011). The long-term no-tillage treatment resulted in higher soil carbon and nitrogen contents, viable microbial biomass, and phosphatase activities at the 0–5 cm depth than the conventional tillage treatment (Mathew et al. 2012).

5.10.2 Cropping Practices

Crop rotations, cover crops, and green manures enhanced soil organic matter, fertility, and tilth. Legume crops, which capture atmospheric nitrogen and "fix" it into forms available to plants, can be used strategically in rotations to meet the needs of nitrogen-demanding crops. Cover crops used after a cash crop capture surplus plant-available nutrients and conserve these for following crops. Cash crops themselves vary in their nutrient demands and considering their needs helps make the most efficient use of the available soil nutrients in a rotation.

5.10.3 Nano-Biofertilizer Application

Nano-fertilizer is eco-friendly and improves soil aggregation, moisture retention and carbon buildup. There is no health hazard and is suitable for all crop varieties including food grains, vegetables and horticulture. Nanotechnology-based products and its applications in agriculture include nano-fertilizers, nano-herbicides, nano-pesticides, recalcitrant contaminants from water, nano-scale carriers, nanosensors, veterinary care, fisheries and aquaculture, detection of nutrient deficiencies, preservation, photocatalysis, nanobarcode, quantum dots, etc. Significant increases in yields have

been observed due to foliar application of nanoparticles as fertilizer (Tarafdar 2012; Tarafdar et al. 2012). It was shown that 640 mg ha^{-1} foliar application (40 ppm concentration) of nanophosphorus gave 80 kg ha^{-1} P equivalent yield of cluster bean and pearl millet under arid environment. One hundred nanometre-diameter fibres can be used as a fertilizer or pesticide absorbent. The technique is called as electrospinning. These high-performance absorbents allow targeted application at desired time and location. Nano-engineered enzymes will allow simple and cost-effective conversion of cellulose from waste plant parts into ethanol. Nanotechnology can reduce the growing risk of rice husk disposal concern as it can be used to make nanosilica used for making other materials e.g. glass and concrete. Nanosensors and nano-based smart delivery systems could help in the efficient use of agricultural natural resources like water, nutrients and chemicals through precision farming. Nanosensors dispersed in the field can also detect the presence of plant viruses and the level of soil nutrients. Nanoencapsulated slow release fertilizers have also become a trend to save fertilizer consumption and to minimize environmental pollution. Nanobarcodes and nanoprocessing could be used to monitor the quality of agricultural produce. Through nanotechnology, scientists are able to study plant's regulation of hormones e.g. auxin—induce root growth and seedling establishment; a nanosensor that reacts with auxin can measure auxin concentration at a particular point using electric signal.

5.10.4 Organic Farming

Soil microorganisms are part of the soil ecosystem and are reported to contribute to soil fertility improvement. The *Rhizobium*/legume symbiosis contributes substantial amounts of biologically fixed nitrogen to cropping systems and significant benefits on yields of crops that follow in rotation. Soil microorganisms such as bacteria and fungi contribute to plant phosphorus nutrition through solubilization of sparingly soluble Al, Fe and Ca phosphates, and mineralization of phosphorus from organic substances. Solubilization is mainly achieved through production of organic acids, chelation and ligand exchange, and other pH lowering mechanisms, whereas mineralization is achieved through production of enzymes such as phytases and phosphatases. Mycorrhizal associations are reported to contribute to plant phosphorus nutrition through increasing root surface area for soil exploration and production of phosphorus solubilizing enzymes and acids. Mycorrhizal fungi and bacteria also solubilize other nutrients such as zinc, copper, potassium and calcium from their precipitated or sparingly soluble forms. Microorganisms also contribute to soil fertility improvement through their roles in composting. Exploitation of microbial activities can contribute to balanced plant nutrition. It can be noted that intensifying soil management practices that maximize microbial activities can go a long way in improving soil fertility with minimal use of chemical fertilizers. A soil's biological properties determine the overall efficiency of nutrient cycling and retention for plant use. Organic farming provides sites for nutrient retention by adding compost and

animal and green manures, which increase organic or humic matter content. In the process, the cation exchange capacity is increased. Additions of organic matter increase the negative charge in soils, increasing the capacity to attract and retain cations. In organic farming, cation exchange capacity of soil is increased, thereby increasing nutrient storage. In a process called mineralization, microbes break down organic plant and animal residues to produce plant nutrients. Soil organisms also promote the development of soil structure by excreting chemicals that bind soil particles together into aggregates. An aggregated soil is said to have good soil tilth. Typically, soils with good tilth have good water infiltration and drainage, and are easy to work. Organic farming deals with "feeding the microbes." Increased microbial activity improves soil physical properties. For example, when microbial activity increases, soil tilth improves. In addition, microbial activity speeds nutrient cycling, increasing the availability of nutrients for plant uptake (when mineralization exceeds immobilization by microbes).

5.10.5 Manures and Fertilizer

The substances which are added to the soil in the form of nutrients for the healthy growth of plants are called manure and fertilizers. Manures are organic substances obtained from the decomposition of dead plants and animal waste. Manures increase the soil fertility by increasing the water and nutrient retaining capacity as well as increasing the population and activity of microbes. The long-term application of excessive chemical fertilizers has resulted in the degeneration of soil quality parameters such as soil microbial biomass, communities, and nutrient content, which in turn affects crop health, productivity, and soil sustainable productivity. Soil microbial activity is important because microbial processes are responsible for decomposition of crop residues and mineralization of organic nutrients to inorganic forms for plant uptake. It is generally accepted that the larger the soil microbial community and the greater the diversity, the greater the potential for nutrient mineralization. Application of manure to soil affects microbial communities. Manure additions to soil have beneficial effects on nutrient cycling, soil microbial biomass, soil microbial activity and enzymatic processes, provided that the manures do not contain high concentrations of undesirable constituents (e.g. potentially toxic trace metals).

5.10.6 Soil Carbon Sequestration

Carbon sequestration and an increase in soil organic matter will have a direct positive impact on soil quality and fertility. Carbon sequestration in agricultural soils counteracts desertification process through the role of increased soil organic matter in structural stability (resistance to both wind and water erosion) and water retention, and increasing water conservation. An increase in carbon sequestration causes

an increase in the operational biodiversity and more effective soil biological functioning, which is normally very low in most agricultural soils. The ability of biochar to curtail leaching, out gassing of greenhouse gases and pollution and improve fertilizer effectiveness occurs through two process. First is the physical adsorption—char's immense capacity to capture and hold nutrient ions. The second process is biological, involving soil microbes that process nutrients into biomolecules and protoplasm.

5.10.7 Biopesticides

Biopesticides, or biological pesticides, are natural pest control agents that are obtained from natural substances. They can come from minerals, plants and bacteria. Biopesticides are less toxic to the environment and natural life. They play an important role in the protection of agricultural foods and protection against unwanted microbial organisms. Furthermore, in biopesticides, the two general microorganisms are the Bacillus species and the plant-incorporated protectants. Of the Bacillus species, the most common used microbial pesticides are strains of *Bacillus thuringiensis* also known as Bt. The different strains of this bacterium produce different mix of proteins, and kill specific insect larvae. Other species include *Bacillus thuringiensis Var. kurstaki, Bacillus popilliae, Bacillus lentimorbus* and *Bacillus sphaericus.* These types of Bt. control moth larvae on plants and the strains are made specifically for the larvae of mosquitoes and flies. The Bt. produces a protein, which binds to the larvae gut receptor and causes it to starve. Whereas, the plant-incorporated protectants, also known as PIP, are pesticidal substances that plants produce from genetic materials that have been added to the plant. The gene from Bt. is introduced to the gene of the other plant, and then the plant can manufacture the substances that destroy the pests. The two pesticides, the Bacillus species and the PIP, work simultaneously in order to produce natural pesticides. These pesticides include plant growth regulators that may help the plant to grow and these mechanisms also include substances that attract and repel pests, such as pheromones or scents that humans smell from the plants. In general, biopesticides are considered to be less toxic than many other conventional pesticides because, they are naturally made pesticides by the plants. When these biopesticides start working, they are usually effective in very small quantities and often decompose fairly quickly. This is beneficial to the environment because this results in avoiding the pollution problems, which most conventional pesticides cause. Additionally, they do not leave harmful residues, can be cheaper than most chemical pesticides and can also be more effective than them in the long term. Biopesticides and biofertilizers are two important cornerstones to improve the quality primarily to achieve food security for the growing population and restore soil fertility. The development of new biopesticides with multiple mode of action against pests and biofertilizers with multi-crop growth promoting activities is most important for sustainable global agriculture.

5.10.8 Soil Amendments

Soil amendments include all inorganic and organic substances mixed into the soil for achieving a better soil constitution regarding plant productivity. Soil amendment does not include mulching, which includes substances lying on top of the soil. It enhances the physical properties of soil, such as water and nutrient retention, permeability and infiltration capacity and aeration. Furthermore, a better soil texture and better root growth avoid soil degradation during heavy rains. By using soil amendment, almost every type of soil can be made fertile; amendments which are not composted and have a high C:N ratio will deplete N from the soil, may cause a salt or an ammonia/ammonium burn, or may cause damage due to heat buildup. Amendments with a low C:N ratio will release N upon further decomposition, thus acting as an organic fertilizer. However, organic matter with a low C:N ratio also should be composed to avoid rapid ammonia/ammonium release and toxicity. Humic acid is a principal component of humic substances, which are the major organic constituent of organic soil amendments. An organic soil amendment (humus) is beneficial to plant growth for longer than recorded history. It was supposed that humus was used directly by plants, but it was shown later that plant growth depends upon inorganic compounds. Organic matter was useful for fertility only as it was broken down with the release of its constituent nutrient elements into inorganic forms. Humus influences soil fertility through its effect on the water-holding capacity of the soil: the spongy structure of organic matter is able to bind water and some inorganic molecules which act as micro- or macronutrients. Consequently, humic acids also slow water evaporation from soils. This is especially important in soils where clay is not present or in a low concentration, in arid areas, and in sandy soils without the capability to hold water. An additional benefit of organic amendments is also the fact that organic matter feeds soil microbes, which in turn release nutrients into the soil, thereby increasing soil fertility. Biofertilizers are living microorganisms that improve the health and quality of different types of soils that help the plants obtain the necessary nutrients. The soil becomes more nutritious and helps the seeds and roots grow to their full potential. Biofertilizers activate the microorganisms that are found in the soil, thus restoring the soils' natural fertility and protecting it against soil diseases and droughts, which stimulates the growth of plants.

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Chapter 6
Trichoderma: A Potent Fungus as Biological Control Agent

Prashant Kumar Sharma and R. Gothalwal

Abstract *Trichoderma* species are free-living fungi that occur in nearly all the soils and other natural habitats. They can be easily isolated from soil and decomposing organic matter. The genus Trichoderma comprises a great number of fungal strains that act as biological control agents, the antagonistic properties of which are based on the activation of multiple mechanisms. A successful biocontrol system is one which is easy and economical to produce, safe, stable in the environment, and easily applied during the conventional agricultural practices. Biofungicides include in a broader sense fungicides of biological origin, i.e., botanical and microbial. The use of microbial fungicides as one of the major components of IPM is gaining acceptance, as these are generally specific, apparently harmless to the beneficial insects, animals, and human beings with no residue problems and environmental hazards. Microbial fungicides are made of microbes such as eco-friendly fungi. Trichoderma strains exert biocontrol against fungal phytopathogens either indirectly, by competing for nutrients and space, modifying the environmental conditions, or promoting plant growth and plant defensive mechanisms and antibiosis, or directly, by mechanisms such as mycoparasitism. These indirect and direct mechanisms may act coordinately and their importance in the biocontrol process depends on the *Trichoderma* strain, the antagonized fungus, the crop plant, and the environmental conditions, including nutrient availability, pH, temperature, and iron concentration. Activation of each mechanism implies the production of specific compounds and metabolites, such as plant growth factors, antibiotics, and carbon and nitrogen permeases. These metabolites can be either overproduced or combined with appropriate biocontrol strains in order to obtain new formulations for use in more efficient control of plant diseases and postharvest applications.

Keywords *Trichoderma* • Phytopathogenic fungi • Biofertilization • Plant protection

P.K. Sharma (✉) • R. Gothalwal
Department of Biotechnology, Barkatullah University, Bhopal, Madhya Pradesh, India
e-mail: prashantbiotech@yahoo.co.in

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6.1 Introduction

Biological control is one of the key components of integrated pest management that envisage the conservation and augmentation of naturally occurring bioagents such as parasitoids, predators, entomopathogens, and antagonistic fungi and bacteria (Singh 2013, 2015). Biofungicides include in a broader sense fungicides of biological origin, i.e., botanical and microbial (Tiwari 2003). The use of microbial fungicides as one of the major components of IPM is gaining acceptance, as these are generally specific, apparently harmless to the beneficial insects, animals, and human beings with no residue problems and environmental hazards. Microbial fungicides are made of microbes such as eco-friendly fungi (Sharma et al. 2013).

Trichoderma was first described more than 200 years ago and was later on envisaged into four genera. The identification and characterization of *Trichoderma* was worked out into a monograph (Rifai 1969), in which this genus was classified into nine aggregates. Rifai recognized several biological species under each aggregate. In India, this genus was isolated by Thakur and Norris (1928) from soils of Madras. It was also reported from various substrates and locations. The identification was mostly based on the morphological characters. Various papers were published in India on the biocontrol efficiency of *T. harzianum, T. koningii, T. longibrachiatum, T. virens, and T. viride*. Even though the species names are mentioned in research papers, invalid names are still used, which brings about a misconception regarding the identification of the species.

Trichoderma genus under Deuteromycotina, Hyphomycetes, Phialosporae, Hyphales, and Dematiaceae has gained immense importance over the last few decades due to its biological control ability against several plant pathogens (Papavizas 1985). Bisset (1991) was unable to define limits of individual biological species and elevated Rifai's species aggregates to species level and recognized two to several species within each of the five sections of the genus. Researchers are interested on this genus because of its novel biological properties and biotechnological applications. Benefits of *Trichoderma* includes the usage in fabric detergent, animal feed, and fuel production as an alternative to conventional bleaching, effluent treatment, degradation of organochlorine pesticides and more importantly as a biocontrol agent as it is harmful as a parasite on mushroom cultivation and on pathogens in organ-transplanted humans (Samuels 1996).

Different species of Hypocrea producing *T. anamorphs* are identified (Rifai and Webster 1966; Bisset 1991; Samuels et al. 1998) under different species names. The species concepts and interspecific relationships between the *Trichoderma* isolates were reviewed along with macromolecular and teleomorphic status (Samuels 1996). The monographic study of Rifai (1969) and revision of the genus by Bisset et al. (1984, 1992) were based only on the cultural and microscopic characters.

The close morphological resemblance that exists between the species of *T. harzianum*, *T. inhamatum*, *T. viride*, *T. asperellum*, *T. koningii* and *T. konilangbra* has been resolved clearly without any controversy using molecular and biochemical analysis by various workers. Even though the studies using

morphological data are used classically, the results obtained from molecular and isozyme analyses with cladistic analysis seem to be more objective in segregating them than traditionally observed and analyzed data (Samuels 1996).

T. parceramosum was considered a different species from *T. ghanense* (Bisset et al. 1992). The molecular studies on this group have revealed that ITS-1 sequences (Kuhls 1997) have a high similarity index based on the RAPD data (Turner 1997). Hence, Samuels et al. (1998) have considered that *T. ghanense* and *T. parceramosum* represent a single species. As *T. ghanense* being older name (Doi 1987), *T. parceramosum* is considered as an illegitimate name. Depending on the molecular data, section *Saturnisporum* represents as an additional species of section *Longibrachiatum* (Kuhls 1997). The ex-type strains of *T. ghanense* of *T. saturnisporum* exhibited same interspecific similarity as the ex-type strains of section *Longibrachiatum* and clustered well with the latter. Hence, both the species of the section *Saturnisporum* are placed along with *Longibrachiatum* section (Turner 1997).

T. longibrachiatum, Trichoderma citrinoviride, T. pseudokoningii, and *T. parceramosum* were originally assigned under section *Longibrachiatum* based on morphological criteria (Bisset et al. 1984). In the same study, *T. reesei* was considered as a synonym to *T. longibrachiatum* on the basis of its morphological characters. Kuhls (1997) studied teleomorph groups and rDNA internal transcribed spacer sequences of all abovementioned species and analyzed the phylogenetic relationships among these species. These workers have found that the section *Longibrachiatum* consists of six groups and their ITS sequence analysis and isozyme studies (Leuchtmann 1996; Samuels 1996) revealed that *T. longibrachiatum* and *T. reesei* showed no similarities, hence, both were considered to be two separate species under the same section. The ribotyping of ITS 1 and 2 regions of different isolates of section *Longibrachiatum* from various biogeographic regions revealed that *T. longibrachiatum* and *T. citrinoviride* overlapped through the geographic ranges, while the *T. longibrachiatum* is common in Africa and India but not in central Asia whereas *T. citrinoviride* is common in southeast Asia and not vice versa (Turner 1997).

T. harzianum is neotypified by an isolate from among the type specimens of the concerned locality and fully redescribed (Gams 1998). *T. harzianum* and *T. inhamatum* comprise group I and *T. atroviride* and *T. viride* group II, based on ITS regions on the rDNA gene cluster and sequence analysis. The strain is an aggressive mushroom competitor and has been initially misidentified as *T. inhamatum* (Section *Trichoderma*) and now has been reclassified as *T. harzianum* (Section *Pachybasium*) after conducting macromolecular studies (Gams 1998).

Trichoderma is easily identified in culture media, by its production of a large number of small green or white conidia from phialides on the profusely or meagerly branched conidiophores. However, the identification of isolates to species level is difficult and confusing due to the complexity and closely related characters of the species. Species concept within *Trichoderma* is very wide and this has resulted in the establishment of many specific and subspecific taxa (Samuels et al. 1998). Kiffer (2009) has recognized a total of 36 species under the genus *Trichoderma*. The *T. stromaticum* has been added to the list with molecular identification.

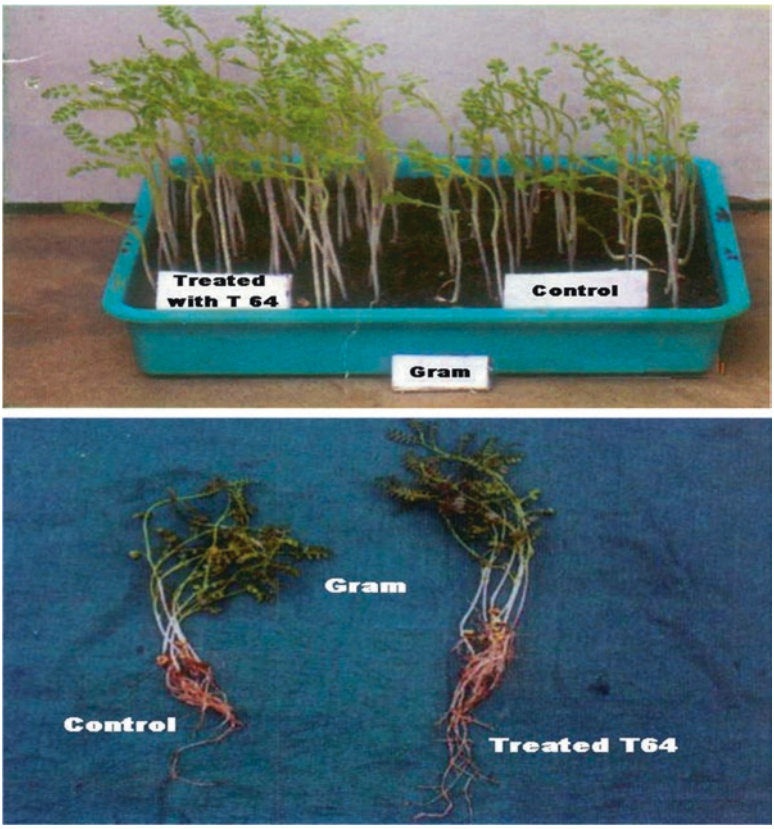

Fig. 6.1 Plant growth promotion ability of *Trichoderma* sp. of Gram (*Cicer arietinum* L.)

Tiwari (2003) reported pathogenic and cultural diversity of *Rhizoctonia solani* causing disease in cereals, oilseeds, pulses, and vegetables. Tiwari (2003) also reported in vitro efficacy of new fungicides against *R. solani* and *Sclerotium rolfsii* with special reference to their nontarget effect on the growth to *T. harzianum* and *Rhizobium leguminosarum.* In vitro and in vivo antagonistic potential of isolates of *Trichoderma* species against *R. solani* was also reported by Sharma and Gothalwal (2010).

The disease controlling potential of *T. harzianum*, *T. viride*, and *T. koningii* is seen in controlling *R. solani* on chickpea (*Cicer arietinum L.*). All species of *Trichoderma* reduced the level of disease when added to soil 1 month before sowing. The potential of antagonistic organisms is affected by several factors i.e., temperatures, pH, soil type, and soil moisture (Sharma et al. 2013). This chapter describes the ability of *Trichoderma* to enhance and regulate the plant growth promotion due to their unique plant growth promoting attributes (Fig. 6.1) and antagonistic abilities.

6.2 Biocontrol Mechanisms

Biological control agents are living organisms whose activities depend mainly on the different physicochemical and environmental conditions to which they are subjected. For this reason, biocontrol exerted by *Trichoderma* strains is sometimes unpredictable. Understanding both the genetic diversity of strains within *Trichoderma* species and their mechanisms of biocontrol will lead to improved application of the different strains as biological control agents. These mechanisms are complex, and what has been defined as biocontrol is the final result of different mechanisms acting synergistically to achieve disease control (Howell 2003).

Biocontrol results from either competition for nutrients and space or the ability of *Trichoderma* biological control agents to produce and/or resist metabolites that either impede spore germination (fungistasis), kill the cells (antibiosis), or modify the *rhizosphere*, e.g., by acidifying the soil, so that pathogens cannot grow. Biocontrol may also result from a direct interaction between the pathogen itself and the biological control agents, as in mycoparasitism, which involves physical contact and synthesis of hydrolytic enzymes, toxic compounds, and/or antibiotics that act synergistically with the enzymes. *Trichoderma* biological control agents can even exert positive effects on plants with an increase in plant growth (biofertilization) and the stimulation of plant defense mechanisms.

Sharma and Gothalwal (2010) isolated a strain of *Trichoderma viride* with high antagonistic potential against *R. solani* and *Sclerotium rolfsii* from soil. It was formulated in talc powder as a biofungicide. The antagonist, *T. viride*, survived in the formulation with 28×10^8 colony forming units even after 4 months of storage. Coating seeds with different doses of the biofungicide increased germination of seed, seedling root and shoot length in Gram (*Cicer arietinum*), Groundnut (*Arachis hypogea*), Onion (*Allium cepa*), Soybean (*Glycine max*), Wheat (*Triticum estivum*), Tomato (*Solanum esculentum*), and Eggplant (*Solanum melongena*). Seed treatment followed by soil application of the biofungicides significantly reduced plant mortality caused by root pathogens and increased yield compared to chemical fungicides and untreated controls. Charati (1998) reported that the seeds treated with talc-formulated *T.viride* and *T. harzianum* at seed gave significant control in comparison with the untreated control. Dutta (1999) tested nine common waste materials on growth and multiplication of the antagonist *T. harzianum*. Naseby (2000) reported that out of five strains of *T. harzianam, T. psuedokoningii, T. koningii, T. longibrachiatum, and T. viride* with known biological control activities were assessed for their effect upon pea growths and their antagonistic activity against large *P. ultimum.*

Six species of *Trichoderma* (*T. hamatum, T. harzianum, T. koningii, T. pseudokoningii, T. longibrachiatum, and T. viride*) were tested for their antagonistic activity against seed-borne *Colletotrichum lindemutthinum* in *Phaseolus vulgaris.* Infected *Phaseolus vulgaris* seeds treated with 0.4 % talc formulation of *T.viride* recorded minimum seed infection and maximum seed germination (Ravi and Thakur 1999).

6.3 Biocontrol by Competition

6.3.1 Fungistasis

Good antagonists are usually able to overcome the fungistatic effect of soil that results from the presence of metabolites produced by other species, including plants. *Trichoderma* strains grow rapidly when inoculated in the soil, because they are naturally resistant to many toxic compounds, including herbicides, fungicides, and pesticides in the soil such as DDT, and phenolic compounds (Chet et al. 1997) and because the strains recover very rapidly after the addition of sublethal doses of some of these compounds. Preparation of *Trichoderma* strains is very efficient in controlling several phytopathogens, such as *R. solani, P. ultimum,* or *Sclerotium rolfsii*, when alternated with methyl bromide, benomyl, captan, or other chemicals (Vyas and Vyas 1995).

Sharma and Gothalwal (2010) reported the disease controlling potential of *T. harzianum* and *T. viride* in controlling *R. solani* on chickpea (*Cicer arietinum L.*) (Fig. 6.2). Both species of *Trichoderma* reduced the level of disease when added to soil 1 month before sowing. It was observed in vitro that isolates T-64 possessed high antagonistic activity while it did not control the disease effectively compared to other isolates, this might be due to weak multiplication in the soil.

6.3.2 Competition for Nutrients

Starvation is the most common cause of death for microorganisms. Hence competition for limiting nutrients results in biological control of fungal phytopathogens (Chet et al. 1997). For instance, in most filamentous fungi, iron uptake is essential for viability (Eisendle et al. 2004) and under iron starvation, most fungi excrete

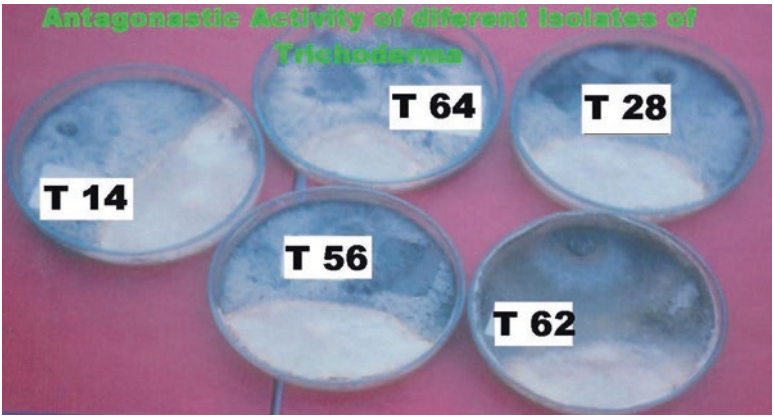

Fig. 6.2 Overgrowth and growth inhibition of *Rhizoctonia solani* and Trichoderma strains

low-molecular-weight ferric-iron-specific chelators, termed siderophores, to mobilize environmental iron (Chet et al. 1997). Subsequently, iron from the ferrisiderophore complexes is recovered via specific uptake mechanisms.

Some *Trichoderma* biological control agents produce highly efficient siderophores that chelate iron and stop the growth of other fungi (Chet and Inbar 1994). For this reason, soil composition influences the biocontrol effectiveness of *Pythium* by *Trichoderma* according to iron availability. In addition, *T. harzianum* controls *Fusarium oxysporum* by competing for both rhizosphere colonization and nutrients, with biocontrol becoming more effective as the nutrient concentration decreases (Sharma and Gothalwal 2010).

Competition has proved to be particularly important for the biocontrol of phytopathogens such as *Botrytis cinerea*, the main pathogenic agent during the pre- and postharvest in many countries (Latorre et al. 2001). The extraordinary genetic variability of this fungus makes it possible for new strains to become resistant to essentially any novel chemical fungicide (Tiwari 2003).

Trichoderma has a superior capacity to mobilize and take up soil nutrients compared to other organisms. The key components of glucose metabolism include assimilation of enzymes and permeases, together with proteins involved in membrane and cell-wall modifications. While the role of the glucose transport system remains to be discovered, its efficiency may be crucial in competition (Delgado-Jarana et al. 2003).

6.4 Biofertilization and Stimulation of Plant Defense Mechanisms

Trichoderma strains are always associated with plant roots and root ecosystems. Some authors has defined *Trichoderma* strains as plant symbiont opportunistic avirulent organisms, able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi and to produce compounds that stimulate growth and plant defense mechanisms (Harman et al. 2004).

6.4.1 Plant Root Colonization

Trichoderma strains must colonize plant roots prior to stimulation of plant growth and protection against infections. Colonization implies the ability to adhere and recognize plant roots, penetrate the plant and withstand toxic metabolites produced by the plants in response to invasion by a foreign organism, whether pathogen or not. There are no data in the literature concerning *Trichoderma* genes specifically expressed during the infection between fungus and plant roots, but there are several reports on altered gene expression during mycorrhizal development (Franken et al. 2002). Mycorrhizal fungi interaction is modulated by plant flavonoids and fungal auxins, followed by morphogenetic events that include appressorium development (Franken et al. 2002; Singh et al. 2011). In addition, genes that encode hydrophobins

Fig. 6.3 Enhanced root development in field crops induced by *Trichoderma* strains T-64

and other cell-wall structural proteins are specifically expressed, or their expression is upregulated (Franken et al. 2002). Hydrophobins are small, functionally proteins, that play fundamental roles in fungal morphogenesis, including infection structures, hyphal aggregation, cell to cell communication, and attachment of hyphae to hydrophobic surfaces and adhesion (Kershaw and Talbot 1998). Strain T-64 stimulates growth of at least Gram (*Cicer arietinum*), Groundnut (*Arachis hypogea*), Onion (*Allium cepa*), Soybean (*Glycine max*), Wheat (*Triticum estivum*), Tomato (*Solanum esculantum*), and Egg plant (*Solanum melongena*) plants, and also protects them against several fungal plant pathogens. Preliminary results have indicated that specific upregulation of hydrophobins during colonization of *Trichoderma* strains and plant roots (Sharma and Gothalwal 2010). The enhancement of root system in groundnut due to *Trichoderma* treatment has been presented in Fig. 6.3.

Some *Trichoderma* strains establish long-lasting colonization of plant root and penetrate into the epidermis. There, they produce or release compounds that induce localized or systemic plant resistance responses (Harman et al. 2004). Plants act against fungal invasion by synthesizing and accumulating phytoalexins, flavonoids and terpenoids, phenolic derivatives, glycones, and other antimicrobial compounds. *Trichoderma* strains are generally more resistant to these compounds than most fungi; nonetheless, their ability to colonize plant root strongly depends on the capacity of each strain to tolerate them (Harman et al. 2004). This resistance, considered an essential requirement for plant colonization, has been associated with the presence of transport systems in *Trichoderma* strains (Harman et al. 2004). Furthermore, spontaneous mutants of Aspergillus *oryzae* that had high resistance to azoles overexpressed transporter genes that were barely detectable in the wild type.

Colonization of plant roots would thus be favored by the isolation of strains that, along with hydrophobins and repellents, also overexpress transporters. Alternatively, the isolation of strains highly resistant to toxic compounds, such as fungicides and/or herbicides, would also increase colonization since such strains frequently display cross-resistance to antimicrobial compounds synthesized by plants.

6.4.2 Biofertilization

Root colonization by *Trichoderma* strains frequently enhances root growth and development, crop productivity, resistance to abiotic stresses, and the uptake and use of nutrients (Arora et al. 1992). Crop productivity in fields can increase up to 300 % after the addition of *T. hamatum* or *T. koningii*. In experiments carried out in greenhouses, there was also a considerable yield increase when plant seeds were previously treated with spores from *Trichoderma* (Chet et al. 1997). The same increase was observed when seeds were separated from *Trichoderma* by a cellophane membrane, which indicates that *Trichoderma* produces growth factors that increased the rate of seed germination (Tiwari 2003). However, there are very few reports on strains that produce growth factors, such as auxins, cytokinins, and ethylene, that have been detected and identified in the laboratory (Arora et al. 1992).

6.5 Rhizosphere Modification

The soil environment influences spore germination, chlamydospore formation, and the production of secondary metabolites, such as siderophores (Eisendle et al. 2004), antibiotics (Chet et al. 1997) and enzymes (Arst and Penalva 2003). There is abundant data in literature describing rhizosphere modifications by biological control agents that impede colonization by pathogens, for instance, antibiotics and toxic metabolites produced by entomopathogenic, mycoparasitic, or mycoherbicide fungi (Vey et al. 2001). Environmental pH is one of the major factors affecting the activity of both *Trichoderma* and pathogenicity factors secreted by different microorganisms. Some antibiotics are degraded by a high pH; air drying and low pH may induce enzyme degradation by acidic proteases (Tiwari 2003) and the growth of many fungi is inhibited by weak acids, such as ascorbic acid, due to a rapid decline in cytosolic and vacuolar pH (Arst and Penalva 2003). Sharma et al. (2013) studied the effect of temperatures, pH, and water potential on biomass production or hyphal extension of *Trichoderma* isolates and found that growth of all three isolates did differ with temperatures and pH.

Therefore, the ability to thrive over a wide range of external pH conditions is an important component of the complex set of characteristics that *Trichoderma*, best adapted to acidic soil, encounters during its interaction with other organisms. One of the mechanisms of *Trichoderma* strains for achieving colonization and pathogen

control in a dynamic pH environment is an appropriate response to each given pH condition. Some strains of *T. harzianum* control external pH strictly, ensuring optimal values for their own secreted enzymes.

Different extracellular proteins are synthesized at different pH. In addition, at the transcriptional level, several proteases, glucanases, cell-wall proteins, and a glucose transporter are pH-controlled, which suggests a pH-dependent transcriptionally controlled response of different enzymes. External pH is also important to pathogens because their pathogenicity factors are produced only within a very narrow range of pHs (Prusky and Yakoby 2003) so that pH modification determines the pathogen's ability to successfully colonize and invade the targeted host. *Trichoderma* strains able to modify external pH and to adapt their own metabolism to the surrounding growth conditions would consequently reduce the virulence of phytopathogens because most pathogenicity factors could not be synthesized. *Trichoderma* ability to change environmental pH may be affected by the surrounding conditions such that a given strain may exert a different kind of control in response to different environmental pH.

6.5.1 Antibiosis

Antibiosis occurs during interactions involving low-molecular-weight diffusible compounds or antibiotics produced by *Trichoderma* strains that inhibit the growth of other microorganisms. Most *Trichoderma* strains produce volatile and nonvolatile toxic metabolites that impede colonization by antagonized microorganism; among these metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-pentyl-α-pyrone, massoilactone, viridian, gliovirin, glisoprenins, heptelidic acid and others has been described (Vey et al. 2001). In some cases, antibiotic production correlates with biocontrol ability, and purified antibiotics mimic the effect of the whole agent. However, there are also examples of antibiotic-overproducing strain, such as gliovirin-overproducing mutants of *Trichoderma virens*, which provide control similar to that of the wild type, and of gliovirin-deficient mutants which failed to protect cotton seedlings from *Pythium ultimum*, whereas the parental strain did (Chet et al. 1997).

In general, strains of *Trichoderma virens* with the best efficiency as biocontrol agents are able to produce gliovirin (Howell 1998). Also, the most effective isolates of *Trichoderma harzianum* against *Gaeumannomyces graminis var. tritici* produce pyrone antibiotics, and the success of the strains was clearly related to the pyrones they produced. The combination of hydrolytic enzymes and antibiotics results in a higher level of antagonism than that obtained by either mechanism alone (Howell 1998; Monte 2001). Synergetic effects between an endochitinase from *Trichoderma harzianum* and gliotoxin, and between hydrolytic enzymes and peptaibols on conidial germination of *B. cinerea* are well known (Howell 1998).

6.5.2 *Mycoparasitism*

Mycoparasitism, the direct attack of one fungus on another, is a very complex process that involves sequential events, including recognition, attack and subsequent penetration and killing of the host. *Trichoderma* sp. may exert direct biocontrol by parasitizing a range of fungi, detecting other fungi and growing towards them. The remote sensing is partially due to the sequential expression of, mostly chitinases, glucanases and protease (Harman et al. 2004). The pattern of induction differs from one *Trichoderma* strain to another. It is believed that fungi secrete exochitinases constitutively at low levels. When chitinases degrade fungal cell walls, they release oligomers that induce exochitinases and attack begins.

Mycoparasitism involves morphological changes, such as coiling and formation of aspersorium-like structures, which serve to penetrate the host and contain high concentrations of osmotic solutes such as glycerol. *Trichoderma* attaches to the pathogen with cell-wall carbohydrates that bind to pathogen lectins. Once *Trichoderma* is attached, it coils around the pathogen and forms the appresoria. The following step consists of the production of peptaibols (Howell 2003), which facilitate both the entry of *Trichoderma* hypha into the lumen of the parasitized fungus and the assimilation of the cell-wall content. The significance of lytic enzymes, reviewed by Viterbo et al. (2002), has been demonstrated by overexpression and deletion of the respective genes. Investigation on the responsible signal transduction pathways of *Trichoderma atroviride* during mycoparasitism has led to the isolation of key components of the cAMP and MAP kinase signaling pathways, such as α-subunits of G proteins (G-α), which control extracellular enzyme, antibiotic production, and coiling around host hypha. In *Trichoderma*, there is biochemical evidence for the participation of G-α in coiling since an increase in coiling ground nylon fibers was detected after the addition of activators of G-protein (Omero et al. 1999).

6.6 Conclusions

Research on the mechanisms responsible for the biocontrol exerted by *Trichoderma* sp. on phytopathogenic fungi has led to a better understanding of such mechanisms, as well as to the isolation of several genes encoding either enzymes and structural or regulatory proteins, or components of signaling pathways that are involved in processes such as the specific recognition of hosts by *Trichoderma* strains. These tools will allow the isolation of improved strains and thus of more efficient formulation to control fungal pathogens in pre- and postharvest periods. This chapter is a general overview of the different reported mechanisms of biocontrol. In addition, some specific mechanisms and/or strategies used or of potential use to improve biocontrol are discussed in relation to Gram (*Cicer arietinum*), Groundnut (*Arachis hypogea*), Onion (*Allium cepa*), Soybean (*Glycine max*), Wheat (*Triticumestivum*), Tomato (*Solanum esculantum*), and Eggplant (*Solanum melongena*) crops.

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Chapter 7
Bioprospecting of Genes from Microbes for Stress Management in Agricultural Crops

Shashi Shekhar, Geetika Gambhir, and Jasdeep Chatrath Padaria

Abstract At present, agricultural systems are under immense pressure to fulfill the increasing demand of food and feed in the context of global climate change with expanding populations. It is an established fact that the global temperature is likely to increase in upcoming decades resulting in the alteration of the edaphic attributes. The change in the edaphic factors due to climatic variations such as annual rainfall, events of drought and flood results in the decrease in soil fertility with water salinization which ultimately results in the reduction of crop yield. Hence in the contemporary era of scientific advancement, it is of central significance to develop mitigation strategies using analytical and forward looking concepts to fulfill the rapidly increasing food demands with ecological sustainability. In recent years, transgenic technology has proven to be very effective in terms of developing stress tolerant crops and use of microbes. This is a relatively simple alternative in terms of cost, unique properties, and ease of handling for broad-spectrum resistance/tolerance against combination of different stresses. Thus, the emphasis is now shifted to the bioprospecting of microbiota to explore the molecular and biochemical potential of microbes towards stress alleviation in crop plants. This book chapter includes an updated progress in microbial gene prospecting and their contemporary use in different plants to enhance their stress tolerance potential. Moreover, the chapter also emphasizes the different metabolic pathways which were previously targeted towards the development of stress tolerant plants and simultaneously proposed theoretical perspective and a baseline knowledge which could be further harnessed in future research towards sustainable agriculture and ecosystem.

Keywords Microbes • Gene prospecting • Climate change • Abiotic stresses

S. Shekhar • G. Gambhir • J.C. Padaria (✉)
National Research Centre on Plant Biotechnology, Pusa Campus,
New Delhi 110012, Delhi, India
e-mail: jasdeep_kaur64@yahoo.co.in

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7.1 Introduction

In the 21st century, the world is confronted with two major challenges which have become a serious threat to the survival of life on planet earth. These include enhanced food production for satisfying the needs of the ever burgeoning human population and the rapidly changing global climate which is causing a major loss in agricultural productivity (Singh et al. 2011a, b, c). The fluctuation in the climatic conditions has always led to constraints in meeting the potential production targets of major agricultural crops. To cope with this double-edged sword situation, food crops that are tolerant to adverse climatic conditions need to be developed. Over the centuries plant breeding approaches have tried to meet these goals and have been successful to a great extent. Unfortunately, in recent years, the available gene pool in major food crops is becoming restricted due to breeding strategies. Hence, the search for newer genetic sources of environmental stress tolerance has become one of the prime goals of agriculture scientists world over. Plant scientists are looking for genes for abiotic as well biotic stress tolerance in organisms inherently adaptable to stresses across species, genera, and even kingdom. Adaptation to the changing environmental conditions is the key for the survival of any organism and forms the hallmark of the biodiversity on the earth (Singh 2015c). Certain microorganisms have the ability to adapt to the adverse climatic conditions in order to survive, whereas many are unable to withstand extreme environments and succumb to the stress pressures (Singh 2015a, b). This inherent property of any microorganism to sustain extreme conditions depends upon its cellular and metabolic pathways (Singh 2013a, b, 2014). Inherently tolerant microorganisms can serve as a good source of genetic repository for not only understanding the stress tolerance mechanisms but also for utilization in the sustainability of climate resilient crops (Singh et al. 2016a, b). Unfortunately due to natural genetic barrier most of the tolerant organisms including microbes cannot be used in breeding programs for improvement of major food crops i.e. rice and wheat. Under these circumstances, the recombinant technology can be successfully utilized to develop transgenics adaptable to climate change induced abiotic stresses.

Microorganisms represent the largest pool of untapped genetic and biochemical diversity on the Earth with a wealth of physiologies and adaptations that enable them to thrive virtually in every environmental niche and inhospitable condition (Singh and Singh 2012). The versatility and adaptive power of the microorganism lies in such a way that their evolutionary head produced a degree of organismic and molecular diversity unparalleled in nature (Singh 2015c). Less than 0.1 % of all microbes are culturable whereas 99.9 % of microorganisms are untapped. Thus microbes could be the potential source for discovery of new biochemical pathways and new genetic capabilities. These studies might provide potential platform for commercially oriented research and development programs to promote the bioprospecting for new, genes, and biochemical pathways which can be further utilized in the genetic manipulation of plants. Sustained growth in agricultural productivity will depend upon continuous improvement of germplasm and improved nutritional

value of crop plants. The traits such as improvement of yield potential, increment in yield stability through stress tolerance, and enhancing the adaptation to high stress condition like heat, salinity, drought and waterlogging are of prime importance. The emphasis is now shifted to the bioprospecting of microbiota for their exact role and function in stress alleviation in crop plants through genetic engineering. Identification of desired genes responsible for the aforesaid attributes may be pursued and explored through gene prospecting and could then be utilized for crop improvement. In the recent years, the challenges of abiotic and biotic stresses can be tackled by prospecting of novel genes from microbes which are better adapted to new climatic and atmospheric conditions (Singh and Singh 2013a, b; Singh and Strong 2016). Thus, implementation of biotechnological approaches to find the candidate genes with enhanced tolerance to combinatorial stresses and to further their utilization in development of stress tolerant food crops would be immensely useful to agriculture.

7.2 Climate Change: Its Comparative Assessment on Agriculture and Environment

The climate change is an additive phenomenon, regulated and governed by different environmental factors in extended periods of time which shows long-lasting effects on the diversity of living beings. The drivers of climate change are interwoven with one another by external and internal forcing mechanisms. These mechanisms include variations in solar radiation, Earth's orbit variations, the albedo effect, mountain building, continental drift, changes in greenhouse gas concentrations, etc. (Singh and Pandey 2013). Certain anthropogenic activities have also been listed as significant causes of recent climate change, referred to as global warming (Singh 2011; Singh and Gupta 2016). At one end, change in climatic conditions is directly influenced by anthropogenic activity while at the opposite end these changes directly influence the sociological development in terms of food, feed, and shelter. As a rapidly growing and emergent population with about 1.2 billion inhabitants in the coming years and decades, India will face the drastic ecological consequences of industrial pollution and global climate change. It is expected that in the coming years, a large population around the globe is likely to suffer from extreme variations in precipitation and temperatures with increased number of natural disasters by the whims of nature. There are different aspects of climate variability which includes temperature, precipitation, drought, and atmospheric carbon dioxide (CO_2), and their interactive impact may lead to the disproportionate production of crops (Ray et al. 2015).

There is a probability that the average temperature of more than 90 % of all habitable continents with many subcontinental regions will rise at a greater extent than the global temperature (Pandey et al. 2014). In coming decades, the major challenge is increasing the crop productivity simultaneously with environmental sustainability. Even the flash of heat stress can reduce crop yield due to lowering the resource use

efficiency (Siebert et al. 2014). Higher temperature and frequent incidences of extreme weather conditions result in significant modification of cropping system and crop yield. It also affects the dynamics of disease severity caused by crop pest and pathogens. It is also expected that disproportionate warming can affect the crop production by altering the ecological sustainability with balance between crop and their associated pathogens. The reduction of crop yield due to pathogenic damage is observed to be more prominent at the early and sensitive stages of a plant's development cycle. In the consideration of negative impact of temperature on the agricultural productivity, the potentiality of extremophiles and their key regulatory metabolic network which helps their sustenance even at very high temperatures can be utilized to impart stress tolerance capacity in plants.

According to the IPCC (2007) forecast, the amount of precipitation is likely to increase at high latitudes while inline pattern of precipitation in subtropical regions will decrease. This type of variability in the precipitation pattern is a result of intensification in the global hydrological cycle. Extremes of daily precipitation are likely to increase in Northern Europe, Southern and Eastern Asia, Australia, and New Zealand, as well as in many other regions (Solomon et al. 2007). Agricultural biodiversity is also threatened due to a decrease in rainfall; rise in sea levels; and increased frequency of severe drought, cyclone, and flood. So there is a constant enhanced demand for irrigated water due to an increase in atmospheric temperature and evapotranspiration rate. The concentration of atmospheric carbon dioxide (CO_2) has also seen a continuous increment since the preindustrial era. In recent times it is more than 100 ppm, leading to a further increase in global warming. In the 21st century, due to the increase of atmospheric CO_2, the buffering capacity of the marine water will be altered with the net result being an increase in the acidity of oceans. As we know that the rate of photosynthesis depends upon the photosynthetic active radiation (PAR) and levels of atmospheric carbon dioxide (CO_2) in an environment.

The term ecological resilience is defined as the ability of an ecosystem to persist despite a disruption and change (Holling 1973), which depends upon the continuity of ecological processes at micro- or macroscales (Peterson et al. 1998). The global change in the climate leads to loss of biodiversity, change in land use/land cover, and modification in hydrological and biogeochemical cycles (Walker and Steffen 1997). These changes will interact with the climate and would be responsible for the alteration of ecosystems in a very intricate and extensive manner. The additive and synergistic impacts of global climate change threaten to reduce ecological resilience at local to global scales, producing ecosystems that are increasingly brittle and sensitive to disruption. Therefore, climate change will cause the dissolution of existing ecosystems and subsequently lead to the formation of new ecosystems. Despite technological advancement, the weather and climate is still a key factor that governs agricultural productivity as well as edaphic factors. The effect of climate change on agricultural productivity is related to patterns of variation in local climate rather than on a global scale. A study suggests that due to climate change, Southern Africa could lose more than 30 % of its main crop by 2030 (Lobell et al. 2008). Along with the abiotic stress imposed by the climate change, many weeds, pests, and fungi thrive under warmer temperature, wetter climates, and increased CO_2 levels. In the

addition to these factors, the agricultural productivity is also directly hampered by seasonal variation, area modification, suitable area for plantation, and change in the plant's pathogen interaction. Thus it is concluded that climate change leads to a modification of the biogeographically agricultural scenario of plantation and cultivation. An analysis of the effect of climate change on agricultural production is highly complex and uncertain due to the interrelationships between the different factors (Sparks and Menzel 2002).

7.3 Stress: Introduction, Type, and Implication

Energy is a prime requirement in order to maintain the structural organization of living beings, and to maintain the order over a period of time. This constant flow of energy provides the dynamic driving force to perform the cellular metabolism, replication, and growth. The maintenance of this steady state of growth and sustainability is called homeostasis. Any change in the surrounding which leads to the disruption or modulation of the homeostasis may be termed as stress. The plant stress implies some adverse effect on the physiology of a plant due to the change in suboptimal conditions which leads to disruption of the plant's homeostatic state.

Plant stress can be primarily classified into two categories:

- A physical or chemical insult imposed by the environment which results in one or more metabolic dysfunctions is termed as abiotic stress.
- A biological insult to which a plant may be exposed during its lifetime is called biotic stress.

The balance between the tolerance and sensitivity determines whether a stress factor has imposed a eustress or distress effect. The duration of stress shows different effects on the plant's life cycle. These effects include long-term and persisting stress causing permanent damage which may lead to cell and plant death as well as short-term stress which induces hardening in the form of acclimatization, adaptation, and repair (Gordon 1992; Lichtenthaler 1996). The differentiation between short-term and long-term stresses is of ultra-importance to decode the mystery behind the plant responses against stress while the extent of variation depends upon the duration and severity of stress (Fig. 7.1).

7.4 Abiotic Stresses

The metabolic pathways related to different abiotic stresses are complex since they are highly interconnected. All abiotic stresses lead to osmotic imbalance which further causes disruption of the cellular ion homeostasis. This change or disruption of homeostasis causes the differential expression of a group of genes which are linked with the determination of growth rate and productivity. The abiotic stress transducer

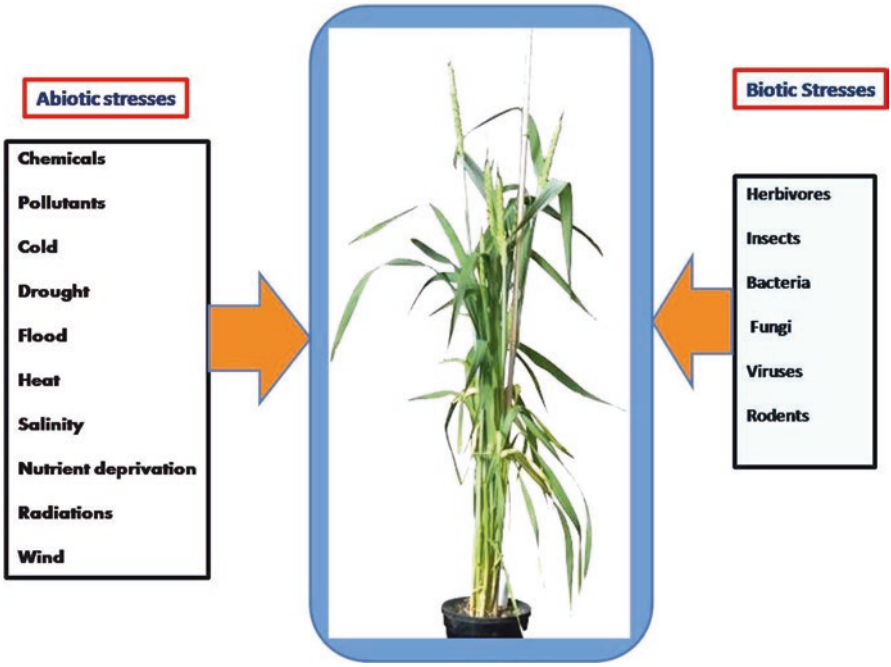

Fig. 7.1 Schematic representation of the abiotic and biotic stresses

and the related signaling mechanism work through several components of signal transduction pathways such as Ca^{2+}, ROS, abscisic acid (ABA), protein kinase, protein phosphatase, and lipid signaling cascades. The perception and response to stress depends upon the differential gene expression and the resulting modification in physiology, metabolism, and growth.

To cope with abiotic stresses, plants perform several mechanisms such as the reduced uptake of toxic ions (Na^+ and Cl^-) into the cytosol and sequestration of the toxic ions either into the vacuole or into the apoplast. Under the salinity stress the osmotic pressure of soil exceeds the osmotic pressure of plant cells which ultimately limits the water and mineral absorption and causes the secondary effects like drought leading to the premature plant death. Further, thermal stress causes reduction in the percentage of seed germination and photosynthetic efficiency which results in yield loss. The heat stress results in dysplastic and indehiscence anther with loss in the function of tapetal cells. In order to manage adaptability of crops to stress, it is imperative that the stress response mechanism in plants is clearly understood due to their interconnectivity at the level of response. Thus the response of different plants to different type of stresses is specific yet highly linked and influenced by each other.

7.5 An Insight: Microbes and Agriculture

Under the subject of stress, either alone or in combination, the plants show differential expression of several genes which are responsible for the stress tolerance or resistance. These genes result in the synthesis and regulation of different metabolites, proteins, and enzymes. The future of microbial gene prospecting is fascinating and boundless due to their interactive relationships with plants. Konstantin Mereschkowski, a Russian Botanist, in 1910 articulated an important theory of evolution referred to as "The theory of symbiogenesis." This theory emphasizes the genetic relatedness of certain microbes and plants. In contrast to this evolutionary theory, exploration of microbial potential towards the optimization of agricultural productivity with ecological sustainability might be better adoption to achieve food and nutritional security.

At present, the understanding about how microbes influence the physiological response of plant under abiotic stress requires an intensive research and analytical approach. Furthermore, many of the underlying physiological and molecular mechanisms still need to be explored in order to optimize the agronomic applications of microbiomes (Singh 2016). The identification and exploration of microbial genetic resources could open a new vista for the development of stress tolerant plants. It can also be harnessed as alternatives for gene pyramiding against different stress simultaneously. Microbial gene prospecting could be utilized in the modulation of nutrient and water uptake, growth promotion, alteration of plant hormonal status, and metabolism with special reference to phytohormone signaling (Fig. 7.2). Furthermore, understanding and improving stress tolerance in plants processes as hormonal status, senescence-hormones, maintenance of source -sink relations, photosynthesis and biomass production-allocation need to be targeted.

7.6 Installation of Climate Resilient Traits in Plants Through Acquisition of Microbial Cellular Machinery

Different pathways which are adversely affected by abiotic stresses and their affects are classified according to their cellular level of responses which are directly influenced by the metabolic pathway of plants (Fig. 7.3). The microbial resources are frequently utilized to enhance the stress tolerance potential of plants under different environmental stresses. Various microbial genes are isolated and characterized in different plant systems to enhance the stress tolerance capacity of plants. In this chapter, we summarize some of the important microbial genes which were validated for increasing stress tolerance ability in different plants (Table 7.1). The utilization of microbial genes in the development of enhanced stress tolerance ability in plants is corroborated with the different cellular and metabolic levels of plants which are affected by stress.

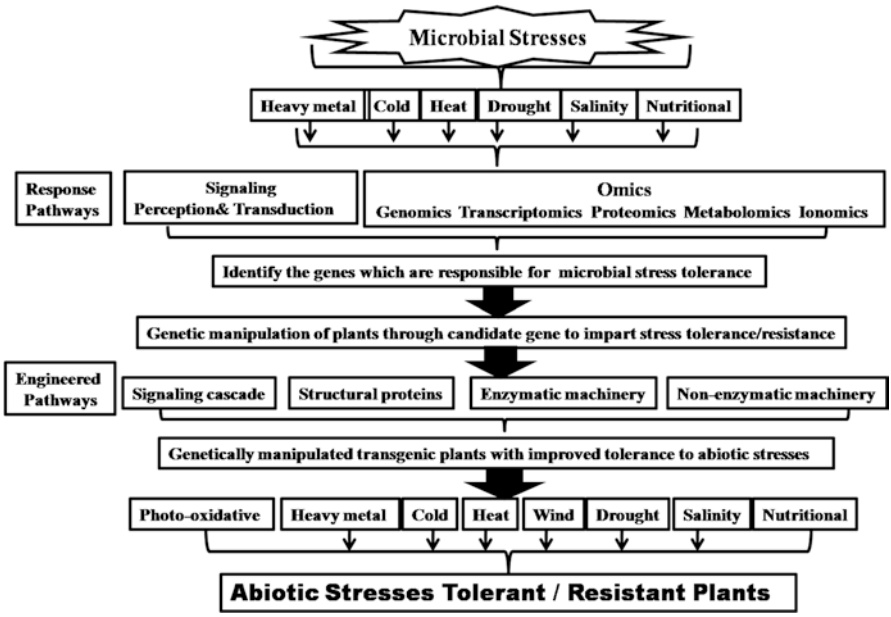

Fig. 7.2 Schematic representation for the development of abiotic stress tolerant/resistant plants through microbial gene prospecting

Ethylene is an important modulator of normal plant growth and development as well as being a key regulator under a wide range of stresses. The catabolic pathway of ethylene in higher plants is well defined and starts from the conversion of methionine to S-adenosyl methionine (SAM). Further, SAM is converted to aminocyclopropane carboxylic acid (ACC) by ACC synthase. Finally, ACC oxidase acts upon ACC to produce ethylene. These last two enzymes are key regulatory players of the ethylene biosynthetic pathway, in which ACC synthase shows inducible activity while ACC oxidase may be constitutive or inducible and vice versa. It is a well-known fact that ethylene biosynthesis appears to increase in any environmental perturbation and increment over its threshold level resulting in inhibition of plant growth or even death. The reduction in the plant growth and development under stress can be altered by the minimization of the ethylene biosynthetic rate above the threshold level which ultimately reduces its deleterious effect on plants. In accordance with the present hypothesis, various research groups (Table 7.1) used the enzyme ACC deaminase which is responsible for the cleavage of the plant ethylene precursor (ACC) into ammonia and α-ketobutyrate, thereby leading to the reduction in the plant ethylene level.

Reactive oxygen species (ROS), also called active oxygen species (AOS) or reactive oxygen intermediates (ROI), are the result of the partial reduction of atmospheric

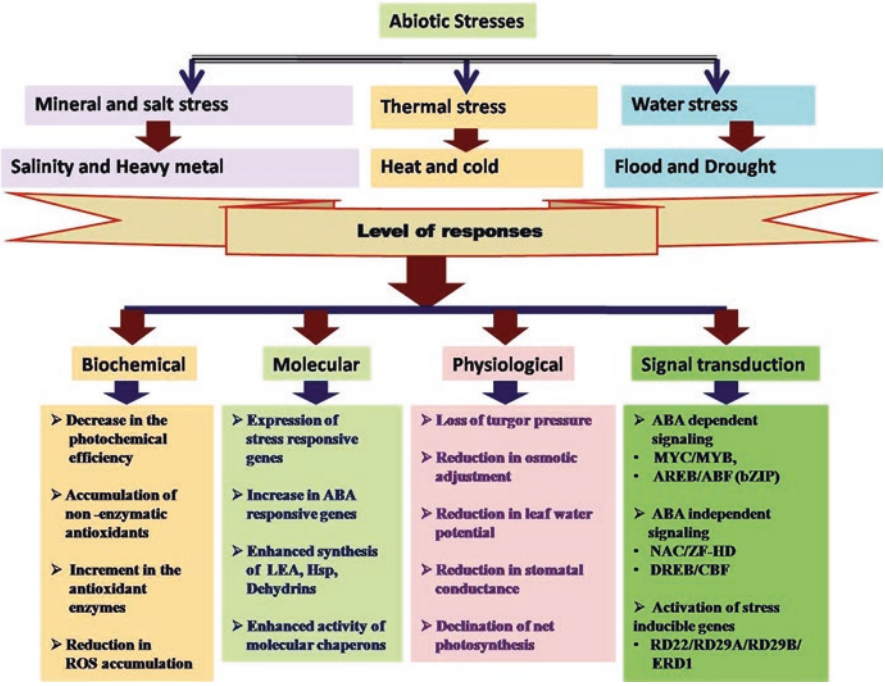

Fig. 7.3 Schematic representation of the different type of abiotic stress and their cellular level of response

oxygen. There are basically four forms of cellular ROS, namely singlet oxygen (O_2), superoxide radical (O_2), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\bullet}OH$), each with a characteristic half-life and an oxidizing potential. ROS can be extremely reactive, especially singlet oxygen and the hydroxyl radical which can oxidize multiple cellular components like proteins, lipids, and nucleic acids, and lead to the alteration in gene expression. It has been hypothesized that ROS production can be the primary symptom of phytotoxicity and this mechanism has been widely studied in plants under abiotic stress. Loss of crop productivity under abiotic stress is indeed related to higher ROS production. In plant cells, the ROS production is strictly regulated by ROS scavenging pathways involving enzymic and nonenzymic antioxidants. Among these, antioxidant enzyme catalase, which converts H_2O_2 to oxygen and water, is considered to be the most important. Unlike the oxygen radicals, H_2O_2 can readily diffuse across biological membranes, thereby causing oxidative stress far from the site of formation. To counter the effect of ROS-mediated stress, different microbial genes were used for the genetic manipulation of plants to enhance tolerance against stress (Table 7.1).

Table 7.1 Different microbial genes being employed for development of abiotic stress tolerant transgenic plants

S No	Name of gene	Sources	Transferred in plant	Role of gene	References
1.	*ACC Deaminase*	Gram negative, Gram positive, rhizobia, endophytes, fungi	Tomato, canola, petunia, tobacco etc.	Decrease level of ethylene in stressed plants	Saleem et al. (2007); Belimov et al. (2001); Ma et al. (2003); Pandey et al. (2005)
2.	Early response to dehydration 15 (*ERD,15*)	*Paenibacillus polymyxa*	*Arabidopsis thaliana*	Against drought tolerance	Timmusk and Wagner (1999)
3.	*Catalase*	*Pseudomonas mendocina, Glomus intraradies*	Lettuce	Alleviate oxidative damage elicited by drought	Kohler et al. (2008)
4.	*Pf1*	*P. fluorescens*	Groundnut	Increase activity of catalase and peroxidase in water-stressed plants	Saravanakumar et al. (2011)
5.	*GB03*	*Bacillus subtilis*	*Arabidopsis thaliana*	Against salinity stress	Ryu et al. (2004)
6.	*ACC deaminase*	*Achromobacter piechaudii* ARV	Tomato and pepper	Reduced ethylene production under transient drought stress	Mayak et al. (2004a)
7.	*ACC deaminase*	*Variovorax paradoxus* 5C-2	Pea plant	Against drought stress	Dodd et al. (2005)
8.	*EPS*	*Rhizobial* strain YAS 34	Sunflower	Against drought stress	Alami et al. (2000)
9.	*EPS*	*P. polymyxa* *Pantoea agglomerans*	Wheat	Against salinity stress	Gouzou et al. (1993); Amellal et al. (1998)
10.	*ACC deaminase*	*Achromobacter piechaudii*	Tomato	Reduced ethylene level and improved plant growth	Mayak et al. (2004b)
11.	*Acc deaminase*	*P. putida* UW4	Canola	Increase plant growth at low temperature under salt stress	Cheng et al. (2007)

12.	*KatE, HPT and NPTII*	*Escherichia coli*	Rice	Against salt tolerance	Prodhan et al. (2008)
13.	*katE*	*Escherichia coli K12*	Jute plant	Improved salt tolerance	Islam et al. (2013)
14.	*Mannitol 1-phosphate dehydrogenase (mtlD) gene*	*mtlD* producing bacteria	Potato	Enhanced tolerance to NaCl stress	Rahnama et al. (2011)
15.	*Choline oxidase (coda)*	*Arthrobacter globiformis*	Tomato	Higher tolerance to salt and water stress	Goel et al. (2011)
16.	*codA*	*A. globiformis*	*Arabidopsis*	Tolerance to high temperature	Alia et al. (1998b)
17.	*Citrate synthetase (Csb)*	*P. aeruginosa*	Tobacco and papaya	Tolerance against heavy metal (aluminum)	De La Fuente et al. (1997)
18.	*DnaK1*	*Aphanothece halophytica*	Tobacco	Conferred high temperature resistance	Ono et al. (2001)
19.	*mtlD* gene	*E. coli*	Tobacco	Tolerance against salt and drought	Shen et al. (1997)
20.	*codA*	*Arthrobacter globiformis*	*Arabidopsis*	Tolerance against salt and cold	Haysashi et al. (1997)
21.	*Choline dehydrogenase (betA)*	*E. coli*	Tobacco	Tolerance against salt	Lilius et al. (1996)
22.	*Trehalose-6-phosphate synthase*	Yeast	Tobacco	Tolerance against drought	Romero et al. (1997)
23.	*Levan sucrase (Sac B)*	*Bacillus subtilis*	Tobacco	Tolerance against drought	Pilon-Smits et al. (1998)
24.	*HAL1*	Yeast	*Cucumis mejo*	Tolerance against drought	Bordas et al. (1997)
25.	Heat Shock Protein soybean 101 kD and At HSPIO1	Yeast	Soybean	Tolerance against heat	Lee et al. (1994)

(continued)

Table 7.1 (continued)

S No	Name of gene	Sources	Transferred in plant	Role of gene	References
26.	*CspA*	*Escherichia coli*	*Arabidopsis*, rice and maize	Tolerance against cold, heat, and water deficits	Castiglioni et al. (2008)
27.	*CspB*	*Bacillus subtilis*	*Arabidopsis*, rice and maize	Tolerance against cold, heat and water deficits	Castiglioni et al. (2008)
28.	*Gut D*	*E. coli*	Maize	Tolerance against salt	Liu et al. (1999)
29.	*ots*A; *ots*B	*E. coli*	Rice	Tolerance to drought, salt and cold	Jang et al. (2003)
30.	*tps1*	Yeast	Tomato	Tolerance to drought, salt, and oxidative stress	Cortina and Culiáñez-Macià (2005)
31.	*tp*	*Pleurotus sajor-caju*	Tobacco	Tolerance to water deficit	Han et al. (2005)
32.	*codA*	*Arthrobacter globiformis*	*Brassica juncea*	Tolerance to salinity	Prasad et al. (2000)
33.	glycine-betaine	*Arthrobacter globiformis*	*Arabidopsis*	Tolerance to cold	Sakamoto et al. (2000)
34.	*MtlD*	*E. coli*	Tobacco	Tolerance to saline stress	Tarezynski et al. (1993)
35.	*HAL1*	Yeast	Tomato	Increased salt tolerance	Gisbert et al. (2000)
36.	*ApoInv*	Yeast	Tobacco	Salt tolerance	Fukushima et al. (2001)
37.	*Acc*	*P. putida* GR12-2	Tobacco	Increased waterlogging tolerance	Grichko and Glick (2001)
38.	*TPS1*	Yeast	Tobacco	Increased drought tolerance	Lee et al. (2003)
39.	*codA*	*Arthrobacter globiformis*	Potato	Increased salinity tolerance	Turhan (2005)

40.	*codA*	*Arthrobacter globiformis*	*Arabidopsis*	Tolerance to salinity and cold stress	Hayashi et al. (2001)
41.	*Parβ*	Yeast	*Arabidopsis*	Protect against Al-toxicity and oxidative stress	Ezaki et al. (2000)
42.	*ArsC*	Bacteria	Tobacco	Tolerance against Cd	Dhankher et al. (2003)
43.	*katE*	*E. coli*	Rice	Tolerance against salt	Nagamiya et al. (2007)
44.	*Mtld gene*	*E. coli*	Peanut	Tolerance against drought	Bhauso et al. (2014)
45.	*katE*	*E. coli*	Tobacco	Tolerance against salt	Shikanaia et al. (1998)
46.	*katE*	*E. coli*	Tobacco	Tolerance against salt	Taweel et al. (2007)
47.	*SOD and Catalase*	*E. coli*	Chinese cabbage	Tolerance against salt	Tseng et al. (2007)
48.	BADH betaine aldehyde dehydrogenase	*E. coli*	Alfalfa [80] (*Medicago sativa* L)	Salinity tolerance	Liu et al. (2011)
49.	CodA/cox choline oxidase	*Arthrobacter globiformis*	Arabidopsis	Increased salt, drought and freezing tolerance	Huang et al. (2000)
50.	ApGSMTApDMT	*Aphanothece halophytica*	Arabidopsis	Tolerance to salinity and chilling	Waditee et al. (2005)
51.	bet	*E. coli*	Cotton	Increased drought tolerance	Lv et al. (2007)
52.	CodA choline oxidase	*A. globiformis*	*Eucalyptus globulus*	Salinity tolerance	Yu et al. (2009)
53.	CodA choline oxidase	*A. globiformis*	Mustard	Heat, cold, and salinity stress tolerance	Wang et al. (2010)
54.	CodA/cox choline oxidase	*A. globiformis*	*Arabidopsis*	Cold tolerance	Alia et al. (1998a)
55.	*ots*A; *ots*B	*E. coli*	Rice	Tolerance to drought, salt, and cold	Garg et al. (2002)

Plant adaptation to abiotic stresses is controlled by a cascade of events at the molecular level. Several defense mechanisms are triggered to reestablish homeostasis and protection of proteins and membranes. Various gene families are responsible for the induction of stress-related defense pathways. These gene families can contain the genes which are involved in the direct protection of important proteins and membranes such as osmoprotectants also known as "compatible solutes" or "osmolytes."

The most common compatible solutes are betaines, sugars (mannitol, sorbitol, and trehalose), polyols, polyamines, and amino acid (proline). These osmolytes seem to perform diverse functions such as osmoregulation, osmoprotection, carbon and nitrogen storage, protection of cellular structures and lipid bilayers (by scavenging active oxygen). They are aptly called as "compatible solutes" since they do not interfere with the biological functions of the cell even at their highest accumulated concentrations. Osmolytes also plays another important role by stabilizing proteins and membrane structures. In accordance with this concept, several research groups genetically transformed various plant species with a microbial gene which regulates and governs the osmolytes biosynthetic pathway.

The "osmophobic theory" suggests that a solvophobic thermodynamic force is responsible for the osmolyte action (Bolen and Baskakov 2001). This theory proposes that the property of unfavorable interaction between the osmolyte and the peptide backbone appears to be selected during evolution for the stabilization of proteins. Protecting osmolytes are ubiquitous in nature, where they play a vital role in stabilizing intracellular proteins against a wide range of adverse environmental conditions.

When plants are under stress conditions, osmotolerance plays an important role in the maintenance of membrane integrity. As not all plant species are capable of natural production or accumulation of these compounds (glycine betaine, proline, and mannitol) in response to stress, extensive research has been conducted to examine various approaches to introduce them into plants. Genetically engineered plants containing transgenes for production of GB, proline, and mannitol have been developed which could ameliorate stress effects by the production of these osmolytes (Table 7.1).

Mannitol 1-phosphate dehydrogenase (*mltD*) is an important enzyme of the mannitol biosynthetic pathway which catalyzes the conversion of mannitol 1-phosphate to mannitol via nonspecific phosphatases. Overexpression of this gene in different plants results in the enhanced tolerance to abiotic stresses.

Glycine betaine (GB) is a quaternary ammonium compound and a compatible solute which accumulates under stress conditions and is synthesized via two distinct pathways from two different substrates, choline and glycine, respectively. The conversion of choline to GB has been studied in a number of organisms and the pathway involves one or two enzymes, depending on the mode of oxidation of choline. The two-enzyme pathway is widespread, occurring naturally in various organisms including microbes. In this pathway, GB is formed as the result of the two-step oxidation of choline via the toxic intermediate betaine aldehyde. In higher plants, the reactions are catalyzed by choline monooxygenase (CMO) and NAD^+-dependent

betaine aldehyde dehydrogenase (BADH), both of which are localized in the stroma of chloroplasts. It provides tolerance to plants under stress condition by stabilizing the quaternary structure of the complex proteins and adjusting osmotic potential in their cytoplasm to maintain water content. In photosynthesis it stabilizes both PS II complexes and Rubisco, at higher level of NaCl and at extreme temperatures. Several research findings reported that overexpression of the gene encoding enzymes for the oxidation of choline in transgenic plants leads to the increased tolerance to different abiotic stresses.

Three major groups of genes are reported to be involved in the stress response. The first group of genes/proteins is involved in signaling cascades and in transcriptional regulation. The second group is those having a role in the protection of membranes and structural proteins and the third group is those involved in water and ion uptake and transport (Wang et al. 2003).

Trehalose, also known as tremalose or mycose, is a nonreducing disaccharide that is present in various biological systems: bacteria, yeast, fungi, lower and higher plants, as well as insects and invertebrates and serves as an energy source, osmolyte or protein/membrane protectant under stress conditions.

In some plants, trehalose does not participate directly in the alleviation of abiotic stress and may act as a signaling molecule. Microarray analyses revealed that both trehalose and trehalose-6-phosphate are affecting the levels of genes involved in abiotic stress.

The pathway of trehalose biosynthesis in plant is TPS-TPP (OtsA-OtsB) which is a two-step process and the most common pathway for trehalose biosynthesis. It is present in both prokaryotes and eukaryotes (archaea, bacteria, fungi, plants, and arthropods) (Paul et al. 2008). In plants, trehalose 6-phosphate synthase (TPS) catalyzes the synthesis of the intermediate trehalose-6-phosphate from glucose-6-phosphate and Uridine Diphosphate (UDP)-glucose, and then trehalose-6-phosphate phosphatase (TPP) catalyzes the dephosphorylation of trehalose-6-phosphate to trehalose.

During abiotic stress (dehydration), membranes are destabilized because of lipid phase transitions and vesicle fusion. Trehalose, even in small quantities, completely inhibits vesicles fusion and depresses the phase transition temperature of dry lipids, maintaining them in liquid crystalline phase in the absence of water (Crowe et al. 1992). It appears that during dehydration or freezing trehalose molecules replace bound water normally associated with biological structures (Donnamaria et al. 1994). Because of its high hydration potential, trehalose may stabilize dry biological membranes and proteins by hydrogen bonding of its hydroxyl groups to the polar groups of proteins and phosphate groups of membranes. Another mechanism by which trehalose protects against desiccation stress is vitrification. Trehalose has the tendency to form a protective glass-like structure that has a low reactivity, making it more stable than other disaccharides due to its nonreducing character. In this hygroscopic glass-like structure, it is extremely stable both at high temperature and when completely desiccated and may hold biomolecules in a form that allows them to return to their native structure and function following rehydration.

7.7 Conclusions

To meet the target of food and feed security for the increasing world population, scientific research with technological developments at many fronts is urgently required. Development of transgenic plants targeting the improvised "Resource Use Efficiency" under environmental stresses is more challenging because these are not single gene governed traits. A cascade of genes is responsible for any abiotic stress tolerance character and there is lot of cross talk between these genes involved in different plant stress tolerance mechanisms. It is argued and hypothesized that designer plant approach may have better applicability in the upcoming year. For achieving this goal, a collaborative and interdisciplinary effort is needed between plant and microbial scientists. As the microbes are ubiquitously distributed in nature so that harnessing their diversified and pivotal potential provides an economical alternative for the development of climate resilient crops.

To achieve the given objective a global approach is a prerequisite to analyze all the distinctive properties of microbes which can be harnessed to fulfill the elevated demand of food, feed, and shelter. In the present era of scientific advancement, it is more feasible to develop different holdup techniques in genomics, metagenomics, transcriptomics, proteomics, metabolomics, and ionomics. Most importantly, long-term efficacy of stress tolerance and impacts associated with use of microbes in genetic engineering of plants need to be assessed thoroughly. Extension of recombinant plant and microbial protocols would facilitate the validation process, and the development of stress tolerant crops. More long-term and standardized studies on plant microbe interactions are still needed along with more worldwide attention towards mitigation of elevated food and feeder demand in the era of global climate change.

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Chapter 8
Improving Soil Fertility and Soil Functioning in Cover Cropped Agroecosystems with Symbiotic Microbes

Yang Zhou, Honghui Zhu, and Qing Yao

Abstract Cover cropping with graminoids or legumes represents an important strategy in agricultural production systems for the improvement of soil fertility and soil functioning. The organic carbon derived from both aboveground littering and root deposition of cover crops can greatly regulate the functional microbial groups involved in the substance cycling of nitrogen, carbon, and phosphorus. This regulation normally improves soil quality from a long-term perspective, and the effects can vary much depending on cover crop species or soil types. On the other hand, symbiotic microbes, such as arbuscular mycorrhizal fungi and rhizobia, can bring great benefits to cover crops and the associated soils. They regulate the soil fertility and soil functioning via the direct effects on native soil microbial communities or the indirect effects through altered plant growth of cover crops. Recently, the synergic effects of cover crops and symbiotic microbes are explored, and the combination of cover cropping and symbiotic microbial inoculation is emerging as a potential technology for sustainable agriculture, mainly in the horticulture area. This chapter reviews the recent progresses in the improvement of soil fertility and soil functioning with cover crops via the soil functional microbial groups, with special focus on the addictive effects of symbiotic microbes.

Keywords Agroecosystems • Cover cropping • Soil functioning • Microbial community • Symbiotic microbes

Y. Zhou
College of Horticulture, South China Agricultural University, Guangzhou 510642, China

Guangzhou Institute of Microbiology, Guangzhou 510070, China

H. Zhu
Guangzhou Institute of Microbiology, Guangzhou 510070, China

Q. Yao (✉)
College of Horticulture, South China Agricultural University, Guangzhou 510642, China
e-mail: yaoqscau@scau.edu.cn

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8.1 Introduction

Intensive agriculture, characterized by high input and high production, has been implemented worldwide, but also been causing much serious environmental problems such as soil degradation, chemical contamination. Therefore, sustainable agriculture now attracts much attention and is well recognized in many agricultural systems (Mader et al. 2002). In sustainable agriculture systems, soil management is one of the most important components (Moreno et al. 2009; Lopes et al. 2011; Hartmann et al. 2015) and mainly involves the utilization of compost and/or manure, cover cropping and reduced mechanical disturbance of soil.

Cover cropping is a well-established cultural practice to manage soil erosion, soil fertility, soil quality, biodiversity, and even pests in an agroecosystem (Lu et al. 2000). The application of cover cropping is greatly important in agricultural production systems for the improvement of soil fertility and functioning (Hartz 2002; Ramos et al. 2010; Sofo et al. 2010). Up to date, cover cropping has shown promising results in the organic transition systems (Katsvairo et al. 2007), particularly for horticultural production. It is now clear that one of the benefits of cover cropping is improvement of diversity and activity of soil microbial community because the soil microbes are indispensable component of soils involved in many ecosystem processes (Barrios 2007). Investigations indicate that microbial activity of organic agroecosystems is always more vigorous than conventional farming with high community and metabolic diversity (Mader et al. 2002; Bengtsson et al. 2005; Pimentel et al. 2005), and the organic carbon (C) derived from both aboveground littering and belowground root deposition of cover crops affected soil microbial population, activities, and biomass (Tian et al. 2011a; Nair and Ngouajio 2012; Souza et al. 2014). Consequently, in terms of soil fertility and function, cover cropping can also regulate soil functioning microbial groups involved in the soil nutrient cycling of nitrogen (N), carbon (C), and phosphorus (P) (Balota and Auler 2011; Tian et al. 2011b; Cui et al. 2015).

In this chapter, we present the recent progress in the improvement of soil fertility and soil functioning with cover crops via promoted soil beneficial microbes and suppressed detrimental organisms. Furthermore, the synergic effects of cover crops and symbiotic microbes are also briefly discussed, indicating the combined practice as a potential technology for sustainable agroecosystems.

8.2 Cover Cropping in Agriculture Ecosystems

As a critical practice for sustainable soil management, cover cropping is widely applied in different agriculture ecosystems (Moreno et al. 2009; White and Weil 2010; Teravest et al. 2011; Maul et al. 2014), such as the cultivation of cash crops

and horticultural crops. In the corn silage systems, soil quality was benefited from cover cropping with significantly increased soil active C fraction and promoted aggregate stability as well as microbial biomass (Jokela et al. 2009). Ramos et al. (2010) reported that cover cropping with oat and oat-vetch (rate 3:1) in almond orchards improved soil quality by increasing the organic matter (OM) content, improving chemical and physical fertility of the soil, and enhancing the soil microbial activity. Based on the improvement of soil quality, it is reported that cover cropping can further improve the orchard productivity and fruit quality in a long term (Chen et al. 2014). In sustainable vegetable production systems, cover cropping is also applied as an alternative soil management with significant edaphic benefits (Hartz 2002). Many studies demonstrate the increased soil quality and high degree of soil microbial functioning activity with cover crops in vegetables production systems (Smukler et al. 2008; Nair and Ngouajio 2012; Souza et al. 2014; Thomazini et al. 2015). Generally, it is widely acknowledged that conventional agricultural practices tend to lead to soil degradation and loss in productivity, while cover cropping in sustainable agroecosystems can alleviate and reverse these trends.

When cover crops regulate the soil microbial activity, cover crops themselves can also benefit from symbiotic microbial inoculation, such as rhizobia and arbuscular mycorrhizal fungi (AMF). The potential synergic effects of them can be expected. Recently, Cui et al. (2015) reported that cover cropping with *Paspalum natatu* in combination of AMF in a subtropical orchard promoted the species richness and diversity of alkaline phosphatase-gene-harboring bacteria, and the activity of alkaline phosphatase. This points out a novel technique to improve the quality of agricultural soils, where cover crops and symbiotic microbes are combined to achieve better effects.

In terms of soil fertility and functioning, soil microorganism plays a vital role in almost all soil processes, that is, microbial community composition and function will largely determine the sustainability of agriculture systems (Van Der Heijden et al. 2008). Within a cover cropping system, more labile C and littering (stable and recalcitrant C) and other nutrients are input into the cultivated layer of soil (Tian et al. 2011b; Wells 2011). Indeed, plants are the primary driver of the alteration of microbial community and the organic C serves as a trigger for the microbes, and the input nutrients can lead to alterations of the microbial community structure and function (Six et al. 2006; Elfstrand et al. 2007; Maul et al. 2014; Tian and Shi 2014; Marinari et al. 2015). Many previous studies reported the microbial responses to cover cropping, which consisted of both beneficial functioning groups and phytopathogenic groups (Mazzola et al. 2002; Patkowska and Konopiński 2013; Wortman et al. 2013). Since managing the soil microbes for the presence of beneficial and absence of detrimental microbial groups is one of the promising approaches to improve sustainable agriculture production (Hartmann et al. 2015), cover cropping may make sense greatly.

8.3 Involvement of Soil Microbes in Cover Cropping

8.3.1 Native Microbes as Affected by Cover Crops

It has been shown that cover cropping as a beneficial soil management in sustainable agroecosystems strongly influences the soil fertility and functioning, and meanwhile, it profoundly modifies the size, composition, and function of soil microbial community, indicating that microbial community is dynamic and responsive to cover cropping. It is well known that microbes influence almost all the soil properties and functional processes, including soil water holding capacity (Bodner et al. 2008) , soil pH (Tian et al. 2005), the cycling of C and N (Steenwerth and Belina 2008a, b), the hydrolysis of organic phosphorus (Maltais-Landry et al. 2014), formation and stabilization of soil structure (Schutter and Dick 2002) in the cover cropped systems, consequently, the microbial community in cover cropped soils is involved in the formation of soil quality (Kong et al. 2011). Due to the OM derived from both aboveground littering and root deposition of cover crops, the alteration of microbial community in the cover cropped system occurs.

8.3.1.1 Microbial Community of General Groups

Previous studies indicated that cover cropping showed large effects on the population size, metabolic activity, and diversity of soil microbial community. Cover crops are common source of organic C in organic farming systems and have been shown to increase the abundance or diversity of Gram-negative bacteria, fungi and AMF, and actinomycetes (Buyer et al. 2010; Ramos-Zapata et al. 2012). Carrera et al. (2007) reported that rapidly growing, culturable, aerobic, heterotrophic bacteria was affected by cover cropping, with detectable copiotrophic bacteria responding the most rapidly and indetectable oligotrophic bacteria responding much more slowly. They explained that the former would be affected by rich organic carbon and nitrogen of root exudates and residual of cover plants. Ferreira and Martin (2012) compared the bacterial community of bulk and rhizospheric soil under cover cropping and found a clear effect of the rhizoplane on the selection of bacterial community with a lower diversity index. It is also reported that the selection of Gram-positive bacteria exists in the bulk soil, while pseudomonas bacteria group was prevalent in the rhizosphere of two common plants in the cover cropping (Marilley and Aragno 1999). Moreover, in an experiment evaluating the responsive microbial groups in cover cropping system, Henneron et al. (2015) revealed, with qPCR, the enrichment of α-proteobacteria and actinobacteria, the decrease of β-proteobacteria, and insensitivity of acidobacteria, γ-proteobacteria, bacteriodetes, firmicutes, and gemmatimonades. In general, cover crops have great potential to enhance microbial activity and also to select soil bacterial community with particular functions.

Fungal community was also affected because of cover cropping (Schutter et al. 2001; Carrera et al. 2007). Molecular analysis showed that the fungal community was influenced more strongly by the cover cropping than tillage practices, with the cropping system characterized by a predominance of Cryptococcus sp. in the phylum Basidiomycota (Nishizawa et al. 2010). In addition, more reports showed the increased fungal biomarker fatty acids (Carrera et al. 2007) and high ergosterol level (Dinesh et al. 2009) in cover cropped soils.

8.3.1.2 Microbial Community of Functional Groups Involved in C and N Cycling

C and N cycling are core biogeochemical processes in soils, and cover crops can activate the soil C and N dynamics and modify the microbial biomass and activity, such as microbial C and N, hydrolytic enzymes, and specific functions. Since the soil functions always depend on the soil enzyme (SE) activities and soil microbes contribute much, if not all, to the SE activities, the research on SE reveals, to a large extent, the diverse functions of soil microbes. Consequently, SE is critical tool to assess the microbial community involved in C and N cycling in cover cropping systems. Microbial activity (such as basal respiration, substrate-induced respiration) and soil OM pools, mineral N pool sizes, and N transformation rates were often measured for C cycling and N cycling, respectively.

Steenwerth and Belina (2008a) investigated the impacts of cover cropping on soil C dynamics and microbiological function in a vineyard grown in California's Mediterranean climate, indicating greater labile C pools, microbiological function and respiration, as well as improved microbial biomass N with greater N mineralization, nitrification, and denitrification (Steenwerth and Belina 2008b). In contrast, however, some experiments also demonstrated the suppression of bacterial nitrifier abundance (Bending and Lincoln 2000), the inhibition of nitrification (Brown and Morra 2009), and the high concentration of NH4+ in response to the input brassicaceae residues. Furthermore, a soil fungal genus *Cylindrocarpon* (Usuda et al. 1995), known to harbor denitrification activity, were significantly decreased with the same treatment in orchard soils (Mazzola and Brown 2010). These results suggest that the responses of microbial activity and functions to cover cropping strongly depend on the identity of cover crops. As to brassicaceae plants, it has been demonstrated that their residues contain glucosinolates and the hydrolytic products of glucosinolates relate to soil N cycling (Brown and Morra 2009; Reardon et al. 2013). Therefore, much caution should be taken when considering brassicaceae plants as potential cover crops.

8.3.1.3 Microbial Community of Functional Groups Involved in P Cycling

Microbial biomass P and phosphatase activity can be used for evaluating the effects of P-cycling functioning groups under cover cropping. Soil P mineralization is controlled, at least in part, by the soil phosphatase that is largely produced by microbes

and plant roots (Spohn and Kuzyakov 2013). Phosphatase activity was significantly affected by cover crops compared with bulk soil that could be viewed as control of no crops (Maltais-Landry et al. 2014). Takeda et al. (2009) reported that soil microbial P and phosphatase activity, both representing the potential of P mineralization, were enhanced in the treatment of cover cropping with rye. In a cover cropped subtropical orchard, Cui et al. (2015) observed an increase of alkaline phosphatase activity only originated from microbes compared with clean culture. Moreover, White and Weil (2010) reported that a ray cover crop increased AMF colonization of maize roots and P acquisition compared to no cover crop, indicating higher activity of AMF groups in the ray cropped system.

Spohn and Kuzyakov (2013) have reported a detailed and elaborate study about spatial organization of microbial phosphatase activity in the rhizosphere. Lupin, a leguminous crop, showed spatial separation of phosphatase activity in the rhizosphere, with acid phosphatase activity (produced by roots and microbes) closely associated with roots while alkaline phosphatase activity (produced only by microbes) distributed more widely. This differential distribution led to a 2.5-times larger area of alkaline phosphatase activity than that of acid phosphatase activity. Moreover, the distribution of phosphatase reflected the spatial differentiation of different ecophysiological groups of organic P mineralizing organisms. It is also reasonable to extrapolate this distribution pattern to a cover cropping agroecosystem with P-limiting soils, where the hydrolysis of organic P by phosphatase is of particular importance for crop production.

8.3.1.4 Pathogenic Microbial Groups

Root diseases by pathogenic fungi can be severe with poor soil conditions, such as low OM content, poor water status. In these cases, cover cropping can be helpful considering their diverse benefits to soils. In brief, cover cropping has potential for suppressing soil-borne pathogen and the severity of their resultant root diseases (Abawi and Widmer 2000). A variety of cover crops (e.g., legume, graminoids, and brassicaceae) have been found to inhibit soil-borne pathogens (e.g., pythium, rhizoctonia, fusarium, and other weak pathogens that survive on organic residues in soil) either in laboratory or in field conditions (Manici et al. 2004; Summers et al. 2014). Hairy vetch as cover crop has been shown to be very effective in suppressing *Thielaviopsis basicola* (Rothrock et al. 1995; Abawi and Widmer 2000). Although they are non-host to arbuscular mycorrhizal fungi (AMF), brassicaceae plants used as cover cropping in agricultural systems effectively suppressed *Pythium* due to the input brassica residues (Reardon et al. 2013). The suppression of pathogen is probably not the result of a direct effect (Zhou and Everts 2004; Buyer et al. 2010; Summers et al. 2014), but of the increase in beneficial groups. In fact, the incorporation of organic C into soils due to cover cropping enhances the microbial biomass and activity, which is beneficial for soil functioning. This improved soil functioning may be helpful in suppressing soil-borne pathogens via several mechanisms, such as increasing microbial competition and antagonism, reducing the colonization of

pathogen and the release of propagules into the soils. Kumar et al. (2004) observed that the control of diseases with a legume hairy vetch was in conjunction with the up-regulation of some specific defense-related genes (e.g., chitinase and osmotin coding genes).

In contrast, however, Manici et al. (2004) reported the increase of *Pythium* populations concurrent with an increase of soil microbial populations. In their experiment, the use of barley mulching and incorporation into soils did not result in suppression of the pathogen. They attributed it to the poor soil conditions with continuous cropping of strawberry for 8 years. Clearly, the suppression of soil-borne pathogens by cover crops may occur only in those well-functioning soils, but not in the deteriorative soils. Considering the fact that cover cropping can also improve the soil quality, it can be expected that cover crops will inhibit the pathogens in the deteriorative soils at late stage when soil functioning is improved.

8.3.2 Symbiotic Microbes Inoculated to Cover Crops

Agroecosystems in general possess a wealth of microbial resources with the potential to be exploited as promoters for soil fertility and plant growth. For example, a plenty variety of microbes can establish intimate symbiotic associations with plants and act as regulators of plant productivity by supplying limiting nutrients. Up to date, the utilizations of beneficial microbes in agriculture mainly include increasing plant P absorption with AMF, improving N status of legumes with rhizobia, and promoting plant performance with other microbes, such as endophytes (Arthurson and Jäderlund 2011). It is reported that mycorrhizal fungi and nitrogen-fixing bacteria are responsible for 5–80 % N and up to 75 % P acquired by plants annually (Van Der Heijden et al. 2008). However, in many cases it often fails to induce desired effects in agricultural systems due to inappropriate tillage. In addition, cell density-dependent quorum sensing is known to regulate many bacterial functions (Compant et al. 2010). The use of symbiotic microbes (e.g., AMF, rhizobia, endophytes) in the sustainable agriculture can enforce the naturally occurring mutualistic symbiosis and further improve the soil fertility and soil functioning. Recently, the novel technology combining the symbiotic microbial inoculation with cover cropping to form synergic effects has emerged and attracted attention in the sustainable agriculture (Cui et al. 2015). This combination integrates the advantages of both sides and modifies plant–microbial interactions to improve the sustainability and resilience of agroecosystems.

8.3.2.1 AMF

AMF are ubiquitous in soils of most ecosystems, and they form mutualistic symbiotic associations with roots of ~80 % terrestrial plants, playing an important role in extensions of plant root systems and increasing nutrient uptake, especially of

phosphorus (Van Der Heijden et al. 1998). Because of these characteristics, AMF are perceived as the most important component of a paradigm for sustainable land management practices (Ryan and Graham 2002). AMF diversity, however, is usually reduced in agricultural farming systems in terms of species composition and species abundance (Boswell et al. 1998; Helgason et al. 1998; Douds and Johnson 2007). Besides, indigenous inoculum potential is often low or ineffective, so the exotic inoculation strategies may be helpful (Sharma et al. 2011a). In order to increase the number of AMF propagules, the root colonization rate and the field productivity in sustainable agriculture, many approaches are applied, such as use of cover crops and inoculation with exotic AMF isolates (Higo et al. 2014; Njeru et al. 2015). Cover crops may serve as hosts for AMF and thus help increase mycorrhizal inoculum potential for crops (Boswell et al. 1998; Kabir and Koide 2002). Moreover, Sieverding (1990) suggested that a few well-selected AMF could increase yields if they are best mutualism to plants. Yao et al. (2008) even observed the differential responses of dominant and subordinate species in an orchard weed community to AMF inoculation, indicating the possibility to improve cover crops using AMF. Consequently, combining AMF inoculation with cover cropping can achieve a win-win situation, with AMF community improved and the cover cropping more effective. Higo et al. (2014) verified the suitability of buckwheat as a cover crop in Florida in conjunction with a comprehensive assessment of its response to AMF inoculation. Sharma et al. (2011a, b) applied symbiotic microbes and cover crop in citrus and mango nursery production. Compared to inoculation with AMF isolates, matrix with the enrichment of appropriate AMF can also be used as "inoculum" in the cover cropping systems (De Bruin et al. 2006). In addition, Higo et al. (2014) reported that the introduction of mycorrhizal crops as preceding crop in cover cropping system improved P uptake and increased AMF inoculation rate, wherein the preceding crops enriched AMF and can be viewed as "inoculation."

When AMF have the potential to improve nutrient uptake of plants (Ryan and Graham 2002; Deguchi et al. 2012), the beneficial interactions between AMF and other soil organisms that potentially enhance mycorrhizal benefits to soil fertility and functioning and crop production can also establish (Douds and Johnson 2007). It is worth mentioning that "mycorrhizal helper bacteria" associated with AM and AMF are selected to promote the establishment of mycorrhizal symbiosis (Garbaye 1994; Hildebrandt et al. 2002, 2006; Long et al. 2008). In general, application of cover cropping in conjunction with AMF inoculation in sustainable agriculture can form synergistic effect which is superior to cover cropping only.

8.3.2.2 Rhizobia

The provision of nitrogen to the soil via symbiotic nitrogen fixation (SNF), mediated primarily by the rhizobia–legume association, is enormous. The efficiency of cover cropping with legumes in sustainable farming depends heavily on the rhizobia

with which the legumes form SNF associates. However, native rhizobia are often less effective in N-fixation than those isolated and screened specially for high N-fixation due to insufficient rhizosphere and/or plant colonization, which is as an important step required for exhibiting beneficial effects (Lugtenberg et al. 2001; Cheminingwa and Vessey 2006; Mothapo et al. 2013). The selected rhizobia strains are often high efficient in colonizing, nodulating, and N-fixing. Therefore, combining cover cropping with legumes with rhizobia inoculation is an available, economical and advantageous soil management in sustainable agriculture. Houngnandan et al. (2000) reported that the use of rhizobia inocula to leguminous cover crops in the savanna in Benin can eliminate the incompatibility between the symbiotic partners.

The elevated plant productivity in rhizobia inoculated cover cropping systems is attributed to increased nodulation and N-fixation efficiency. It is generally believed that the absence of an effective indigenous population of rhizobia and low P levels are the major limiting factors to SNF (Vera-Nunez et al. 2008). Obviously, inoculation with rhizobia will increase the rhizobia population size, and thus promote the nodulation and SNF to some extent.

Interestingly, previous studies indicated that AMF colonization was stimulated to exploit available P by rhizobia inoculation, which may be, in turn, an elevator to SNF (Linderman 1988; Arthurson and Jäderlund 2011). Thus, the dual inoculation with rhizobia and AMF in cover cropping systems can optimize the functions of both inoculants (Li et al. 2009). The typically synergistic effect of dual inoculation has been ever demonstrated repeatedly. For example, Gong et al. (2012) indicated the promoted soil quality by a pioneer leguminous cover crop dual-inoculated with Glomus mosseae and Rhizobium. The mechanisms underlying these associations, however, are still not well understood at present. Further insights into the associated mechanisms may facilitate the development of mixed inoculation techniques that can be used efficiently in cover cropping systems for sustainable agriculture.

8.3.2.3 Endophytes

Endopytes are microbes that reside within the living tissues of host plants without substantively harming them, and in contrary, the endophytes reported to date are mostly beneficial to host. Ecologically, endophytes are ubiquitous and diverse in most plant species and play diverse indispensable functions in nature for plant productivity, mainly through improving nutrient assimilation and metabolism, secreting plant growth hormones, modulating the genes expression of defense and other secondary metabolic pathways of the host. For cover crops, ryegrass is the most reported species to harbor endophytes exerting multifaceted functions (Casas et al. 2011; Card et al. 2014; Ma et al. 2015; Rahman et al. 2015). The ecological significance and application of endophytes in cover cropping systems, however, are far from understanding, and more work in this field is to be carried out.

8.3.3 *Different Microbial Community Associated with Different Cover Crop Species*

The use of cover crops has been shown to improve the soil microbial activities, soil fertility, and functioning. These effects, however, can greatly vary depending on the cover crop species (Kramberger et al. 2009; Zhou et al. 2012). Graminoids (e.g., oat, ryegrass) and legumes (e.g., clover, vetch) are two main groups of the most frequently utilized species in cover cropping systems. When graminoids normally produce large amounts of biomass (particularly the root biomass) and be efficient in nutrient assimilation, increase overall soil OM (Thorup-Kristensen 2001; Snapp et al. 2005; Kramberger et al. 2007, 2008), legumes often effectively fix N biologically and improve the soil N fertility, leading to increased N availability (Vaughan et al. 2000; Sainju et al. 2002). Other cover crop species, such as brassicaceae (e.g., radish), are also used mainly because of the beneficial hydrolytic products of glucosinolates for suppression of pathogens (Weil and Kremen 2007).

Due to different biological properties of cover crop species, the quantity and quality of nutrient derived from cover crops differ significantly (Lehmann et al. 1999; Dinesh et al. 2006; Van Eekeren et al. 2009). Moreover, the aboveground can strongly influence the belowground and further the microbial community diversity and ecosystem processes in rhizosphere (Qiao et al. 2012). Therefore, it is not surprising that the responses of microbial community to different cover crops vary much (Sarathchandra et al. 1997; Buyer et al. 2010; Diouf et al. 2010). In comparison with gramineous cover crops, leguminous cover crops often have lower total root biomass and lower C/N ratio of the biomass, and these result in a lower bacterial and fungal biomass (De Neergaard and Gorissen 2004; Van Eekeren et al. 2009). De Neergaard and Gorissen (2004) reported that more C was allocated to belowground pools in graminoids, and the C remained mobile within the plant for a longer period than legumes (a faster mineralization rate for legumes). They revealed higher microbial biomass for graminoids using the method of pulse-labelled 14C. Sanchez et al. (2003) indicated that graminoids are the most manageable groundcover for fruit production but can compete with trees for soil N, whereas legumes appear to have no adverse effect on trees and increase the active N pool by residues entering soils. Strock et al. (2004), however, indicated that cereal rye possessed a greater capacity to conserve residual soil nitrogen and reduced the potential for N leaching compared with leguminous cover crops. Kusliene et al. (2014) reported that different short-term C (fresh root exudate) utilization patterns and similar medium-term responses of microbial community between two cover crops, with more active microbial utilization of root exudate under ryegrass than that of white clover. Buyer et al. (2010) showed that vetch and rye, respectively, as cover crop have unique root and shoot effects on soil microbial community, with vetch shoot increasing the amount and proportion of Gram-negative bacteria, fungi, and AMF in the rhizosphere of tomato plants measured by phospholipid fatty acid analysis.

In an integrated view, a mixture of graminoids and legumes as cover crop can be more beneficial for soil fertility, soil biota, and ecosystem services (Sainju et al. 2007), mainly due to the optimized appropriate C/N ratio of input residues, which

has been recommended to the sustainable systems recently. An array of studies has indicated the improved C, N, P cycling by the mixture of graminoids and legumes than monoculture (De Neergaard and Gorissen 2004; Van Eekeren et al. 2009; Tang et al. 2014). In this case, however, the competition between graminoids and legumes should be carefully regulated to avoid one side (normally the graminoids) prevailing over the other.

8.4 Mechanisms Underlying the Interaction Between Soil Microbes and Cover Crops

The interaction between plants and surrounding soil microbial community has been an attractive topic of intense research; however, the mechanisms underlying the complex interaction are still not fully understood. Plants as producers absorb nutrients mostly supplied by decomposers, whereas decomposers (mostly the soil microbes) acquire C from OM primarily supplied by producers (Schimel and Bennett 2004). When focusing on the cover cropping systems, it is easy to elucidate the interaction in two aspects: improved soil chemical properties by cover crops and promoted plant growth by microbes.

8.4.1 Increased C, N Input and Nutrients Accumulation into Soils by Cover Crops

The quantity and quality of OM entering the soils, such as root exudates and depositions and residues of cover crops, have a strong influence on the total C and active C pool, which often increase the total organic C, dissolved organic C, and microbial biomass C (Steenwerth and Belina 2008a; Dinesh et al. 2009; Liang et al. 2014). With summer and winter cover cropping in a vineyard, multivariable analysis showed that extractable organic C was the most associated indicator with microbial community, suggesting the reliance of microbes on nutrient pools mostly provided by root deposition and plant residues (Mackie et al. 2014). Furthermore, Xavier et al. (2013) analyzed the effect of soil management on the OM fraction, and found that use of legume as cover crop caused the predominance of humic acids and presented great C-humic/C-fulvic acids ratio, which reflected the accelerated humification process and more stable soil OM due to leguminous cover crops. Cover crops can enhance soil N dynamics and microbial functions of N mineralization, nitrification, and denitrification, as well as the involved microbial activity, which often increase total N, microbial biomass N, and SNF activity, nitrification, and denifrification (Steenwerth and Belina 2008b; Dinesh et al. 2009; Zhou et al. 2012).

It is well acknowledged that soil C and N cycling are coupled, regulating and maintaining the productivity and stability of agroecosystems (Bowles et al. 2014). In the cover cropping system, soil organic C and N concentrations can be conserved

by reducing losses of mineralization and erosion, and by sequestering atmospheric CO_2 and N_2 (Sainju et al. 2002). Soil C and N and their active mineralizable forms are available food for organisms, and the enhanced C and N pools are likely to increase soil microbial biomass and activity. Moreover, variation in the amount of C and N contributed by cover crops can alter C/N ratio of the soil. In the cover cropped soils, microbial community typically become N-limited because of the increased available organic C. As a result, N mineralization is expected to increase, which is normally associated with active N-fixation, OM mineralization, nitrification, and denitrification. Steenwerth and Belina (2008b) reported that potential nitrification, N mineralization, and denitrification were generally two to fourfold greater in cover cropped treatment. Gu and Mazzola (2003) showed the benefits of cover cropping via increased N-fixation and enhanced soil OM. On the other hand, N addition and availability into soils, such as legume residues, often stimulate C mineralization. Kong et al. (2011) inferred that the greater soil C in cover cropped system may be the result of more available N that stimulated C processing (i.e., C utilization by microbes that include C incorporation into microbial biomass) within the microbial community and, therefore, increased the stabilization of microbial-derived C in the soils compared to conventional systems. This may have great agricultural and ecological significance because the cover cropping with legumes could reduce the reliance on fertilizer addition for crop production (Jackson 2000) and alleviate the potential negative impacts on water N eutrophication (Harrison et al. 2005).

It is noted that the cover cropping can reduce nitrate leaching in the improvement of N cycling. Cereal rye (Strock et al. 2004; Reeleder et al. 2006), bracken (Smart et al. 2007), a mixture of legume and nonlegume (Sainju et al. 2007) have been reported to plant as cover crops to reduce N loss in the sustainable systems, especially in the off-season months. Staver and Brinsfield (1998) have observed a low nitrate concentration in rye root zone leachate less than 1 mg/L. This is because the cover cropping keep the land covered with growing vegetation for a longer period, which can reduce N run-off from the soil profile through N uptake.

In addition to C and N, other soil nutrients can also be derived from the mineralization of soil OM, and thus cover cropping also represents an efficient tool to improve accumulation of these soil nutrients (Bünemann et al. 2004; Guo et al. 2008).

8.4.2 Promoted Plant Growth and Activity by Soil Microbes

Presently, the effects of soil microbes on cover crops are not fully understood (De Bruin et al. 2006). It is possible that interaction with soil microbes positively affects the establishment and growth of cover crops since beneficial microbes, such as AMF and plant growth-promoting rhizobacteria (PGPR), can have strong effects on plant growth and stress resistance. In contrast, however, the pervasive influences by other microbes are still less appreciated and understood (Morrissey et al. 2004). Microbial contribution to promoted plant performance may be in direct and indirect ways. For example, AMF can directly influence the architecture of the host root

systems (Berta et al. 2002; Yao et al. 2009), which are closely related to the nutrient uptake of host plant. Colonization by Azospirillum brasilense resulted in a proliferation of root hairs and plant growth of a nonlegume species (Fallik et al. 1994). As to the indirect ways, PGPR has been reported to exert diverse beneficial effects on host plants, such as suppressing soil-borne pathogens (Summers et al. 2014), improving mineral nutrients (Liang et al. 2014) and phytohormone synthesis (Vessey 2003).

8.4.3 Other Mechanisms

The roots of cover crops can secrete some secondary metabolites that influence the microbial community and the interaction with plants. Higo et al. (2014) reported that allelochemicals of the cover plants affected the composition of AMF communities. The roots of leguminous cover crops secreted strigolactones and flavonoids, such as daidzein and coumestrol, to induce the nodulation and mycorrhization, namely the activities and functions of rhizobia and AMF (Shaw et al. 2006; Sugiyama and Yazaki 2012).

8.5 The Ecological Significance of Microbe–Cover Crop Interaction in a Wider Context

Agricultural systems have important functions from an ecological perspective and provide services that are essential to maintain the sustainability of development on a local and global scale. Inappropriate anthropogenic activities, however, have shown to bring some serious problems, such as the global warming due to emission of greenhouse gases. Fortunately, some soil management practices (e.g., cover cropping) have potential to mitigate these problems. In the cover cropping systems, the microbe–cover crop interaction can have important ecological significance.

8.5.1 Alleviation of Soil Erosion

Soil erosion is the most widespread form of soil degradation and can cause negative impacts including the removal of topsoil and the subsequent reduction of crop productivity (Lal 2003; Baumhardt et al. 2015). These impacts are more significant in hilly areas. Cover cropping can mitigate soil erosion and degradation by stabilizing soil aggregates, improving soil structure, enhancing air and water exchange, increasing nutrient cycling, and promoting soil microbial activity (Baumhardt et al. 2015). Recently, the application of cover cropping to prevent soil erosion in the hilly areas has been reported (Arnhold et al. 2014; Li et al. 2014; Morvan et al. 2014; Davidová

et al. 2015). Apart from the water-induced soil erosion, cover cropping also helps control the wind-induced soil erosion by promoting stable aggregates (Baumhardt et al. 2015) because elevated soil OM promotes soil aggregation via the enhanced fungal hyphae binding the particles together (Cambardella and Elliott 1993; Six et al. 2000).

8.5.2 Reduced Emission of Greenhouse Gases

In terms of global warming, cover cropping can exert certain impacts in two contrasting aspects: (1) reducing global warming by sequestrating atmospheric CO_2 and N_2 (Sainju et al. 2002; Six et al. 2004; Aguilera et al. 2013); (2) increasing the emission of greenhouse gases (e.g., CO_2, N_2O, CH_4) due to the accelerated minerlization of soil OM (Palm et al. 2010; Kim et al. 2013; Cuello et al. 2015). Agricultural soils can be regarded as the sink to sequester atmospheric C and N, which are driven by photosynthesis, microbial fixation of C and N, nitrification, denitrification (Dalal et al. 2003; Burger et al. 2005; Piva et al. 2012; Kim et al. 2013), and consequently, the concentrations of CO_2 and N_2O in atmosphere can be reduced. In cover cropping systems, graminoids sequester more CO_2 while legumes fix both C and N to the plants and soil microbes. Other studies, however, indicated that cover cropping may increase the emission of greenhouse gases (Palm et al. 2010; Kim et al. 2013) because the mineralization of soil OM is speed up. Given the higher contribution of N_2O to global warming than CO_2 and CH_4 (Solomon 2007), the impact of cover cropping on N_2O emission has been investigated. Palm et al. (2010) found that leguminous cover crops resulted in high N_2O emission. In a meta-analysis, Basche et al. (2014) concluded that cover crops do not always reduce direct N_2O emission in the short term. Clearly, more work is needed to address the paradox in the contribution of cover cropping to global warming.

8.5.3 Restoration of Polluted Soils

The application of cover crops in the rehabilitation of heavy metal (HM) contaminated soil, often called phytoremediation, is an emerging area of interest because it provides an ecologically sound and safe method for restoration and remediation (Wu et al. 2006; Vamerali et al. 2010). Rhizosphere influences the dynamics of nutrients and contaminants through the increased microbial activity, the release of root exudates and the alteration of pH, and finally improves the degradation of contaminants (Karthikeyan and Kulakow 2003; Kirkpatrick et al. 2008). Bolan et al. (2013) showed that the rhizosphere soil of cover crops contained higher levels of microbial activity and 2.4 to 5.1 fold increases in the rate of reduction of As and Cr than the non-rhizosphere soil, indicating a significant role of increased microbial activity in the phytoremediation of HM-polluted soils. Interestingly, some functional microbial groups depending on root exudates and OM can effectively degrade

organic pollutants, among which polycyclic aromatic hydrocarbons (PAH) are the most serious (EEA 2007). Kirkpatrick et al. (2008) indicated that the number of total hydrocarbon-, alkane-, and PAH-degrading microbes were 3–5 times higher in the soils cover cropped with sudangrass than the non-covered soils.

8.6 Conclusions and Future Research Perspectives

It is now clear that, in the sustainable agroecosystems, cover cropping has the potential to improve the soil fertility and functioning by promoting the microbial biomass and activity. Although a series of progress and diverse knowledge has been achieved in this field, there are still many gaps and blanks to be filled. In a macroscopic view, more work is needed to investigate the difference among cover crop species, especially between graminoids and legumes. Graminoids and legumes are two important groups in agricultural systems, and they are so different in many aspects (e.g., root system architecture, root exudates, biomass C/N ratio, nutrient acquisition). These differences can certainly lead to the differences in rhizosphere microbial community composition and function. In a microscopic view, more work is needed to probe the functional microbial groups in the rhizosphere of cover crops. Nutrient cycling is central to the soil fertility and functioning, which is achieved by different functional microbial groups. Deep insight into these functional groups, however, is scarce presently, retarding the full understanding of cover cropping and the further application of it in agroecosystems and even other ecosystems. Moreover, differential regulation of microbial community by aboveground littering and root deposition is also attractive and of practical significance, when mowing is taken into consideration in cover cropping systems. Methodologically, with the recent technical progress, microarray, metagenomics, metatranscriptomics, metaproteomics, and metabonomics can be used to better understand and deeply explore the microbial community in cover cropped soils (Myrold et al. 2014; Ofek-Lalzar et al. 2014). Besides, from principles to practices, the application of symbiotic microbes in cover cropping systems can represent a promising technique in sustainable agriculture.

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Chapter 9
Actinobacteria in Agricultural and Environmental Sustainability

L. Shivlata and Tulasi Satyanarayana

Abstract The advent of green revolution or high input agrotechnologies have led to self-reliance in food production. Modern agriculture methods are getting increasingly dependent on the steady supply of synthetic inorganic fertilizers and pesticides, which are products of fossil fuels. There is an increasing concern about the excessive dependence on the supply of chemical fertilizers and pesticides, and the adverse effects of the indiscriminate use of synthetic inputs in soil productivity and environmental quality. The cumulative effect of environmental degradation due to application of agrochemicals has led to a decline in food production during the last two decades. In order to overcome these adverse effects, there is an urgent need to develop new strategies for ensuring further growth in agricultural output. By adapting a strategy involving integrated supply of nutrients from a combination of chemical fertilizers and pesticides, organic manures, and biofertilizers and biopesticides, the soil can be saved from further impoverishment and environmental degradation. The use of microbes as bioinoculants for promoting plant growth and/or bioremediation purposes gives a new dimension to agricultural and environmental biotechnology. Actinobacteria are considered as the most prominent source of bioactive compounds (antibiotics, enzymes, and plant growth modulators) facilitating plant growth promotion and plant disease suppression. Attempts are being made to utilize actinobacteria that produce antibiotics and agro-active compounds as biofertilizers and biopesticides; these aids in mitigating the use of harmful chemical fertilizers and pesticides. Besides making agriculture systems sustainable, soil inhabiting actinobacteria play important roles in various ecological processes such as organic matter decomposition and toxic pollutant and heavy metal bioremediation, thus contributing to the restoration of soil fertility and environmental sustainability.

Keywords Actinobacteria • Antibiotics • Biofertilizers • Biopesticides • Organic matter decomposition • Environmental sustainability

L. Shivlata • T. Satyanarayana (✉)
Department of Microbiology, University of Delhi South Campus,
Benito Juarez Road, New Delhi, Delhi 110021, India
e-mail: tsnarayana@gmail.com

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9.1 Introduction

Ever increasing population and over exploitation of natural resources are the two major causes of disturbance in the structure of world economy that has resulted in drastic setbacks on overall growth and development (Bretschger 2013). The world population has already reached 6.8 billion and is estimated to cross nine billion by 2050 (Alexandratos and Bruinsma 2012). Intensive agricultural technologies have been adopted to feed the escalating population since 1960s. These conventional agricultural technologies include hybridized seed distribution, modern irrigation practices, use of improved crop varieties, synthetic fertilizers and pesticides, etc. With advances in modern technologies, crop productivity rates have increased to meet the global food demand and provide future food security through green revolution. The green revolution is an agrotechnology-based solution to the worldwide food scarcity that arose after the Second World War. It has brought tremendous breakthroughs in agricultural economy in the last few decades (Pingali 2012) and has led to a phenomenal increase in food production per capita, especially in the yield of staple foods (rice, maize, and wheat). This revolution has been successful in making many nations self-sufficient in food grains. The development of agricultural self-reliance system in many countries has ensured a long-term food production in an economically viable way (Herdt 1998). Despite impressive results, these modern practices have caused certain negative impacts on ecological units encompassing changes in physiochemical properties of soil, depletion of stratospheric ozone, and destruction of food chains. An excessive use of synthetic fertilizers and pesticides pollute land area and ultimately water resources giving rise to algal blooms, nitrate poisoning, emergence of pesticide-resistant insects and pathogenic fungi (Ntalli and Menkissoglu-Spiroudi 2011), and thereby making crop production more susceptible to abiotic and biotic stresses (Babalola 2010). Other severe repercussions include ecological infrastructure damages, unfavorable climate change, deforestation, and soil erosion (Zacharia 2011), thus disturbing the overall sustainability of agriculture system and environment, which ultimately leads to major health concerns, extinction of wildlife, and other life forms (Carson 1962).

Environmental degradation is one of the biggest concerns that must be addressed at the global level. This is mainly due to ever increasing human population, urbanization, and industrialization. The effluents from various industries contaminate the atmosphere as well as aquatic and terrestrial zones of the biosphere, thus, influencing both biotic and abiotic environmental factors. Disturbances in the environment lead to undesirable and deleterious outcomes triggering unseasonal rainfall, atmospheric pollution, soil degradation, and deterioration of soil microbiota that affect land fertility and agro-economy.

Environment and agriculture are two interlinked systems. The perturbation of environment causes negative impacts on agriculture system and vice versa. These two systems determine the economic status and progressive structure of a nation. The preservation of sustainability in agriculture and environment is an important concern in the current scenario that needs special attention. Consequently, robust

organizational systems need to be developed, which control land use and coordinate soil and water management to a sustainable level. Sustainable agriculture aims primarily at making nutritious food available for the present and future generation to conserve soil fertility and natural resources. To fulfill these, sustainable soil management, development of pest-resistant crop plants, improvement in agricultural services, and search for alternatives to hazardous chemicals are the current focus.

Soil management practices increase nutrient content and water holding capacity of soil that permits proliferation of beneficial microbes and restricts the entry of toxic compounds into the food chain. Soil microbes regulate nutritive and physical status of the soil (Anderson and Domsch 1989) and make an essential contribution in humus formation and soil texture improvement, thereby making soil more suitable for sustainable cropping practices. In the present scenario, pesticide-resistant plant pathogens and abiotic stresses are emerging factors that severely affect the agricultural production. Genetically modified and improved crop varieties are being used to relieve the effects of biotic and abiotic stresses (Rai et al. 2011). On the other hand, the emergence of resistant pathogenic strains is comparably high warranting a search for an alternate solution. The application of microbial inoculants has proven to be effective for suppression of pathogenic fungal growth (Toyota and Watanabe 2013). In addition, some microbes have an inherent trait of triggering the plant immune system in order to defend herbivore insect attack (Van Wees et al. 2008). Thus, these microbes can be employed as alternatives to harmful pesticides. Some extremotolerant microbes are capable of supporting plant growth in adverse environments (Yandigeri et al. 2012; Selvakumar et al. 2015), and these could serve as suitable candidates to cope with abiotic stresses (drought, salinity, and nutrient deficiency) to enable exploitation of unsuitable soils (saline coastal sediments and desolate areas) for cultivation purposes.

Microbial flora also has a key role to play in biogeochemical cycles, which regulates recycling of principal elements (carbon, nitrogen, sulphur, and phosphorus) between biotic and abiotic factors. Recycling of essential elements facilitates growth and survival of microbes and others in the ecological niches (Rousk and Bengtson 2014). Moreover, microorganisms participate in plant growth promotion via plant–microbe associations. This association can be cooperative or antagonistic. Mutualistic association is broadly classified into two major types: bipartite communities (nitrogen-fixing nodular symbioses or arbuscular mycorrhiza) and multipartite communities (endophytes and epiphytes) (Tikhonovich and Provorov 2011). Beneficial microbes provide solubilized minerals to plants and fixed nitrogen to enhance fitness of plants, and thus, bepragmatic biofertilizers. The application of biofertilizer and biopesticide is more promising as they have negligible detrimental effects on the environment. Likewise, the multiple benefits of microbial inoculants offer an effective way for sustainable agriculture (Jha et al. 2013).

Microbes are a boon to keep the environment clean. They possess an immense tolerance to toxic environment and exhibit metabolic potential to degrade xenobiotics. They play a role in providing a cleaner and healthier environment for mankind through pollution control. They are of paramount importance in the degradation of recalcitrant organic compounds (Daubaras and Chakrabarty 1992), detoxification of

heavy metals (Lovley and Coatest 1997) and waste treatment. Several microbes and their enzymes have found application in the process of bioremediation. To improve their degradation capabilities, potential microorganisms have been genetically improved for combating environmental problems (Sayler and Ripp 2000). Microbes such as bacteria, actinobacteria, fungi, and algae have been tested for their utility in agricultural and environmental sustainability. The domain bacteria include a large number of biotechnologically important strains. One such good example is of the phylum *Actinobacteria* that constitutes a large number of antibiotic producing, disease suppressing, and plant growth-promoting genera (Hamedi and Mohammadipanah 2015). Their ability to secrete a large number of bioactive compounds, high catabolic rate, and omnipresence in the environment make them potential candidates for agriculture and environmental biotechnology. Moreover, their metabolic diversity, characteristic growth pattern, and tolerance to noxious environmental pollutants enable them to remediate extremely polluted sites (http://www.biotecharticles.com/Environmental-Biotechnology-Article/Actinomycetes-and-Bioremediation-1091.html). In this chapter, an attempt has been made to describe the utility of actinobacteria in the conservation or restoration of agricultural and environmental sustainability.

9.2 Actinobacteria: Biological Properties and Prospects

Actinobacteria is an interesting prokaryotic phylum that includes physiologically, taxonomically, and morphological diverse genera (Atlas 1997). This includes a heterogeneous group of Gram-positive/Gram-variable, aerobes or anaerobes, motile/nonmotile and sporulating/non-sporulating prokaryotes. The majority of actinobacteria possess DNA with high GC content (>50 %) and a few with low GC (Ghai et al. 2012, 2013). Actinobacteria are mostly heterotrophs that thrive on complex organic matter, but the oligotrophic mode of nutrition has also been documented in a very few actinobacteria (Yoshida et al. 2014; Toth 1996). These are often regarded as the prokaryotic equivalent of fungi or filamentous bacteria as most of them grow as branched filamentous hyphae resembling fungi and show similar nutritional preferences. They also share certain characteristic features with bacteria in being unicellular and having prokaryotic nuclei, cell wall composition, and antibiotic susceptibility patterns. Genome size of actinobacteria is in the range of 0.93 Mb (*Tropheryma whipplei*) and 11.9 Mb (*Streptomyces bingchenggensis*) (Verma et al. 2013). Some actinobacteria harbor circular (*Nocardia*)/linear (*Streptomyces*) plasmids. Actinobacteria have been considered as an intermediate group between bacteria and fungi. Subsequently, the precise taxonomic status of actinobacteria had been approved and categorized as a separate phylum *Actinobacteria* within the domain Bacteria. The phylum *Actinobacteria* is one of the most dominant taxonomic units of the domain Bacteria (Ventura et al. 2007) that constitutes six major classes (*Actinobacteria, Acidimicrobiia, Coriobacteriia, Nitriliruptoria, Rubrobacteria, and Thermoleophilia*).

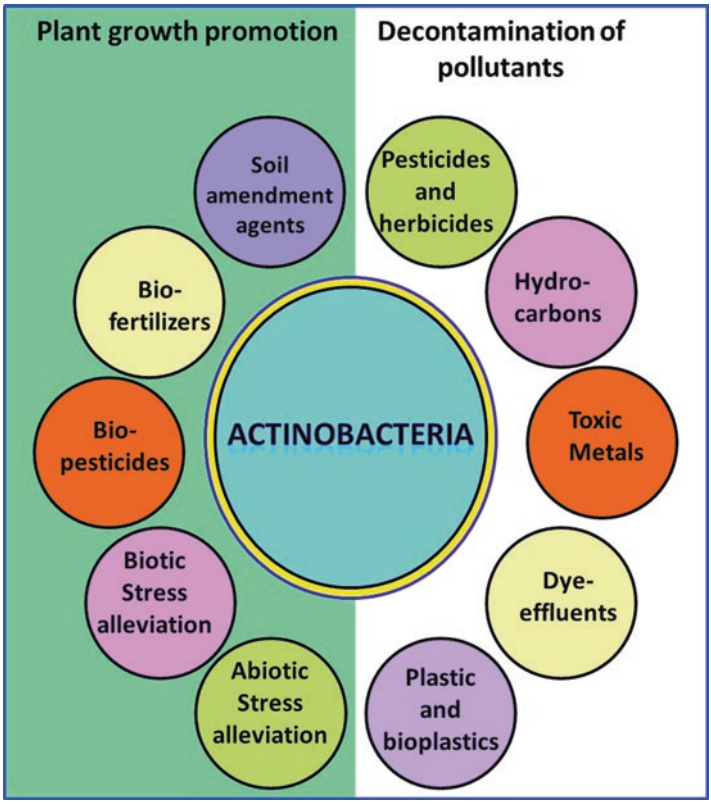

Fig. 9.1 A schematic diagram showing the role of actinobacteria in agriculture and environment sustainability

Actinobacteria encompass a large group of industrially and agriculturally significant species. They are a prolific source of novel secondary metabolites [antimicrobial, antitumor, anti-inflammatory agents (Brana et al. 2015), antioxidants (Karthik et al. 2013)], and other pharmaceutically valuable compounds. Actinobacterial species have a tremendous economic importance in both agriculture and environmental ecology (shown in Fig. 9.1). The phylum *Actinobacteria* includes a considerably high number of plant growth-promoting genera than bacteria (Hamedi and Mohammadipanah 2015). Plant growth-promoting actinobacteria secrete a vast array of chemical modulators, which either directly stimulate plant growth or act indirectly by supporting other plant beneficial microbes. Soil-dwelling actinobacteria either kill or inhibit the growth of plant pathogens via antibiotic production, thereby ensuring the good health of plants. The term "wonder drug" was proposed for antibiotics, as these diminish the threat caused by plant and animal pathogens (Demain 1999). Actinobacteria comprises the largest number of antibiotics producing genera, which produce approximately 45 % of the total antibiotics known (Raja and Prabakarana 2011). They secrete some volatile tertiary alkaloids such as geosmin

(Gerber and Lechevalier 1965) and 2-methylisoborneol (2-MIB) (Gerber 1969), which account for the earthy smell of soil (Wilkins 1996) and indicate fertility and nutrient levels of the soil to farmers. Some actinobacteria display mutualistic relationship like actinorhizal (Verghese and Misra 2002), actinolichen (Lazo and Klein 1965) and endophyte associations (Taechowisan et al. 2005) to promote plant fitness viaplant morphogenesis. Other ecophysiological roles of actinobacteria include nitrogen fixation, phosphate solubilization, and production of phytohormones (auxins and cytokinin) and siderosphores (Palaniyandi et al. 2013b), which add further value to the significance of actinobacterial taxa from the perspective of agriculture.

The effectiveness of actinobacteria is not only limited in formulation of biofertilizers or biopesticides, but they also appear ideal for myriad applications in environmental biotechnology. Their adaptive morphology as well as exceptional metabolic versatility enables them to establish their populations to all kinds of extreme environments including highly polluted locations. Pizzul et al. (2006) evaluated the significant role of actinobacteria in decontamination of polyaromatic hydrocarbons. Studies on the evaluation and characterization of pollutant degrading actinobacteria are currently increasing and these are gaining considerable attention in developing bioremediation tools because of their favorable characteristics such as filamentous structure, sporulation, drought resistance, and having an ability to secrete hydrolytic enzymes.

9.3 Role of Actinobacteria in Sustainability of Agriculture System

Besides being a potential source of antibiotics, actinobacteria are a prominent source of agro-active products (Tekaya et al. 2012). Actinobacteria are naturally associated with plants and display several beneficial effects on plant growth. Their distinctive features make them highly useful in the conservation of soil quality, control of plant diseases, and regulation of plant metabolism. The inoculants of some actinobacteria are being employed in soil amendment, biocontrol and as biofertilizers. The mechanisms and applications, through which they regulate and improve the health of plants, are described below.

9.3.1 Soil Amendments

Conventional farming practices rely on chemical inputs (fossil fuel derivatives) and highly mechanized approaches which have proven to be effective in feeding an exponentially increasing population. These rapid agricultural innovations have been successful in maximizing the crop yield though at the cost of natural ecosystems. They bring about a radical change in environmental biotic and abiotic factors that lead to soil and land degradation, water scarcity, and resource depletion. These

modern practices often require high cost energy inputs and nonrenewable resources affecting the landscape economy. These concerns have prompted the agriculture policies to shift towards organic farming to preserve the ecological integrity that includes the health of soils, ecosystems, and people. Organic agriculture techniques include sustainable practices such as crop rotation, composting, and biological pest controls. Composting is microbial degradation of complex organic matter into nutrient-rich humus that nurtures plants and helps in restoration of productivity of eroded soils (Barker 1997). Humus contributes to the formation of dense aggregates by gluing soil particles together and thereby improving water retention capacity of the soil. Similarly, the filamentous structure of actinobacteria is also involved in the formation of stable soil aggregates (Barea et al. 2005). Humus results from hydrolytic microbial actions on lignocellulosic materials during the composting process. Actinobacteria secrete various types of peroxidases (le Roes-Hill et al. 2011) among which, lignin peroxidases facilitate humification and composting via hydrolysis of lignin into humic acid-like complexes. Compost not only acts as a good soil conditioner to improve soil texture, but also supplements the nutrient content of the soil. Microbes (bacteria, actinobacteria, and fungi) show cumulative actions to break down the complex organic matter during the composting process. Actinobacteria and bacteria belonging to the phylum Firmicutes (Fracchia et al. 2006) primarily dominate composts. Cultivation- dependent and -independent methods have revealed the dominant and active participation of actinobacteria in composting (Dees and Ghiorse 2001). The composition of actinobacterial community changes during various stages of composting, for example, the presence of both mesophilic (*Corynebacterium*, *Rhodococcus* and *Streptomyces*) and thermotolerant species (*Saccharomonospora viridis*, *Thermobifida fusca* and *Thermobispora bispora*) have been recorded at different phases of compost formation (Steger et al. 2007).

9.3.2 Nutrient Availability

The high metabolic rate and hydrolytic enzyme secretion (amylase, chitinase, cellulase and peroxidases) makes actinobacteria potential decomposers that mineralize complex organic matter into simpler assimilative forms. They release solubilized carbon compounds in large quantities into the soil. In addition to carbon sources, other macro- and micro-nutrients are also essential for plant growth. Agricultural practices such as irrigation and natural phenomena like rain result in unwanted washing away of essential minerals from the cropland making soil unproductive. Numerous chemical fertilizers are used to the soil directly or onto the plant foliage to improve crop yield and quality. These fertilizers get immobilized in soil (Reddy et al. 2002) or percolate into deeper soil horizons and become unavailable for plant uptake. The agricultural runoff contaminates ground water as well as fresh surface water resources such as pond and river by leaching the hazardous chemicals (Shigaki et al. 2006). Therefore, innovative agricultural research inclines towards cleaner and safer cropping practices such as utilization of microbes as biofertilizers. Several

actinobacterial species with efficient biological activities such as nitrogen fixation, phosphate solubilization and siderophore production have been isolated and screened from soil, rhizosphere, roots and aerial parts of plants (Table 9.1).

9.3.2.1 Nitrogen Fixation

Nitrogen is a versatile element available in both organic and inorganic forms that play various structural and functional roles in all living organisms. It is a critical limiting factor for plant morphogenesis. Despite being a highly abundant gas (approximately 78 % of total atmospheric gases), molecular nitrogen (N≡N) is quite stable and inert which is an unsuitable form for plant use. Some microbes, known as diazotrophs, possess nitrogenase activity, and are capable of fixing the atmospheric N_2 into ammonium (NH_4^+), which is transformed into nitrate (NO_3^-) or organic nitrogen forms for their own growth or for plant assimilation. They maintain symbiotic association with plants by providing nitrogenous compounds and in turn utilizing carbon compounds like sugars of plant origin. They live as either endobionts of plants or free living. Diazotrophic growth metabolism is also displayed by some actinobacterial species. For example, *Frankia* species have been well characterized which make an association with dicot plants (belonging to 24 genera and eight families) and cause nodulation on plant roots, known as actinorhizal association (Yamaura et al. 2010). Nitrogen fixation activity has also been noticed in non-*Frankia* actinobacteria including *Arthrobacter* sp. (Cacciari et al. 1979), *Mycobacterium flavum* (Fedorov and Kalininskaya 1961), *Corynebacterium autotrophicum* (Berndt et al. 1978), *Microbacterium* isolates (Ruppel 1989), *Agromyces* and *Propionibacteria* (Sellstedt and Richau 2013). The actinobacterial species belonging to the family *Thermomonosporaceae* and *Micromonosporaceae* are also capable of fixing atmospheric nitrogen (Valdes et al. 2005). A thermophilic actinobacterium, *Streptomyces thermoautotrophicus* is an obligate chemoautotroph, which has been isolated from burning charcoal pile (Gadkari et al. 1990). This actinobacterium has a tendency to fix atmospheric nitrogen during autotrophic growth (Ribbe et al. 1997). Actinobacterial species also facilitate nitrogen availability by promoting the growth of other plant symbionts (Palaniyandi et al. 2013b). Actinobacterial species such as *Streptomyces*, *Micromonospora*, and *Actinoplanes* have been shown to promote the root nodulation of *Frankia* sp. (Solans 2007) and *Sinorhizobium meliloti* (Solans et al. 2009). Several other actinobacteria are known to colonize the mycorrhizae, and strengthen the plant mycorrhiza association (Table 9.1) by promoting the growth of fungal hyphae or germination of fungal spore. Mycorrhiza is a plant–fungal association in which fungal species mineralize the nutrients and make them available to the plant and utilize the sugars released by plant roots.

Table 9.1 List of plant growth-promoting actinobacteria

Actinobacteria	Inhabitation types	Host plant/ mycorrhizal association	References
Nitrogen-fixing actinobacteria			
Frankia sp.	Root colonizing or free living	Plants of families (*Betulaceae*, *Casuarinaceae*, *Coriariaceae*, *Datiscaceae*, *Elaeagnaceae*, *Myricaceae*, *Rhamnaceae*, and *Rosaceae*)	Benson and Silvester (1993)
Micromonospora sp.	Root colonizing	*Casuarina equisetifolia*	Valdes et al. (2005)
	Root nodule colonizing and rhizosphere inhabitant	*Pisum sativum*	Carro et al. (2012)
Streptomyces thermoautotrophicus	Soil inhabitant	–	Ribbe et al. (1997)
Phosphate solubilizing actinobacteria			
Streptomyces sp. CTM396	Agricultural soil and rock processing site inhabitants	–	Farhat et al. (2015)
Citricoccus zhacaiensis B-4	Rhizosphere inhabitant	Banana plant	Selvakumar et al. (2015)
Cellulosimicrobium sp. S16	Rhizosphere soil inhabitants	Potatoes plant	Nabti et al. (2014)
Streptomyces badius	Mangrove isolate	–	Bhardwaj et al. (2012)
Leifsonia soli	Rhizosphere inhabitant	Teak plant	Madhaiyan et al. (2010a)
Microbacterium azadirachtae	Rhizoplane inhabitant	Neem seeding	Madhaiyan et al. (2010b)
Thermobifida sp.	Rhizosphere inhabitant	Clover plant	Franco-Correa et al. (2010)
Kitasatospora sp.	Rhizosphere inhabitants	Maize crop	Oliveira et al. (2009)
Streptosporangium isolates	Casts isolates of tropical earthworms	–	Mba (1997)
Plant mycorrhiza growth influencing actinobacteria			
Streptomyces sp.	Mycorrhizal inhabitants	Norway spruce	Schrey et al. (2012)
Rhodococcus sp. strain EJP75	Ectomycorrhizal colonizing	*Pinus sylvestris*–*Lactarius rufus* association	Poole et al. (2001)

(continued)

Table 9.1 (continued)

Actinobacteria	Inhabitation types	Host plant/ mycorrhizal association	References
Actinomycetes	Soil inhabitant	–	Carpenter-Boggs et al. (1995)
Streptomycescoelicolor 2389	–	Sorghum–*Glomus intraradices* LAP8 association	Abdel-Fattah and Mohamedin (2000)
Streptomyces strains MCR9, MCR26 and *Thermobifida* strain MCR24	Rhizosphere inhabitant	Clover plants–*Glomus mosseae*	Franco-Correa et al. (2010)
Siderophore producing actinobacteria			
Streptomyces sp.	–	–	Imbert et al. (1995)
Rhodococcus erythropolis IGTS8	–	–	Carrano et al. (2001)
Nocardia tenerifensis NBRC 101015	–	–	Mukai et al. (2009)

9.3.2.2 Phosphate Solubilization

Like carbon and nitrogen, phosphorus is also a crucial macro-element, which is necessary for growth and development of all living organism. It is an integral part of various biological molecules such as nucleic acids, phospholipids and energy-rich compounds (ATP, NADH, and NADPH). It has an important role in numerous metabolic pathways such as cell division, signal transduction, macromolecular biosynthesis, photosynthesis (Shenoy and Kalagudi 2005; Fernandez and Schaefer 2012), and constitutes approximately 3 % of total dry cell weight (Bhardwaj et al. 2012). It is second most crucial component after nitrogen for plant growth (Donahue et al. 1990). Despite the presence of high quantity (400–1200 mg/kg) of phosphorus in soil (Fernandez and Novo 1988), only a small proportion (1 mg/kg or less) is accessible to the plant (Goldstein 1996). The availability of phosphorus is mainly limited by two processes (1) immobilization of soluble phosphorus in soil particles (2) adsorption of phosphorus onto compounds (aluminum oxide, iron oxide, and aluminum silicate) in acidic soil (Whitelaw 2000) or calcium carbonate in alkaline soil (Gyaneshwar et al. 2002). Several phosphate solubilizing microbes have been characterized which transform insoluble phosphorus into solubilized form through processes such as acidification, chelation (Delvasto et al. 2006), and hydrolytic enzyme production. Mutualistic actinobacterial species are the key participants in the biogeochemical cycling of phosphorus in marine environments (Sabarathnam et al. 2010). Various rhizosphere inhabitants and endophytic actinobacteria have phosphate solubilizing capability, among which, a comparatively high abundance of *Streptomyces* species occur in phosphate mobilizing sites (Hamdali et al. 2008; Jog et al. 2014; Franco-Correa et al. 2010). Their additional antimicrobial activities make them more competent to function as Plant

Growth-Promoting Agents (PGPA). Few other non-*Streptomyces* species have also been reported (Table 9.1) to facilitate plant growth by mineralizing insoluble phosphorus into soluble forms for plant uptake.

9.3.2.3 Enhancement of Iron Uptake

Plant growth and development also requires additional elements such as Fe, Co, Cr, Cu, Zn, Mn, and Mo in very small quantities. Iron is a major limiting element which functions as a cofactor of several enzymes and reaction center of numerous proteins involved in energy metabolism. In the soil, it mainly exists in various oxide forms such as insoluble (Fe^{+3}) and soluble (Fe^{+2}) forms. Many microbes have a tendency to catalyze the reduction of Fe^{+3} into a soluble form (Fe^{+2}) which is assimilated by plant or plant beneficial microbes (Francis et al. 2010). Actinobacteria such as *Arthrobacter* spp. (Valencia-Cantero et al. 2007) and *Kocuria rosea* HN01 (Wu et al. 2014) are capable of catalyzing the reduction of ferric iron to a soluble form and facilitate plant growth in alkaline soils. The actinobacteria enhance iron availability (listed Table 9.1) by producing siderophores. Siderophores are small organic molecules, which chelate the iron moieties and sequester them in the rhizospheric zone of the plant. Furthermore, siderophore production enables *Streptomyces* species to hinder the germination of basidiospores of pathogenic fungus, *Moniliophthora perniciosa* (Macagnan et al. 2006). Actinobacterial siderophores can also promote the proliferation of beneficial actinobacteria exhibiting antagonistic activity against pathogens (Palaniyandi et al. 2013b), thereby involving in the regulation of health of plants.

9.3.3 Alleviation of Biotic and Abiotic Stresses

Plants are constantly subjected to various biotic and abiotic stresses in their natural environment. These stresses cause severe impact on agricultural crop productivity. Biotic plant attackers include microbial pathogens (bacteria, fungi, and viruses), weed plants and insects, which lower crop yields and their market value. Abiotic stresses are due to environmental factors (drought, temperature, nutrient deficiency, and salinity). To cope up with these stresses, plants have developed various strategies such as synthesis of phytohormones (salicylic acid, jasmonic acid, abscisic acid, and ethylene). These phytohormones are involved in providing protection against both biotic and abiotic stresses (Fujita et al. 2006). A diverse array of microbes help plants to mitigate the negative impact of various stresses caused by abiotic factor (Grover et al. 2011). Actinobacteria are the prominent species that participate in providing protection to plants by killing or suppressing the growth of microbes directly via antibiosis, parasitism or in an indirect manner (induction of the plant immune system) (Palaniyandi et al. 2013b). The mechanisms, through which the actinobacteria show plant disease suppression and biotic or abiotic stress alleviation, are described below.

9.3.3.1 Biotic Stress Alleviation

Microbial pathogenicity and emergence of pesticide-resistant pathogens bring about new challenges to agro-economy. Biotic stresses, primarily plant diseases, are a significant impediment in attaining the actual potential crop yield. The incidence of several plant diseases brings nearly 9–16 % losses in total production yields of many important crops (rice, wheat, barley, maize, potato, and cotton) (Chakraborty et al. 2000). Several side effects of using chemical pesticides are prompting researchers to find eco-friendly solutions to combat the severe damages caused by plant pathogens. Actinobacteria have a great potential in controlling plant pathogens. Many genera including *Arthrobacter* (Mitchell and Hurwitz 1965) *Cellulomonas* (Wadi and Easton 1985), *Actinoplanes*, *Micromonospora* (Filnow and Lockwood 1985), and *Streptomyces* (Al-Askar et al. 2015) are capable of reducing the growth of plant pathogens. Palaniyandi et al. (2013b) described basic mechanisms of disease suppression by actinobacteria that include: (1) production of antibiotics or cell wall degrading enzymes, (2) exhibition of hyperparasitism on plant parasitic microbes as well as competition with disease causing microbes in order to colonize the plant rhizosphere, and (3) induction of plant immune system. Antibiotic production is a major mechanism that gives the actinobacterial group prominence in agricultural crop protection. Actinobacteria colonize as a major microbial population in rhizosphere of many plant species. Their high dominance has been recorded in soil amended with *Brassica* plant residues. An increased actinobacterial population had resulted in a significant suppression of *Rhizoctonia solani* damping-off disease (Ascencion et al. 2015) in the *Brassica* amended soil. Many *Streptomyces* species are known for having a pronounced competence for controlling the growth of plant pathogens (Table 9.2). For instance, *Streptomyces griseorubens* E44G showed a high antifungal effect, thus, could inhibit the mycelial growth of *Fusarium oxysporum* f. sp. *lycopersici* (Al-Askar et al. 2015) which is a seed-borne fungal species responsible for causing damages to tomato crop. The growth of another soil-borne pathogen, *Sclerotium rolfsii*, has been controlled by *Streptomyces* sp. (Errakhi et al. 2007). A novel actinobacterium, *Streptomyces* sp. N2, had a broad-spectrum inhibitory effect against various phytopathogenic fungi such as *Pyricularia grisea*, *Fusarium oxysporum* f. sp. *niveum, F. oxysporum* f. sp. *vasinfectum*, *Penicillium italicum*, *Colletotrichum gloeosporioides*, and *Rhizoctonia solani.* In vivo, this actinobacterium showed significant inhibitory action only against *Rhizoctonia solani* causing anthracnose disease of grapes (Xu et al. 2015). Li et al. (2014b) isolated a *Streptomyces* sp. strain CNS-42 from the plant *Alisma orientale,* which displayed a broad-spectrum antimicrobial activity against pathogenic bacteria and fungi. The strain CNS-42 produced a compound staurosporine that shows both antifungal and plant growth-promoting activity. Another *Streptomyces* species, *Streptomyces araujoniae* ASBV-1^{T} was reported to produce a multiantibiotic complex (containing monactin, dinactin, trinactin, tetranactin, and valinomycin) that eradicates fungal pathogens by disturbing the integrity of cell structure (Silva et al. 2014) via formation of ionophores in the cell membrane. Actinobacteria reported to exhibit plant disease suppression activities are listed in Table 9.2.

Table 9.2 Biocontrol of plant pathogens by actinobacterial species

Actinobacteria	Bioactive compounds/enzymes	Plant pathogen	Economic plant	References
Streptomyces sp.	Antifungal agent	*Sclerotium rolfsii*	Sugar beet	Errakhi et al. (2007)
Streptomyces sp. N2	Antifungal metabolite (3-methyl-3,5-amino-4-vinyl-2--pyrone, $C_6H_7O_2N$)	*Colletotrichum gloeosporioides*	Grape fruits	Xu et al. (2015)
Streptomyces ambofaciens S2	Antifungal compounds	*Colletotrichum gleosporioides*	Red Chilli fruits	Heng et al. (2015)
Streptomyces sp. strain CNS-42	Antifungal agents (staurosporine)	*Fusarium oxysporum* f. sp. *cucumerinum*	Cucumber	Li et al. (2014b)
Streptomyces (*S. canus, S. fradiae, S. avermitilis,* and *S. cinnamonensis*) and non-*Streptomyces* sp. (*Leifsonia poae*)	–	*Xanthomonas axonopodis*	Pomegranate	Poovarasan et al. (2013)
	Antifungal compounds	*Fusarium oxysporum* and *Alternaria solani*	Guava	Mohandas et al. (2013)
Propionicimonas sp. ENT-18	Albocycline	*Sclerotinia sclerotiorum*	–	Zucchi et al. (2014)
Streptomyces hygroscopicus	–	*Colletotrichum acutatum, C. gloeosporioides and Fusarium avenaceum*	Apple	Grahovac et al. (2014)
Streptomyces araujoniae ASBV-1 T	Multiantibiotic complex	*Botrytis cinerea*	Strawberry pseudofruit	Silva et al. (2014)
Production of cell wall degrading and other antagonistic enzymes				
Streptomyces sp.	Chitinase	*Lasiodiplodia theobromae*	Rubberwood	Sajitha and Florence (2013)
Streptomyces sp. 9p	Chitinase and β-1,3-glucanase	*Alternaria brassiceae* OCA3	Chilli	Srividya et al. (2012)
Streptomyces sp. PTK19	Chitinase	*Fusarium oxysporum* PTK2	–	Thiagarajan et al. (2011)
Streptomyces vinaceusdrappus S5 MW2	Chitinase	*Rhizoctonia solani*	Tomato	Yandigeri et al. (2015)
Streptomyces goshikiensis YCXU	Volatile antifungal compounds and enzymes (β-1,3-glucanase, chitinase, and urease)	A broad range of phytopathogenic fungi and in vivo suppression of *Fusarium* sp.	Watermelon	Faheem et al. (2015)

(continued)

Table 9.2 (continued)

Actinobacteria	Bioactive compounds/enzymes	Plant pathogen	Economic plant	References
Streptomyces phaeopurpureus ExPro138	Proteases	*Colletotrichum coccodes*	Tomato	Palaniyandi et al. (2013a)
Streptomyces sp.	Chitinase, phosphatase, and siderophores	*Xanthomonas oryzae* pv.*oryzae*	Rice	Hastuti et al. (2012)

EL-Tarabily et al. (1997) screened 45 *Streptomyces* and non-*Streptomyces* sp. for their in vitro and in vivo fungal inhibition activity. Among them, seven species (*Streptomyces janthinus*, *Streptomyces cinerochromogenes*, *Streptoverticillium netropsis*, *Actinomadura ruhra*, *Actinoplanes philippinensis*, *Streptosporangium albidum*, and *Micromonospora carbonaceae*) showed inhibitory action against fungal pathogens (*Pythium* sp.) by producing non-volatile metabolites. *Pythium* species are widely known as causative agents of disease cavity spot in carrots, which decrease the quality of carrots resulting in substantial economic losses. *Actinoplanes philippinensis* and *Micromonospora carbonaceae* showed hyperparasitism on growing hyphae and oospores of *Pythium coloratum.* They colonized heavily on the outer surface of mycelium and resulted in cytoplasmic collapses of oospores. In another report, 64 out of total 317 actinobacterial cultures (isolated from roots and rhizospheric soils of leguminous plants) were reported to exhibit antagonism against soybean pathogen *Xanthomonas campestris* pv. *glycine* (Mingma et al. 2014) causing bacterial pustule. Among them, *Streptomyces* sp. RM 365 showed highest inhibition rate against *Xanthomonas campestris* pv. *glycine*. This actinobacterium did not display any antagonistic activity against *Rhizobium* sp. (plant growth-promoting bacterial species), thus, can be a potential candidate for the development of a biocontrol agent (BCA) to control the plant bacterial pustule. *Streptomyces phaeopurpureus* ExPro138, isolated from rhizosphere of yam plant, was shown to produce multiple proteases and inhibit the growth of foliar fungal pathogen (*Colletotrichum coccodes*) in early stage by disrupting various processes such as spore germination, spore adhesion, and appressorium formation (Palaniyandi et al. 2013a). Marine isolates belonging to the genera *Streptomyces*, *Nocardiopsis*, and *Saccharopolyspora* also displayed antagonism against phytopathogens like *Colletotrichum falcatum*, *Thielaviopsis paradoxa,* and *Fusarium semitectum* (Vijayakumar et al. 2012). *Streptomyces* sp. PM9 was effective candidate for controlling microbial disease in forest plants (Salla et al. 2014). This actinobacterium brought changes in the secondary metabolism of economically valuable plants (*Eucalyptus grandis* and *Eucalyptus globulus*) by (1) boosting up the plant immune system by triggering the enhanced production of key enzymes (polyphenol oxidase and peroxidase) of plant defense mechanism and (2) inducing synthesis of total

phenolic and quercetinic flavonoid fraction. The strain PM9 also produces indole-3-acetic acid to stimulate high rooting of plants. Mohamed et al. (2013) reported that *Streptomyces noboritoensis* produces bioactive compounds and their usefulness was assessed for suppressing the growth of bacterial or fungal contaminants during in vitro micropropagation of banana. The two actinobacterial species, *Curtobacterium flaccumfaciens* and *Rhodococcus* sp., were isolated from ascocarps of *Tuber magnatum* collected from a natural truffle ground in Western Serbia (Pavic et al. 2013). Both showed β-glucanase activity, siderophore production, and ammonification of organic matter. Besides enhancing the nutrition content of soil, they were also capable of promoting growth of other plant beneficial fungal species such as *Trichoderma* species. Valois et al. (1996) showed that multiple glucanases (β-1,3-, β-1,4-, and β-1,6-glucanases) producing actinobacteria triggered lysis of the cell wall of *Phytophthora fragariae* and reduced root rot when co-inoculated with raspberry plantlets. *Streptomyces* species (*Streptomyces canus, S. fradiae, S. avermitilis,* and *S. cinnamonensis*) and non-*Streptomyces* species (*Leifsonia poae*) colonizing the mycorrhizae (*Glomus mosseae*) of plant pomegranate (*Punica granatum* L. cv *Bhagwa*) were shown to exhibit antibacterial activity against *Xanthomonas axonopodis* which causes bacterial blight of pomegranate and decreases its export drastically (Poovarasan et al. 2013). Among them, *Streptomyces canus* was capable of promoting the plant growth by producing gibberellic acid (GA3) and auxin (indole 3-acetic acid). Mohandas et al. (2013) isolated same *Streptomyces* and non-*Streptomyces* species from the mycorrhizal (*Glomus mosseae*–guava plant association) zone. Out of five, *S. canus*, *S. avermitilis*, and *L. poae* exhibited higher activity of siderophore production and phosphate solubilization. All isolates possessed chitin degradation activity. Chitinase producing actinobacteria mainly provide protection against fungal pathogen because chitinase breaks down chitin, a major component of fungal cell wall. Some endophytic *Streptomyces* species provide protection to plants against pathogenic actinobacterial species such *Streptomyces scabies* by activating the salicylic acid (SA)-mediated plant defense system (Lin et al. 2012). Besides the above, an unusual plant protecting mechanism was identified in an actinobacterium (*Rhodococcus erythropolis*) which was capable of degrading the signaling molecules such as *N*-acyl-homoserine lactone, and thus disturbed quorum sensing-based communication of Gram-negative soft-rot bacteria, thereby providing protection against Gram-negative bacterial pathogens (Latour et al. 2013). In this actinobacterium, the degradation of signaling molecules is stimulated upon activation of γ-lactone degradation pathway that generally requires the presence of incuder (γ-lactone) or cheap stimulating compounds such as γ-caprolactone. The biocontrol system of *R. erythropolis* could be activated by using a stimulator in order to guard crop plants from microbial attack.

Priyadharsini and Dhanasekaran (2015) reported that some actinobacterial species exhibit allelopathic activity against weed plants such as *Cyperus rotundus*. Other reports also suggested that *Streptomyces* species are a potent source of herbicide and inhibit the growth of *Echinochilora crusgalli* (Dhanasekaran et al. 2010) as well as *Cyperus rotundus* (Dhanasekaran et al. 2012). Many *Streptomyces* species

exhibit insecticidal or pesticidal activity. For instance, *Streptomyces hydrogenans* DH16 exhibited antifeedant, pupicidal, larvicidal, and growth inhibitory effects against pest, *Spodoptera litura.* This pest causes defoliation in plants and damages crop yield severely (Kaur et al. 2014). *Streptomyces* species also displayed insecticidal activity against lepidopteran insects (*Helicoverpa armigera*, *Spodoptera litura,* and *Chilo partellus*) (Vijayabharathi et al. 2014). A compound with antibacterial and insecticidal properties was purified from *Streptomyces bikiniensis* A11 (El-khawaga and Megahed 2012). This compound belonging to the class of aminoglycoside antibiotics was found to be very effective against cotton leaf worm *Spodoptera littoralis*, which is one of the most destructive agricultural lepidopteran pests.

9.3.3.2 Abiotic Stress Mitigation

In addition to soil topology, other abiotic factors such as nutrient content, temperature, and moisture are also key determinants influencing global crop productivity. For example, drought has an immense impact on agriculture crop yield, and has generally been considered as one of the major destruction factors of the entire crop system. Actinobacterial species such as *Citricoccus zhacaiensis* B-4 (MTCC 12119) was reported to show plant growth modulation. The strainB-4 (MTCC 12119) enhanced biopriming of onion seeds even under water stress conditions (Selvakumar et al. 2015). This actinobacterium showed other activities such as IAA and GA3 production, phosphate and zinc solubilization, NH_3 production, and 1-aminocyclop ropane-1-carboxylate (ACC) deaminase activity to assist the plant growth by alleviating stress caused by water deficit condition. ACC deaminase activity has been reported from a number of plant growth-promoting actinobacteria. The enzyme ACC deaminase hydrolyzes a substrate (ACC) that is a precursor of ethylene. Ethylene is a well-known stress hormone, which negatively modulates the plant growth during stress conditions (Glick 2005). The concentration of ethylene increases during both biotic and abiotic stresses, which shrinks plant growth and activates other stress alleviating mechanisms. ACC producing actinobacteria therefore enhance plant growth by reducing the effect of stress environment. *Streptomyces* sp. strain PGPA39 isolated from agriculture soil was found to produce ACC deaminase and endorse the growth of "MicroTom" tomato plants under salt stress (Palaniyandi et al. 2014). Besides ACC deaminase activity, this strain produced indole 3-acetic acid (IAA) and was also capable of solubilizing tricalcium phosphate, thus, enhancing both nutrient availability and plant tolerance capacity. El-Tarabily (2008) isolated 64 *Streptomyces* isolates from rhizosphere of tomato plant, which were screened for evaluating their ACC deaminase and plant growth modulation. Among them, two strains *S. filipinensis* no. 15 and *S. atrovirens* no. 26 showed both ACC deaminase activity and high rhizosphere competence. However, an increased plant growth promotion was observed in the plants co-cultured with *S. filipinensis* no. 15 as compared to *S. atrovirens* no. 26. Since the former strain also produces the phytohormone IAA that gives an additional benefit to the plants for their growth.

High salt concentration is another growth limiting factor, which disrupts plant metabolism leading to crop destruction. Basic mechanisms of alleviation of saline stress are production of IAA, secretion of siderophore and ACC deaminase activity. For instance, *Streptomyces* isolate (C) exhibited high IAA production, siderophore biosynthesis, and phosphate solubilization under high salt environment (Sadeghi et al. 2012), which makes it a good candidate as a bioinoculant for enhancing nutrient content in saline crop field. A rhizosphere inhabitant, *Kocuria turfanensis* strain 2 M4 produces IAA and was isolated from rhizospheric soil of the halotolerant plant *Suaeda fruticosa*, colonizing in the saline desert of Little Rann of Kutch, Gujarat, India (Goswami et al. 2014). Srivastava et al. (2014) demonstrated that *Streptomyces rochei* SM3 activates ethelyne-mediated defense pathway and phenylpropanoid pathway in chickpea and therefore discharged stresses caused by both biotic (*Sclerotinia sclerotiorum*) and abiotic (NaCl) factors. Hence, this could be a potential candidate for the development of a plant growth-promoting agent (PGPA).

9.4 Bioformulation of Actinobacteria Inoculant as Biofertilizer and Biopesticide

Bioformulation is a preparation of microbial cell inoculants or microbe-derived products with economical carrier materials (Arora et al. 2010). The use of a suitable carrier material improves shelf-life and stability of microbes and their bioactive compounds during storage and field implementation. Several plant growth-promoting microorganisms including bacteria, actinobacteria, and fungi have been formulated and tested in crop fields. Microbes with multiple mechanisms of disease suppression and plant growth promotion are better candidates for the development of biofertilizers and biopesticides. It is obvious from the foregoing discussion that actinobacteria have several attributes useful for biocontrol and plant growth promotion. Many *Streptomyces* species and their bioactive compounds have, therefore, been formulated and commercialized as biofertilizers and biopesticides for crop protection and enhancing yield (Table 9.3).

9.5 Role of Actinobacteria in Environment Sustainability

Besides plant growth promotion and disease suppression, actinobacteria play a vital role in various biological degradation processes. They have a high competence for degrading recalcitrant polymers such as toxic chemicals (pesticides, insecticides, and herbicides), dyes, bioplastics, and oil and petroleum products. Their imperative role in heavy metal detoxification has also been documented. In general, laboratory studies related to microbial degradation are not always successful during in situ bioremediation, since microbial cells used are subjected to both biotic and abiotic

Table 9.3 Commercially available bioformulants of actinobacteria (adapted from Palaniyandi et al. (2013b).

Commercialized product name	Actinobacterium and/or bioactive compounds	Applications
Actinovate® AG	*Streptomyces lydicus* WYEC108	BCA
Micro108® soluble	*S. lydicus* WYEC108	BCA
Action Iron®	*S. lydicus* WYEC108	BCA and PGPA
Thatch Control	*S. violaceusniger* strain YCED 9	BCA
Mycostop®	*S. griseoviridis* strain K61	BCA
YAN TEN *Streptomyces saraceticus*	*S. saraceticus* KH400	BCA
AFFIRM^{WDG}	Polyoxin D (*S. cacoi* var. *asoensis*)	BCA
PH-D® Fungicide	Polyoxin D (*S. cacoi* var. *asoensis*)	BCA
Keystrepto™	Streptomycin (*S. griseus*)	BCA
Agri-Mycin 17 WP	Streptomycin (*S. griseus*)	BCA
Strepto	Streptomycin (*S. griseus*)	BCA
Plantomycin WG	Streptomycin (*S. griseus*)	BCA
Ag-Streptomycin	Streptomycin (*S. griseus*)	BCA
Plantomycin	Streptomycin (*S. griseus*)	BCA
Kasumin™	Kasugamycin (*S. kasugaensis*)	BCA
Biomycin	Kasugamycin (*S. kasugaensis*)	BCA
Omycin	Kasugamycin (*S. kasugaensis*)	BCA

environmental challenges that may decrease their survival rate and degradation efficiency. Therefore, microbes isolated from polluted sites are better candidates for bioremediation. Many investigations support the fact that actinobacterial species show supremacy in heavily contaminated zones (Gremion et al. 2003; Chikere et al. 2009). Actinobacteria have considerable tolerance or acclimatization potential for the toxic compounds or metals, which help them to grow in highly polluted sites as well as to clean the environment. The use of actinobacteria and their enzymes as a bioremediation tool may thus provide an effective gateway to the field of environmental biotechnology.

9.5.1 Bioremediation of Pesticides/Insecticide-Polluted Sites

Cultivable land area and water resources are becoming scarce in modern industrial times, which drastically affect the world agro-economy. The new challenges of modern agricultural system include producing more and more food commodities to feed the ever increasing population with limited resources. Considering the agricultural intensification aspects, the use of various organic or inorganic agrochemicals has been allowed for cropping. Most chemical compounds are hazardous and persist for longer in the environment raising serious concerns such as their toxicity to

nontarget organisms. This creates a need to decontaminate toxic pollutants and restore the environmental sustainability. A high catabolic rate, adaptability, rapid germination of spores, and fast-growing hyphae make actinobacteria potent candidates to remediate polluted sites (Fuentes et al. 2010). Their filamentous structures penetrate and facilitate colonization into the deep soil horizon, therefore, minimizes the mixing step of bioremediation process (Ensign 1992), which is an advantage for using actinobacteria in bioremediation. In addition, the indigenous actinobacterial population is comparatively high (10^4–10^6 per gram of soil) and has been enumerated as the second most abundant inhabitants after bacteria in soil (Goodfellow and Williams 1983). In general, indigenous microbes of contaminated environment are considered as good candidates for bioremediation (El Fantroussi and Agathos 2005) since they are already acclimatized to tolerate (Shelton et al. 1996). The actinobacteria isolated from a polluted site are capable of secreting a large number of extracellular enzymes such as monooxygenase and dioxygenase that catalyze the mineralization of xenobiotic pesticides with diverse chemical compounds. A large group of actinobacterial species was observed as the active participants of biodegradation processes in freshwater and marine sediments. They constitute an approximately 21.7 % fraction of the total genera identified by a metagenomic approach (Fang et al. 2014). Actinobacteria utilize pesticides either as a carbon and energy source or co-metabolize the harmful chemicals without gaining any advantage. Co-metabolism is the degradation of toxic chemicals by hydrolytic enzymes produced by microbes for metabolism of other energy yielding biomolecules. *Brevibacterium linens* DSM20425 was shown to co-metabolize the toxic pesticide 2,4,5-T into 3,5-dichlorocatechol (Horvath 1971). Pesticide degradation is most often completed through synergistic actions of microbial consortia than via a single isolate. In mixed microbial populations, microorganisms either directly degrade toxic compounds or hasten the biotransformation efficiency of other microbes. Byss et al. (2008) showed the synergistic action between actinobacteria and *Pleurotus ostreatus* in bioremediation.

Organophosphate pesticides (OP) are chemicals with O-P bonds, used worldwide as pesticide, insecticide, and herbicide accounting for more than 34 % of the total world market (Singh and Walker 2006). It includes chlorpyrifos (*O*,*O*-diethyl *O*-(3,5,6-trichloro-2-pyridinyl) phosphorothioate), parathion (*O*,*O*-diethyl *O*-4-nitrophenyl phosphorothioate), malathion (*O*,*O*-dimethyl *S*-1,2-di(ethoxycarbonyl), ethyl phosphorodithionate), and diazinon (*O*,*O*-diethyl *O*-(2-isopropyl-6-methylpyrimidine-4-yl(phosphorothioate)). Most of them have been found to interfere with the function of acetylcholinesterase (a key enzyme of neurotransmission) (Hassall 1990), thus act as broad-spectrum insecticides. However, only <0.1 % fraction of total employed pesticide is used up in killing or suppression of growth of the target organisms (Pimentel 1995), while the rest remains in the environment and contaminates both soil and water ecosystems, leading to major environmental and human health problems. The extensive use of OP has become a major cause of over 2,00,000 deaths annually worldwide (Singh et al. 2009). These polluted sites require to be decontaminated by chemical, physical, and biological methods. Microbial degradation is considered as a better

option to clean up the polluted sites since microbes can detect even the presence of small quantity of pesticides and detoxify them.

Chlorpyrifos (CP) is a broad-spectrum chlorinated organophosphorus insecticide that has been used for over 40 years to increase crop productivity. According to the statistics data of the Committee of the Ministry of Agriculture and Land Reclamation (2011), approximately 1280 tons of CP is consumed annually in agriculture fields in Egypt. Persistence of CP is between 10 and 120 days in soil, but can extend up to 1 year in some environmental conditions. CP residues have been detected in various ecosystems, which led to disturbance in biogeochemical cycles (Chishti et al. 2013). They need to be completely detoxified as soon as possible after their application. Briceno et al. (2012) isolated two potent *Streptomyces* strains, which could metabolize up to 90 % of toxic CP within 24 h of incubation, and yield 3,5,6-trichloro-2-pyridinol (TCP). However, the release of TCP into environment is another major ecological problem because of its higher solubility and mobility than the parent compound (CP). It exhibits antimicrobial activity inhibiting the proliferation of CP degrading bacteria (Singh and Walker 2006), thus the complete degradation of CP is required. An actinobacterium, *Gordonia* sp. JAAS1 capable of degrading the CP and its hydrolytic metabolite such as TCP into diethylthiophosphoric acid (DETP) was isolated from a paddy field (previously exposed to CP treatment) (Abraham et al. 2013). Recently, a kinetic study of parathion degradation by *Streptomyces venezuelae* ACT 1 has been done, which revealed that the actinobacterium strain ACT 1 has a high ratio of degradation and chemical oxygen degradation (COD) reduction rate (Naveena et al. 2013). The high biodegradability enhances industrial importance of this strain, especially in the treatment of pesticide-contaminated wastewater. Conversion of methyl parathion into PNP has been achieved by using *Nocardiopsis* sp. DD2, isolated from the coastal area, Gujarat, India (Pravin et al. 2012). The strain DD2 showed a broad catabolic activity and was capable of degrading other organophosphate pesticides such as endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9 a-hexahydro-6,M9-methano-2,4,3-benzo-dioxathiepine-3-oxide). A constitutive expression of enzymes degrading parathion has been observed in *Arthrobacter* sp. (Nelson 1982) that utilizes parathion as a sole source of carbon and energy. *Arthrobacter* species are also capable of degrading another OP such as diazinon, but it requires the process of cometabolism by *Streptomyces* species to initiate the degradation process (Gunner and Zuckerman 1968).

PNP is another environmental pollutant, extensively used as a raw material in the manufacturing of dyes, explosives, drugs, and herbicides. It is also released as an end product of microbial degradation of pesticides such as parathion and methyl parathion (Ningthoujam et al. 2012). A comparatively high solubility of PNP (16 g/L) in water enhances its infiltration through soil strata, leading to the contamination of both surface and ground water (Kulkarni and Chaudhari 2006). High concentration of PNP is extremely hazardous to human health and affects severely both microbial flora and fauna (PAN 2008). It has been listed as a major pollutant by the U.S. Environmental Protection Agency (EPA) (http://www.epa.gov/waterscience/methods/pollutants.htm). Several actinobacterial species such as *Citricoccus nitrophenolicus* (Nielsen et al. 2011), *Rhodococcus* sp. HS6-1 and *Brevibacterium* sp. (Ningthoujam 2012)

have been reported to metabolize PNP and lower its toxicity. The last two actinobacterial strains were shown to degrade up to 350 and 270 mg/L PNP, respectively. Hanne et al. (1993) isolated two PNP degrading soil actinobacteria (*Arthrobacter aurescens* TW17 and *Nocardia* sp. strain TW2). *Arthrobacter* strain harbors genes, which encode enzymes involved in biodegradation on an extrachromosomal plasmid. The enzyme production in both strains is inducible and requires the presence of pesticides.

Glyphosate is a well-known organophosphonate herbicide (Pn) with C-P linkage which inhibits the function of a critical enzyme (5-enolpyruvyl shikimic acid-3-phosphate synthase) of the biosynthetic pathway of aromatic amino acids (Steinrucken and Amrhein 1980). The C-P bond makes glyphosate more stable and resistant to the microbial degradation. Only two *Arthrobacter* species had been reported to utilize glyphosate as the sole source of phosphorus (listed in Table 9.4). Metabolic pathways of glyphosate differ in both the actinobacteria. *Arthrobacter* sp. GLP-1 produces two distinct C-P lyases, which act on glyphosate to yield sarcosine. Sarcosine is further degraded to glycine (incorporated in purine and pyrimidine) and C_1-unit (utilized for the synthesis of aminoacids) (Kertesz et al. 1991). A very dissimilar glyphosate metabolism was observed in *A. atrocyaneus* ATCC 13752 that catabolizes glyphosate into aminomethylphosphonic acid (AMPA) and C_2-units (Pipke and Amrhein 1988). Complete degradation of AMPA to CO_2 occurs in this actinobacterium.

Organochlorine pesticides such as Endosulfan (6,7,8,9,10,10-Hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepine-3-oxide), Lindane (gamma-hexachlorocyclohexane (γ-HCH)), Chlordane (1,2,4,5,6,7,8,8-Octachloro-3a,4,7,7a-tetrahydro-4,7-methanoindane), Metolachlor [(RS)-2-Chloro-*N*-(2-ethyl-6-methyl-phenyl)-*N*-(1-methoxypropan-2-yl)acetamide],atrazine(2-chloro-4-isopropylamino-6-ethylamino-*s*-triazine), Methoxychlor [1,1,1-Trichloro-2,2-bis(4-methoxyphenyl) ethane], DDT (Dichloro diphenyl trichloroethane), PCNB (pentachloronitrobenzene), 2,4-D [(2,4-Dichlorophenoxy)acetic acid], 2,4,5-T [(2,4,5-Trichlorophenoxy)-acetic acid], and pentachlorophenol are the most toxic and environmentally destructive synthetic chemicals. Most of them have been banned in many countries because of their long lasting persistence, high toxicity and ability to bioaccumulate in the living tissues (Hirano et al. 2007). Actinobacteria have a good potential to detoxify or feed on the hazardous organochlorine pesticides (listed in Table 9.4). Martens (1976) had isolated several endosulfan degrading actinobacteria. The detailed investigations on actinobacteria confirmed that the genus *Streptomyces*is capable of catabolizing a wide range of organochlorine pesticides, specifically, DDT, PCNB (Chacko et al. 1966), metolachlor (Liu et al. 1990), Dalapon (Kaufman 1964), diuron (Castillo et al. 2006), atrazine (Fadullon et al. 1998), lindane, chlordane, and methoxychlor (Fuentes et al. 2010).

The degradation of mono-, di-, and tri-chlorinated pesticides is commonly observed among actinobacteria. The enzymatic system involved in biodegradation of 2,4-D has been extensively studied in two actinobacterial strains including *Nocardioides simplex* 3E (Kozyreva and Golovleva 1993) and *Arthrobacter* strain

Table 9.4 List of actinobacteria degrading pesticide and insecticide

Chemicals	Actinobacteria	Site of isolation	Pesticide degradation capacity	References
Organophosphorus pesticides				
Chlorpyrifos	*Streptomyces* sp. strain AC5	Soil samples, southern Chile	90 % of 25 mg/L or 50 mg/L	Briceno et al. (2012)
	Streptomyces sp. strain AC7			
	Streptomyces thermocarboxydus strain A-B	Agricultural wastewater, Egypt	77.57 % in 28 days	Eissa et al. (2014)
	Gordonia sp. JAAS1	Agricultural soil	110 mg/L	Abraham et al. (2013)
Parathion	*Streptomyces venezuelae* ACT 1	Marine water sample	Hydrolase activity rate as 0.273/h	Naveena et al. (2013)
Methyl parathion	*Nocardiopsis* sp.	Coastal area, India	–	Pravin et al. (2012)
Diazinon	*Streptomyces* sp. AC1–6	Agricultural soil	40–50 % and 70–90 % after 24 and 96 h of incubation, respectively	Briceno et al. (2015)
	Streptomyces sp. ISP4			
	Arthrobacter sp. and Streptomyces	–	84 % in 7 days	Gunner and Zuckerman (1968)
Organophosphonate pesticides				
Glyphosate	*Arthrobacter* sp. GLP-1	–	–	Pipke et al. (1987)
	Arthrobacter atrocyaneus ATCC 13752	–	–	Pipke and Amrhein (1988)
Organochlorine pesticides				
Chlordane	*Streptomyces* sp. A5	Contaminated environment, Argentina	56 % in 28 days	Cuozzo et al. (2012)
Lindane	*Streptomyces*sp. M7	Pesticide-contaminated sediments, Argentina	50 % in 3 days	Benimeli et al. (2007)
	Streptomyces strain PS1/5	–	–	Speedie et al. (1987)
Metolachlor	*Streptomyces* sp.	Soil	70 %	Liu et al. (1990)

Atrazine	*Streptomyces* sp. PS1/5	Soil, Beltsville	~70 %	Fadullon et al. (1998)
	Frankia alni ACN14a	–	–	Rehan et al. (2014)
2,4-D and 2,4,5-T	*Nocardioides simplex* 3E	–	100 %	Kozyreva and Golovleva (1993)
DDT and PCNB	*Nocardia* and *Streptomyces* species	–	–	Chacko et al. (1966)
Dalapon	*Arthrobacter*, *Nocardia*, and *Streptomyces* species	–	–	Kaufman (1964)
Aldrin	*Mycobacterium, Nocardia, Streptomyces*, and *Micromonospora* species	–	–	Ferguson and Korte (1981)
Pentachlorophenol (PCP)	*Janibacter* sp. FAS23	Saline sediment of arid land, southern Tunisia	300 mg/L	Khessairi et al. (2014)
	Mycobacterium chlorophenolicum PCP-1	–	40 µmol (g of dry cells)$^{-1}$ h^{-1}	Wittmann et al. (1998)
	Rhodococcus chlorophenolicus PCP-I	Biofilter filled with soft wood bark chips	–	Apajalahti et al. (1986) and Apajalahti and Salkinoja-Salonen (1984)
	Arthrobacter strain ATCC 33790	–	–	Edgehill (1994)
	Kocuria sp. CL2	Secondary sludge of pulp and papermill, India	58.64 % of sludge (>100 mg/L)	Karn et al. (2011)
	Saccharomonospora viridis	Mushroom compost	100 %	Webb et al. (2001)
Diuron	Actinomycete strain CCT	Soil	–	Esposito et al. (1998)
Polychlorinated biphenyls	*Janibacter* sp. MS3–02	Soil sample, Spain	70–100 % in 7 days	Sierra et al. (2003)
Monochlorinated dibenzo-*p*-dioxin	*Janibacter* sp. strain YA	River sediment	>90 % in 18 h	Iwai et al. (2005)

(continued)

Table 9.4 (continued)

Chemicals	Actinobacteria	Site of isolation	Pesticide degradation capacity	References
Chlorinated dibenzo-*p*-dioxin	*Terrabacter* sp. strain DBF63	–	–	Habe et al. (2001)
	Rhodococcus opacus SAO 101	Forest soil, Japan	–	Kimura and Urushigawa (2001)
Benzonitrile herbicides				
Bromoxynil	*Aminobacter* sp. MSH1	Plant nursery	20–30 %	Frkova et al. (2014)
Ioxynil				
Dichlobenil				
Synthetic pyrethroid insecticides				
Deltamethrin	*Streptomyces aureus* strain HP-S-01	Activated sludge, China	50–300 mg/L deltamethrin in 7 days	Chen et al. (2011)
Cypermethrin	*Streptomyces* sp. HU-S-01	Wastewater sludge, China	1.236 µmol/min	Lin et al. (2011)

(Loos et al. 1967). The former actinobacterium was also found to degrade another pesticide 2,4,5-T (Golovleva et al. 1990). The *Arthrobacter* strain was also capable of utilizing two other organochlorine pesticides, viz. 4-CPA (4-chlorophenoxyacetate) and MCPA (2-methyl-4-chlorophenoxyacetate) as the sole source of carbon and energy. Stability of organochlorine compounds depends on the degree of chlorination. The polychlorinated compound, pentachlorophenol (PCP), is a highly stable compound, widely used as biocide (bactericide, fungicide, and algaecide), wood and leather preservative (Kao et al. 2004). PCP acts as an inhibitor of oxidative phosphorylation, therefore, is toxic to almost all living organisms (Shen et al. 2005) causing severe disease symptoms in humans. This compound has also been listed as a toxic pollutant (EPA 1987). Aerobic degradation of this recalcitrant chemical by diverse genera of actinobacteria has been confirmed (listed in Table 9.4). *Streptomyces rochei* 303 is the only actinobacterium reported till date that can metabolize a broad spectrum of chlorophenols ranging from mono- to pentachlorophenols (Golovleva et al. 1992). Mono- and polychlorinated dibenzo-p-dioxin degradation is known to be catalyzed by *Janibacter*, *Rhodococcus*, and *Terrabacter* species (shown in Table 9.4). They metabolize and incorporate the carbon moieties of the toxic compounds into their cell biomass.

Benzonitrile herbicides include dichlobenil (2,6-dichlorobenzonitrile), ioxynil (3,5-diiodo-5-hydroxybenzonitrile), and bromoxynil (3,5-dibromo-5-hydroxybenzonitrile). The massive use of these chemicals contaminates soil and ground water (US-EPA, Herbicide Report, 1974). The use of dichlobenil is restricted in the European Union since its hydrolytic metabolite [2,6-dichlorobenzamide (BAM)] is highly toxic. The complete degradation of dichlobenil by *Aminobacter* MSH1 was reported (Frkova et al. 2014). However, the use of this actinobacterium in bioremediation application is limited because it can only partially hydrolyze the other two benzonitrile herbicides (ioxynil and bromoxynil) and yields toxic end products which may pose an environmental risk.

Streptomyces sp. has also been found to metabolize the synthetic pyrethroid insecticides (shown in Table 9.4). Synthetic pyrethroid insecticides are pyrethrin analogues derived from plants (Laffin et al. 2010). Their photostability, low mammalian toxicity, and quick insecticidal capability enhanced their market value (approximately 25 % of the total world insecticide market) and replaced toxic organophosphate pesticides (Katsuda 1999; Zhang et al. 2010). Synthetic pyrethroid insecticides were earlier considered as thenontoxic insecticides (Dorman and Beasley 1991). According to recent studies, these have been found to be carcinogenic and a major causative agents of chronic diseases (Wang et al. 2009). For instance, cypermethrin was found to disturb the food chain of aquatic ecosystem (Pearce 1997). *Streptomyces aureus* strain HP-S-01 isolated from activated sludge is capable of degrading deltamethrin and its toxic metabolite (3-phenoxybenzaldehyde) (Chen et al. 2011). 3-phenoxybenzaldehyde possesses antimicrobial activity and hinders further biodegradation (Laffin et al. 2010). This actinobacterium can also efficiently degrade other synthetic pyrethroids such as cyfluthrin, bifenthrin, cypermethrin, fenvalerate, fenpropathrin, and permethrin.

9.5.2 Biodegradation of Hydrocarbon Containing Contaminants

As compared to bacteria and fungi, actinobacteria exhibit greater potential for degradation of hydrocarbons (Idemudia et al. 2014). The concentration of hydrocarbon pollutants including complex organic compounds, petroleum and oil products are steadily increasing in the environment due to their excessive use. Hydrocarbons are toxic to microbes, plants, and other living organisms (Andreoni et al. 2004) causing a potential risk to the environment. Bioremediation and phytoremediation, the use of microbes or plants to remove toxic hydrocarbon compounds, are getting attention in the recent years (Chibuike and Obiora 2014; Arthur et al. 2005). Phytoremediation method relies on a mutualistic relationship between plants and microbes. Actinobacterial species (showing close similarities with *Arthrobacter* species) form a dynamic part of microbial communities associated with phytoremediation of hydrocarbon-polluted sites (Phillips et al. 2008). The taxonomically diverse actinobacterial genera colonize as dominant populations in hydrocarbon-polluted sites. They constitute antimicrobial group capable of degrading a wide range of hydrocarbons (listed in Table 9.5). Hydrocarbonoclastic bacterial communities isolated from mangrove sediment, Guanabara Bay (Brazil) include bacterial species as well as actinobacterial species (belonging to the genera *Micrococcus, Cellulomonas*, *Dietzia*, and *Gordonia*) (Brito et al. 2006). These are capable of degrading an assortment of hydrocarbon pollutants. Hydrocarbon degrading microorganisms have also been isolated from seawater, Semarang port, Indonesia. This microbial community consisted of approximately 23 % of actinobacterial species (Harwati et al. 2007). Culture-dependent microbial diversity analysis revealed that actinobacterial species (*Micrococcus, Nocardia, Gordonia, Micromonospora*, and *Rhodococcus*) and bacterial species form a potential microbial group for degrading spent lubricating oil (Idemudia et al. 2014). These actinobacterial species showed approximately 1.035–7.53 % degradation of oil. Actinobacterial isolates belonging to the genera *Rhodococcus* and *Gordonia* were capable of degrading both long chain *n*-alkanes and *c*-alkanes of petroleum compounds (Kubota et al. 2008). Diverse salt-tolerant actinobacterial species, *Streptomyces albiaxialis* (Kuznetsov et al. 1992), *Rhodococcus erythropolis* and *Dietzia maris* (Zvyagintseva et al. 2001), *Rhodococcus* sp. and *Gordonia* sp. (Borzenkov et al. 2006), *Dietzia* sp. and *Actinopolyspora* sp. DPD1 (Al-Mueini et al. 2007) were documented to possess an efficiency to degrade crude oils under moderate to high saline environment. Bjorklof et al. (2009) reported that *Mycobacterium* species were a dominant population in the hydrocarbon-contaminated soil. Actinobacterial species (*Rhodococcus* sp., *Nocardia* sp., *Arthrobacter* sp., *Gordonia* sp., *Mycobacterium* sp., *Corynebacterium* sp., and *Micrococus* sp.) contributed significantly to the biodegradation of crude oil (Chikere et al. 2009). A high potential for biodegradation makes actinobacteria a prospective clean-up solution for remediation of hydrocarbon-contaminated sites.

Table 9.5 List of hydrocarbons degrading actinobacteria

Actinobacteria	Hydrocarbons	Site of isolation	References
Janibacter anophelis strain JY11	Phenanthrene, anthracene, and pyrene	Polluted soil sample, Jinan Oil Refinery Factory, China	Zhang et al. (2009)
Streptomyces sp., *Rhodococcus* sp., and *Nocardia* sp.	Crude oil, Anthracene, Coronene, Napthacene, Acenapthene	Soil samples, Mathura Oil Refinery, Lucknow	Shekhar et al. (2014)
Rhodococcus erythropolis BZ4, *R. cercidiphyllus* BZ22, *Arthrobacter sulfureus* BZ73, *Pimelobacter simplex* BZ91	*n*-alkanes, phenol, anthracene, pyrene	Petroleum hydrocarbon-contaminated alpine soil, Italy	Margesin et al. (2013)
Dietzia strain DQ12-45-1b	Petroleum hydrocarbons (C6–C40) and crude oil	Oil production water sample, China	Wang et al. (2011)
Micrococcus luteus GPM2603 and *Cellulomonas variformis* GPM2609	Pristine and pyrene, respectively	Mangrove sediments, Brazil	Brito et al. (2006)
Gordonia alkanivorans HKI 0136T	Hexadecane	Tar-contaminated soil, Rositz	Kummer et al. (1996)
Micrococcus luteus	Naphthalene and benzene	Oil-contaminated tropical marine sediments, south Singapore	Zhuang et al. (2003)
Dietzia sp. strain GS-1	Disodium terephthalate	Soil sample	Sugimori et al. (2000)
D. maris and *Rhodococcus erythropolis*	*n*-alkane and *iso*-alkanes	–	Zvyagintseva et al. (2001)

9.5.3 Detoxification of Heavy Metals

All living organisms require a small quantity of heavy metals including iron, zinc, copper, manganese, cobalt, and nickel for their physiological growth and development (Park et al. 2006), but these metals become toxic at higher concentrations. The presence of very small quantity of other heavy metals causes toxic effects on both prokaryotes and eukaryotes. On the basis of physiological viewpoints, heavy metals are classified into two major categories (1) harmful at high concentrations (e.g., Fe, Zn, Cu, Mn, Co, Ni, and Cr) (2) highly toxic or nonessential (Hg, Cd and Pb) (Valls and Lorenzo 2002). Industrial activities and abandoned mining represent major sources of discharge of copious amounts of heavy metals into the environment leading to human health risks and serious ecological complications. At present, environmental metal-toxicity is increasing alarmingly which calls for an

immediate action. Currently, development of phytoextraction (Gremion et al. 2003) and microorganism-based remediation methods (Colin et al. 2012) have been in focus for toxic metal detoxification as they are cost-effective and efficient. Actinobacteria is an ecologically important group that is conferred with specific cellular machinery to respond to both metal deprived and overloaded condition. The exact mechanisms for metal homeostasis by actinobacteria have not been adequately understood. In southwest Slovakia, a heavy metal-contaminated farmland was predominantly colonized by actinobacterial species after proteobacteria (Karelova et al. 2011). High dominance of actinobacterial species occurs in heavy metal-contaminated bulk and rhizospheric zone of many metal accumulating plant species (Gremion et al. 2003). In general, microbial populations isolated from metal-contaminated sites are preferred for the development of metal remediating tools. Actinobacteria includes a number of heavy metal-resistant species (Table 9.6) that are capable of bioaccumulation of toxic elements and decontamination of metal-polluted sites. The species of *Streptomyces* has been considered as potential sources for remediating sites that were co-polluted with Cr and lindane (Aparicio et al. 2015). *Frankia* species show an elevated level of tolerance to several metals and metalloids (Pb^{2+}, Al^{3+}, SeO_2^{3-}, Cu^{2+}, AsO_4, and Zn^{2+}) (Richards et al. 2002). Metal resistance in *Frankia* species aids colonization of actinorhizal host plants in highly contaminated or nutrient-poor soils (Schwencke and Caru 2001). *Arthrobacter* sp. U3 isolated from metal-contaminated environment was capable of detoxifying a hazardous metal (Hg) up to 80 % in a bioremediation site (Giovanella et al. 2015). The soil inhabiting and nonpathogenic *Arthrobacter* species offer their exploitation in environment cleanup and remediation process.

9.5.4 Biodegradation of Plastics/Bioplastics

The term "white pollution" refers to solid waste including polythene and plastic bags, disposed into the environment, which affect the soil ecosystem adversely. These plastic products are made of polystyrene, polypropylene, polyvinyl chloride, and other polymers that are highly resistant to microbial degradation, thereby leading to severe urban environmental consequences. The problems related to white pollution have encouraged research into finding or developing biodegradable plastics (Steinbuchel 2001). Several microbes synthesize biopolymers in the form of intracellular storage granules (Luengo et al. 2003). These have been explored for the manufacturing of biodegradable plastics. Microbe-derived biopolymers are majorly poly (3-hydroxyalkanoate) (PHA) and poly (3-hydroxybutyrate) (PHB) (Bugnicourt et al. 2014). Bioplastics are receiving considerable attention since they can easily be degraded by microbes in the environment. Diverse thermophilic and thermotolerant actinobacterial species have been reported with the capability to degrade bioplastics and rubbers (Shivlata and Satyanarayana 2015). Several mesophilic *Streptomyces* species producing polyhydroxyalkanoate and poly (3-hydroxybutyrate) depolymerases and other non-*Streptomyces* species have been shown to degrade bioplastics

Table 9.6 List of metal detoxifying, dye decolorizing, and bioplastic degrading actinobacteria

Actinobacteria	Toxic metal or effluents	Site of isolation/ collection	References
Metal detoxifying actinobacteria			
Streptomyces roseisederoticus (V5)	Cr, Cd, Zn, and Pb	Rhizosphere region of *Casuarina equisetifolia*	Vinod et al. (2014)
S. flavochromogenes (V6)			
S. vastus (V7)			
S. praguaeneses (V8)			
Streptomyces and *Amycolatopsis* species	Cr, Cd, Zn, and Pb	Abandoned mining areas	El Baz et al. (2015)
Streptomyces werraensis LD22	Cr, Pb, Ni, and Zn	Chicken and goat feces	Latha et al. (2015)
Actinobacteria including both *Streptomyces* and non-*Streptomyces* species (*Micromonospora*, *Actinoplanes*, *Norcardia* and other rare genera)	Hg, Cd, Cu, Pb, As, Ni, and Zn	Tin tailings and forest soil	Hema et al. (2014)
Arthrobacter sp. U3	Hg	Metal-contaminated industrial effluents	Giovanella et al. (2015)
Bioplastic degrading actinobacteria			
Streptomyces roseolus SL3, *Streptomyces pulveraceus, Streptomyces atratus, Streptomyces anulatus, Streptomyces beijiangensis,* and *Streptomyces omiyaensis*	Polyesters including P(3HP), P(3HB), P(HB-HV), and PCL	Soil, sludge, and water sample	Gangoiti et al. (2012)
Streptomyces venezuelae SO1	Medium-chain-length PHA	Soil sample	Santos et al. (2013)
Arthrobacter globiformis SBI-5	Polyurethane	Oil-contaminated connecticut soil	El-Sayed et al. (1996)
Corynebacterium sp.	Polyurethane	Degraded polyester polyurethane samples	Kay et al. (1991)
Rhodococcus equi TB-60	Urethane	Soil samples	Akutsu-Shigeno et al. (2006)
Actinomadura sp. AF-555	P(HB-HV)	Soil sample	Shah et al. (2010)
Kibdelosporangium aridum JCM 7912	Poly(L-lactide)	Japan collection of microorganisms	Jarerat et al. (2003)
Amycolatopsis orientalis IFO 12362	Poly(L-lactide)	Institute for Fermentation, Osaka	Jarerat et al. (2006)
Saccharothrix waywayandensis JCM 9114	Poly(L-lactide)	Japan collection of microorganisms	Jarerat and Tokiwa (2003)
Dyes decolorizing actinobacteria			
Saccharothrix aerocolonigenes TE5	Reactive azo dyes	Soil contaminated with textile effluents	Rizwana and Palempalle (2015)

(continued)

Table 9.6 (continued)

Actinobacteria	Toxic metal or effluents	Site of isolation/ collection	References
*Micrococcus glutamicus*NCIM 2168	Reactive green 19A	Culture Collection Center, National Chemical Laboratory, Pune	Saratale et al. (2009)
Streptomyces species	Azo blue and azo orange dyes	Textile industry effluent, Kerala	Pillai et al. (2014)
Rhodococcus qingshengii JB301	Triphenyl methane dyes	Sawdust	Li et al. (2014a)
Amycolatopsis orientalis	Amido black	Soil sample	Chengalroyen and Dabbs (2013)
Streptomyces chromofuscus A11	Azo dye isomers	American type culture collection	Pasti-Grigsby et al. (1996)
Dietzia sp. PD1	Congo red and indigo carmine	Textile effluent, Kolkata	Das et al. (2016)

P(3HP) poly(3-hydroxypropionate), *P(3HB)* poly(3-hydroxybutyrate), *P(HB-HV)* poly(3-hydroxybutyrate-co-3-hydroxyvalerate), *PCL* poly-ε-caprolactone

(Table 9.6). Bioplastics are also derived from renewable resources including vegetables, cornstarch, and agricultural by-products. Synthesis of bioplastics from green renewable resources is of current interest. Recently, a mesophilic actinobacterium, *Streptomyces coelicolor* CH13 degraded a blended cassava starch/natural rubber biopolymer (Watcharakul et al. 2012). An endophytic actinobacterium, *Nocardiopsis* sp. mrinalini9 capable of degrading polythene, plastic and diesel, was isolated from leaves of *Hibiscus rosasinensis* (Singh and Sedhuraman 2015). *Rothia* sp. belonging to the phylum *Actinobacteria* was isolated from a deteriorating epoxy resin statue (Pangallo et al. 2015). A chemical compound dibutylin (DBT) is a most widely used plastic stabilizer, which causes neurotoxic, hepatotoxic, and immunotoxic effect on humans. It is also released into the environment as a by-product of degradation of tributylin (used as antifouling agent in boat paints) (Antizar-Ladislao 2008). *Streptomyces* spp. isolated from plant waste composting heaps have been shown to be capable of degrading up to 90 % of DBT (added at 20 mg/L) after 1 day of incubation (Bernat and Dlugonski 2009).

9.5.5 Decolorization of Dyes

After the discovery and successful commercialization of the world's first synthetic dye (mauevin), more than 10,000 synthetic dyes have been developed and are being used in textile and dyestuff manufacturing (Robinson et al. 2001). Other applications of synthetic dyes include paper printing, manufacturing of food coloring

additives and cosmetics. More than 7×10^5 metric tons of synthetic dyes are produced annually (Zollinger 1987). In general, complete utilization of coloring dyes does not occur during the dyeing processes. Approximately 10–15 % of total dyes used are lost as effluent from industries due to inefficiency of the processing operation. Dye-containing effluents discharged from industries enter into water bodies and disturb the aquatic ecosystem. Textile industries consume a substantial amount of water for wet processing of textiles and release a large quantity of liquid effluent pollutants into the environment. Approximately 2.8×10^5 tons of dye effluents are discharged from textile industries per annum (Jin et al. 2007), representing the largest source of water pollution. Consumption of dye-polluted water causes toxicity and carcinogenicity in all living beings (Ratna and Padhi 2012). The presence of colored dye molecules in water bodies reduces the penetration of sunlight and decreases photosynthetic activity of aquatic flora, thereby deteriorating water quality such as decreasing the dissolved oxygen concentration (Vandevivere et al. 1998). In addition, their acute toxic effects on aquatic fauna have also been demonstrated (Olaganathan and Patterson 2013). The presence of toxic dyes in the environments everly damages economically important plants growing in the vicinity of such polluted areas (Kapustka and Reporter 1993). Therefore, there is an urgent requirement of proper treatment of industrial effluents prior to their discharge into the environment. Several physical and chemical methods have been used for the treatment of wastewater effluent. Physiochemical methods are too expensive and inefficient to perform complete removal of dyes from wastewater (Saratale et al. 2011). These limitations inspire to search for an alternate effective way to decontaminate the water resources. Microbial or enzymatic decolorization methods are therefore being developed, as these are economic and eco-friendly as opposed to physiochemical decomposition methods (Rai et al. 2005). Actinobacteria are considered as potent decomposers, and they mineralize a diverse array of recalcitrant pollutants including toxic dyes (shown in Table 9.6). Ball et al. (1989) reported that three actinobacterial species, *Streptomyces badius* 252, *Streptomyces* sp. strain EC22, and *Thermomonospora fusca* MT800, have the ability to decolorize the polymeric dye Poly R. Fourteen lignocellulolytic *Streptomyces* species were screened for their ability to decolorize dyes (Poly B-411, Poly R-478 and Remazol Brilliant Blue R). A strong positive correlation was found between ligninolytic capability and dye decolorization of two dyes (Poly B-411 and Remazol Brilliant Blue R). *Streptomyces* species produced extracellular peroxidases involved in decolorization of dyes (Pasti and Crawford 1991). There is another report that also supports the fact that lignin solubilizing *Streptomyces* species, *S. violaceoruber*, decolorized 63 % of Poly R-478 after 24 h of incubation (Abou-Dobara and Omar 2014). Zhou and Zimmermann (1993) demonstrated that actinobacteria removed dyes from effluents through either adsorption or degradation process. Actinobacteria catalyzing the reactions of hydroxylation, dealkylation, and oxidation were able to degrade the xenobiotic pollutants (Goszczynski et al. 1994).

Complete degradation of Reactive Green 19A (50 mg/L) was achieved by using *Micrococcus glutamicus* NCIM 2168 within 48 h of incubation (Saratale et al. 2009). Actinobacteria are known to decolorize dyes in all states as in pure culture or

co-culture or mixed culture. Saratale et al. (2010) developed a bacterial consortium by co-culturing two pure cultures of *Proteus vulgaris* NCIM-2027 and *Micrococcus glutamicus* NCIM-2168. This consortium degraded azo dyes more efficiently than the individual strains. Another actinobacterium *Rhodococcus globerulus* capable of decolorizing azo dyes was found to be an active participant in the microbial consortium with two bacterial strains (Joshi et al. 2008). *Streptomyces* sp. C1 isolated from thermophilic phase of composting showed decolorizing activity by producing an enzyme known as laccase-like multicopper oxidase (Lu et al. 2013). *Streptomyces psammoticus* was also shown to secrete an extracellular laccase that finds application in decolorization of dyes (Niladevi and Prema 2008). Therefore, whole cells of actinobacteria or their enzymes can be used for decolorization of dye-contaminated effluents.

9.6 Conclusions and Future Perspectives

Actinobacteria have potential applications in both agricultural economy and environmental biotechnology. Use of actinobacteria as microbial inoculants for enhancing crop productivity and environmental pollution control would be a beneficial approach to keep both agriculture and environment clean and safe. In order to exploit actinobacteria, there is a need to carry out detailed investigations on their physiology and molecular mechanisms. Detailed investigations are called for understanding the physiological and molecular basis of plant–actinobacteria interactions. In view of the major impact of actinobacteria in environmental sustainability, the elucidation of metabolic pathways involved in the biodegradation of toxic pollutants would be useful.

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Chapter 10
Atmospheric Carbon Sequestration Through Microalgae: Status, Prospects, and Challenges

S.P. Shukla, S. Gita, V.S. Bharti, G.R. Bhuvaneswari, and W.A.A.D.L. Wikramasinghe

Abstract Microalgae are considered to be suitable candidates for atmospheric carbon sequestration by virtue of attributes such as faster growth, ability to grow in low-quality water, and tolerance towards a wider range of temperature, salinity and nutrient-deficient environment. Further, the downstream processing of microalgal biomass yields a variety of value-added products including biodiesel which is considered to be a lucrative alternative to fossil-based fuels. In this review, the potentialities of microalgae for atmospheric carbon sequestration are discussed with reference to present status of microalgae biomass production systems, strategies for enhancing the growth of natural populations of microalgae in marine environment, status of knowledge about downstream processing of biomass for biodiesel production and its implications on global warming mitigation. In concluding part, the prospects and challenges pertaining to microalgal biomass production and its utilization are highlighted. Based on an overview of the state of knowledge, few recommendations are submitted for the consideration of the scientific community.

Keywords Carbon sequestration • Algae • Down-stream processing • Value-added product • Prospects

10.1 Introduction

An unprecedented increase in atmospheric carbon dioxide during postindustrial era has posed a serious challenge to the global ecosystems and their sustainability (Stewart and Hessami 2005; Kumar et al. 2010). An appreciable number of research and development initiatives were taken during recent past directing the efforts for reducing the greenhouse gases including the carbon dioxide through innovative

S.P. Shukla (✉) • S. Gita • V.S. Bharti • G.R. Bhuvaneswari • W.A.A.D.L. Wikramasinghe
Aquatic Environment and Health Management Division, Central Institute of Fisheries Education, Versova, Mumbai, Maharashtra 400061, India
e-mail: spshukla@cife.edu.in

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technologies (Tsai et al. 2015; Singh and Strong 2016; Singh and Gupta 2016). Among the mitigation strategies, the prominent ones are:

1. Chemical reaction based approaches such as washing with alkaline solution (Diao et al. 2004), multiwalled carbon nanotubes (Su et al. 2009), and amine-coated activated carbon (Plaza et al. 2007).
2. Direct injection to underground (Herzog 2001) or to the ocean (Israelsson et al. 2009).
3. Biological carbon dioxide fixation through photosynthetic microorganisms, algae and terrestrial plants (Skjanes et al. 2007; Singh et al. 2016).

Among the carbon fixing organisms, microalgae and cyanobacteria have few advantages such as faster growth as compared to terrestrial plants and 10–50 times higher carbon dioxide fixation efficiency (Costa et al. 2000; Singh and Singh 2013; Singh 2014). There is a considerable interest worldwide in microalgae-based carbon dioxide sequestration because the biomass of microalgae and cyanobacteria could be used for wide-ranging valuable products such as biofuels, medications, cosmetics, and nutraceuticals (De Morais and Costa 2007; Singh et al. 2011a, b, c; Singh 2013, 2015a, b). Apart from the precursors of value-added products, microalgal carbon dioxide fixation is environmentally sustainable as it can be combined with various environment-friendly processes such as wastewater treatment (Mallik 2002) and heavy metal removal (Jacome Pilco et al. 2009).

10.2 Biotic and Abiotic Factors Influencing Algal Growth

Microalgae thrive under the influence of varying environmental factors, biotic and abiotic in nature. The most relevant environmental factors that directly influence the growth of algae are:

10.2.1 Light

Sunlight is the major source of energy for microalgal growth. Under the circumstances where light is the only limiting factor, microalgal productivity shows a linear increase with increasing light conversion efficiency. Microalgal growth is generally not inhibited up to 400 μmol/m^2/s light intensity. The saturation level for *Chlorella* and *Scenedesmus* is exhibited at 200–400 μmol/m^2/s. Many of the microalgal species can utilize either the organic compounds for their growth (Heterotrophy) or the organic compounds and photosynthesis products for their growth (Mixotrophy). The light requirement of such species varies from obligate photoutotrophic algae.

10.2.2 Carbon Dioxide Concentration

Microalgae have a greater ability to fix atmospheric carbon dioxide (Yang and Gao, 2003). Usual sources of carbon dioxide for microalgae are: (1) atmospheric carbon dioxide, (2) carbon dioxide from industrial exhaust gases (e.g., flue gases), and (3) carbonate fixed in the form of soluble carbonates ($NaHCO_3$, Na_2CO_3). Carbon dioxide level in the atmosphere (≈0.0387 % v/v) is insufficient for optimum growth of algae. Therefore, the sources such as flue gases (containing >15 % carbon dioxide) emanating from the combustion process are a suitable source of carbon dioxide for the upscaling of algal biomass production (Carvalho and Malcata, 2001).

10.2.3 Nutrient Requirements

In addition to carbon, microalgae require adequate quantity of nitrogen. As a constituent of the nucleic acid and protein, nitrogen is directly associated with the primary metabolism of microalgae. Ammonium rather than nitrate is the preferred source of nitrogen for most of the fast growing microalgal species. Phosphorus in the form of phosphate is one of the crucial elements that limits growth, therefore, the system used for the growth should be efficient in recycling of the phosphorus.

10.2.4 Temperature and pH

Temperature and pH directly regulate the cellular and biochemical processes in microalgae. In general, an increase in temperature within certain range increases the metabolic rates of microalgae. However, the optimum temperature and tolerance to low and high temperature varies among microalgae depending upon the environmental conditions prevailing in their native biogeographic region. pH of the aquatic bodies directly influences the rate of biochemical reaction and activation/deactivation of the enzymes associated with various metabolic processes in microalgae. Among microalgae, majority of species are adapted to neutral pH; however, species such as *Spirulina (Arthrospira) platensis* are tolerant to higher pH.

10.2.5 Gas Transfer and Mixing Rates

Introduction of gases in bioreactors has a crucial role in determining the final yield of the biomass. The primary and secondary metabolism depends upon the supply of gases such as CO_2, SO_x, and NO_x which are the sources of carbon and nitrogen for

the growing microalgal cells. Mixing of the cultures in the bioreactor through propellers, blades, brushes, and fine bubble diffusion enhances the yield of biomass (Xu et al. 2009). The adequate mixing of culture is required to avoid the nutrient concentration and for the exposure of all cells to light for a minimum time required for active photosynthesis. Therefore, lack of a proper mixing device in the bioreactor may result in accumulation of nutrients in a particular stratum of the water column in the bioreactor. Other advantages of mixing include availability of adequate light to the microalgal cells and maintenance of pH of the culture by assuring the equal distribution of carbon dioxide in the water column and striping of accumulated oxygen in the culture tank which otherwise becomes toxic to growing cells.

10.3 Bioreactors for Microalgae Biomass Production

10.3.1 Open Ponds

Open ponds are the most common and extensively studied algal cultivation system (Boussiba et al. 1988; Tredici and Materassi, 1992). The open ponds are mainly of two types: (1) natural water bodies such as lakes, lagoons, and ponds and (2) artificial ponds or containers such as cemented raceways and fiber reinforced plastic (FRP) tanks. One of the major advantages of open ponds is that they are more feasible to construct and operate than closed systems. However, poor light utilization by the cells, evaporative losses, diffusion of carbon dioxide to the atmosphere, and requirement of large areas of land are major limitations of open systems. Contamination by predators and other fast growing heterotrophic organisms and low biomass productivity due to inefficient stirring are few drawbacks of open systems. Therefore, the commercial production of algae in open systems is restricted to only those species that can grow under extreme conditions.

10.3.2 Flat-Plate Photobioreactor

Flat-plate photobioreactors are suitable for higher yield of biomass than open systems due to larger illumination of the surface area (Milner, 1953). There are several reports on cultivation of algae and their growth performance in flat-plate photobioreactors (Tredici and Materassi 1992; Zhang et al. 2002; Hoekema et al. 2002). In general, flat-plate photobioreactors are made of transparent materials for maximum utilization of solar energy. Accumulation of dissolved oxygen in the medium is relatively low compared to tubular photobioreactor, therefore, a higher yield of biomass can be obtained in the flat-plate bioreactors (Richmond 2000; Tredici & Chini Zitelli 1998; Tredici 2010).

10.3.3 Tubular Bioreactor

Tubular bioreactors are one of the suitable type of reactors for outdoor mass cultivation of algae. The configuration of tubular bioreactor varies from horizontal, inclined to vertical (Lee and Low 1991; Ugwu et al. 2002). Tubular bioreactors are usually constructed with either glass or plastic tubes where the culture is circulated either with an oil-free pump or through airlift system (Molina et al. 2001). One of the major limitations of tubular photobioreactor is poor mass transfer i.e., oxygen buildup. Accumulation of oxygen in culture is a major hurdle in scale-up of the biomass production in tubular bioreactors.

The configuration of the bioreactor determines the final yield of biomass. Therefore, the configuration which facilitates optimum utilization of carbon dioxide and solar energy coupled with efficient gas transfer and mixing provides higher yield. Various designs of bioreactors have been developed during recent past for the cultivation of microalgae (Carvalho et al. 2006, Shukla et al. 1997, Shukla et al. 2013). The open raceway ponds are most commonly used bioreactors in the developing countries including India. Other types of bioreactors employed are vertical tubular and horizontal tubular reactors, helical tubular reactors, fermenter type reactors, flat-plate reactors, and hollow membrane reactors (Torzillo et al. 1986; Richmond et al. 1993; Molina et al. 2001). Temperature control in tubular bioreactors is difficult; however, a desired temperature can be maintained with little more investment by adding a thermostat to the system. Few other minor limitations of tubular bioreactors are adherence of cells to the wall of the tubular portion and the gradient of oxygen and carbon dioxide transfer along the tubes (Ugwu et al. 2003; 2008).

10.3.4 Vertical-Column Photobioreactors

Vertical-column photobioreactors are compact, low-cost, and easy to operate bioreactors (Kaewpintong et al. 2007). The bubble-column and airlift photobioreactors (up to 0.19 m in diameter) can attain a final biomass yield and specific growth rate that are comparable to the values reported for narrow tubular bioreactors (Sanchez-Miron et al. 2002).

10.3.5 Internally Illuminated Photobioreactors

Internally illuminated photobioreactors are equipped with impellers for agitation of the algal cultures. The spargers supply the air and carbon dioxide to the culture. Internally illuminated bioreactors can be modified in such a way that they can utilize

Table 10.1 Advantages and limitations of various types of algal culture systems

Culture system	Advantages	Limitations
Open ponds	Relatively economical, easy to clean up after cultivation	Partial control of culture conditions is possible, growth for longer period difficult, poor productivity, higher land requirement and low water use efficiency, frequent contamination, limited strains can be cultured
Vertical-column photobioreactors	High mass transfer, good mixing with low shear stress, low energy consumption, high potential for scalability, easy to sterilize, suitable for immobilization of algae, reduced loss of biomass due to photoinhibition and photooxidation	Small illumination surface area, construction requires sophisticated materials, shear stress to algal cultures, decrease of illumination surface area upon scale-up
Flat-plate photobioreactors	Large illumination surface area, suitable for outdoor cultures, good for immobilization of algae, good biomass productivities, relatively cheap, easy to clean up, low oxygen buildup	Scale-up requires many compartments and support materials, difficulty in controlling temperature
Tubular photobioreactor	Large illumination surface area, suitable for outdoor cultures, fairly good biomass productivity	Gradients of pH, dissolved oxygen accumulation, fouling of tubes, adherence to wall, space requirement is larger

both solar and artificial light system (Ogbonna et al. 1999). This helps in switching on to the artificial light whenever the solar light intensity is reduced due to cloudy weather or during night. Use of optic fibers to concentrate and distribute light in the cylindrical photobioreactors is gaining prominence in the design (Matsunaga et al. 1991). Unlike the other photobioreactors, one of the major advantages of internally illuminated photobioreactor is that it can be heat-sterilized under pressure, and thus contamination can be minimized. Further, supply of light can be ensured during day and night by integrating artificial and solar devices. A comparison of various types of bioreactors and their productivities has been presented in Tables 10.1 and 10.2.

10.3.6 High Rate Ponds (HRPs)

HRPs are raceway type ponds with a depth of 0.2–1 m. The culture in the pond is mixed by a paddle wheel. The horizontal water velocity is approximately 0.15–0.3 m/s. The configuration of an HRP may vary depending upon the number of loops (single or multiple) around a central dividing wall. The pond bottom may be either lined or unlined depending upon soil conditions. CO_2 is added into a counter-current gas sparging sump (~1.5 m depth) creating a turbulent flow in the raceway pond (Figs. 10.1 and 10.2).

Table 10.2 Biomass yield of the algal species in outdoor photobioreactors

Photobioreactor	Volume (L)	Photosynthetic strain	Productivity (g/L/d)	References
Airlift tubular	200	*Porphyridium cruentum*	1.5	Camacho Rubio et al. (1999)
Airlift tubular	200	*Phaeodactylum tricornutum*	1.2	Acien-Fernandez et al. (2001)
Airlift tubular	200	*P. tricornutum*	1.9	Molina et al. (2001)
Inclined tubular	6	*Chlorella sorokiniana*	1.47	Ugwu et al. (2002)
Undulated row tubular	11	*Arthrospira platensis*	2.7	Carlozzi (2003)
Outdoor helical tubular	75	*P. tricornutum*	1.4	Hall et al. (2003)
Parallel tubular	25,000	*Haematococcus pluvialis*	0.05	Olaizola (2000)
Bubble column	55	*H. pluvialis*	0.06	Garcia-Malea Lopez et al. (2006)
Flat plate	440	*Nannochloropsis* sp.	0.27	Cheng-Wu et al. (2001)

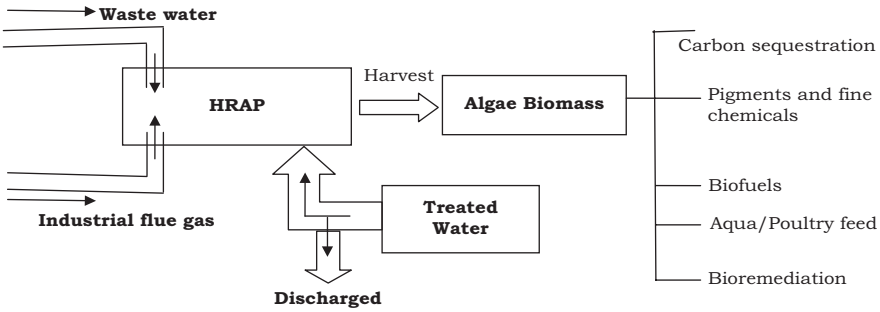

Fig. 10.1 A conceptual process for producing microalgal biomass through HRP

10.3.7 Continuously Stirred Tank Reactor (CSTR)

CSTR is used for continuous culture of microalgae. It runs at a steady state with continuous flow of culture medium in the culture vessel, and a simultaneous withdrawal of algal suspension from the unit for harvesting and further applications. The culture is continuously stirred through a motor-based stirrer (Fig. 10.3).

CSTR has following advantages:

- Continuous operation is possible, Good temperature control, Simplicity of construction, Low operating cost, Easy to clean, etc.

There are few disadvantages also such as:

- Lowest conversion per unit volume, Loss of yield due to settling of algal cells at the bottom or walls if agitation is not adequate, etc.

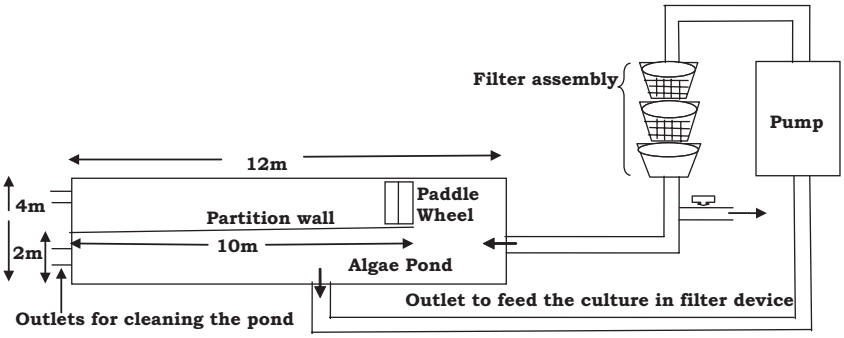

Fig. 10.2 Schematic diagram of a raceway pond

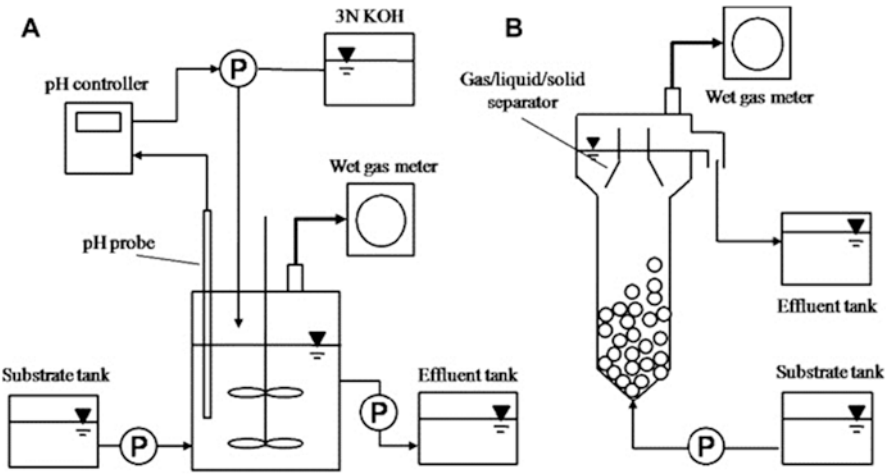

Fig. 10.3 CSTR for algae production for biogas using wastewater and groundwater (from online information about US patent no. US 20120202242 A1)

10.4 Algae Biomass Production Using Wastewater

Utilization of amended or unamended wastewater as growth medium is a promising avenue for algal biomass production for atmospheric carbon sequestration. Carbon sequestration by algae varies in a species dependent manner. According to Arjun et al. (2012), species like *Chlorella minutissima* are identified in the wastewater oxidation ponds in India, which will be a good candidate for setting up wastewater HRAPs. In general, the productivity on a dry weight basis ranges from 23 to 700 mg/L/d depending upon the algal species and type of cultivation system used.

10.5 Advantages of Carbon Sequestration Through Algae

Microalgae are one of the most productive biological systems for generating biomass and capturing carbon. Algae can produce two- to tenfold more biomass per unit land area than the fastest growing terrestrial systems. There are several reasons for the greater biomass yields of algae compared to higher plants. Generally, algae have higher photosynthetic efficiency than land plants because of greater abilities to capture light and convert it to usable chemical energy. Under ideal growth conditions, algae channelize most of their energy into cell division (6–12 h cycle), allowing a rapid biomass accumulation. Also, unlike higher plants, unicellular algae do not partition large amounts of biomass into supportive structures such as stems and roots that are energetically expensive to produce and often difficult to harvest. In addition, algae have carbon-concentrating mechanisms that suppress photorespiration. Algal biomass can be harvested at any time of the year, rather than seasonally.

With the ability to capture as high as 90 % carbon dioxide, microalgae can potentially be exploited for CO_2 capture and sequestration. The use of algae for CO_2 sequestration has several advantages: mitigating CO_2, the major source of global warming, as well as producing biofuels and other interesting secondary metabolites. In general, 1 kg of algal dry cell weight utilizes around 1.83 kg of CO_2. However, the major problems associated with the biological use of flue gas CO_2 are the high temperature of flue gas and the presence of NOx, SOx as well as other impurities of the fossil fuel used. Comparison of the growth characteristics and carbon dioxide fixation of few microalgal species under different CO_2 concentrations, temperatures, and NOx/SOx contents is listed in Table 10.3.

10.5.1 Carbon Sequestration in Natural Ecosystems

10.5.1.1 *LOHAFEX* (*Loha* Fertilization Experiment)

This was joint venture between Alfred Wegener Institute for polar and marine research (AWI), Germany and National Institute of Oceanography, Goa. The project aimed to conduct iron fertilization in southwest Atlantic sector of Southern Ocean on German polar vessel "Polarstern." Artificial iron fertilization was carried out by releasing a solution of ferrous sulfate in iron-deficient area in the sea. Iron concentration in the fertilized patch after homogenization is equivalent to those recorded from natural iron fertilization caused by dust settling from the atmosphere, contact with continental and inland margins and from melting of the icebergs. The density of bloom through iron fertilization was same as attained by natural bloom.

Table 10.3 Comparison of the growth characteristics and carbon dioxide fixation of few microalgal species under different CO_2 concentrations, temperatures, and NOx/SOx contents (Chisti 2007; Ho et al. 2011)

Microalgal species	CO_2 (%)	Temperature (°C)	NOx/SOx (mg/L)	Biomass yield (mg/L/d)	CO_2 consumption rate (mg/L/d)	References
Nannochloris sp.	15	25	0/50	350	658	Negoro et al. (1991)
Nannochloropsis sp.	15	25	0/50	300	564	Negoro et al. (1991)
Chlorella sp.	50	35	60/20	950	1790	Maeda et al. (1995)
Chlorella sp.	20	40	ND	700	1316	Sakai et al. (1995)
Chlorella sp.	50	25	ND	386	725	Sung et al. (1999)
Chlorella sp.	15	25	0/60	1000	1880	Lee et al. (2002)
Chlorella sp.	50	25	ND	500	940	Yue and Chen (2005)
Chlorogloeopsis sp.	5	50	ND	40	20.45	Ono and Cuello (2007)
Chlorococcum littorale	50	22	ND	44	82.0	Ota et al. (2009)

10.5.1.2 OMEGA (Offshore Membrane Enclosure for Growing Algae)

The OMEGA system consists of large plastic bags with inserts of forward osmosis membranes that grow freshwater algae in processed wastewater. Algae absorb carbon dioxide from the atmosphere and nutrients from the wastewater to produce biomass and oxygen. With the growth of algae, nutrients are contained in the enclosures, while the cleansed freshwater is released into the surrounding ocean through the forward osmosis membranes. OMEGA can remove contaminants from the coastal areas by removing the nutrients that cause them. Forward osmosis membrane uses less energy as compared to other harvesting processes.

10.6 Major Products from Algae

Algae are source of a variety of industrial products including biodiesel. The products obtained from algae are applied for various purposes. The pigments such as astaxanthin and carotenoids are used as antioxidants and natural colorant. PUFA (Polyunsaturated fatty acids) are used in health supplements. Poly-β-hydroxybutyrate

is used in plastic industries and polysaccharides such as agar-agar, alginates, and carrageenan are employed as thickening agent in food processing industries (Pulz and Gross 2004).

10.6.1 Algal Biodiesel

Biomass produced by mass cultivation of algae contributes to sequestration of atmospheric carbon dioxide and therefore in mitigation of global warming also. Algae are one of the richest sources of oil as the yield per hectare is approximately 72-folds higher than *Jatropha*, the most common higher plant used for biodiesel production. Algae require minimum quantity of water and chemicals for their growth and the biomass can be produced and harvested daily except for few days of rainy season. The cost of production can be further curtailed by using wastewater, inland saline water, and sea water. The oil content of algae can be enhanced with relative ease by manipulating the culture conditions which is not feasible in case of oil yielding plants such as *Jatropha* and *Pongamia*. All the above attributes make algae a potential candidate for atmospheric carbon sequestration and biodiesel production.

An added advantage of algal biodiesel production is that there is no conflict of resources like water, fertile land, and fertilizers with food crops and therefore food security is not at the stake in case of algal biodiesel production.

10.7 Sustainability of Algal Biodiesel Production

There are few major concerns about the sustainability of algal biodiesel production, especially the resource utilization, employment creation, and social impacts of algal biodiesel production need to be addressed for optimizing the benefits. The potential sustainability issues may come to the forefront when the industries producing algal biodiesel develop and start growing. Therefore, it is prudent to evolve a strategy to avoid or mitigate the factors which may influence the sustainable production of biodiesel from algae. Major sustainability of algal biofuels is given in Table 10.4.

10.8 Energy Security from Biodiesel

The contribution of algal biofuels, including biodiesel, in energy security will mainly depend on the resources (land and water) available for algal biofuel production. The productivity of algal cultivation system, the yield of the fuel from per unit biomass, and development of an integrated biofuel production system with a potential for scaling up the production are important aspects of sustainability.

Table 10.4 Some important criteria for sustainable algal biofuels

Sustainability criteria	Explanation
Cost of production	Cost-effectiveness with respect to other fuel alternatives, effects on the standard of living and economic health, and effects on fiscal balances
Employment	Employment creation
Energy balance	Energy output in fuel per unit of energy input to make the fuel over its life cycle
Resource utilization including land and water	Land and water requirements to produce one unit of fuel
Pollutant emissions including greenhouse gases and other pollutants	Emissions (for example, CO_2 and sulfur oxides) over the life cycle of one unit of fuel
Biodiversity	Effects on ecological species and communities (for example, habitat destruction or fragmentation)
Competition for resources being used for other human activities	Effects of resource use (for example, water and nutrients) for biofuel production on other activities (for example, farming food crops and animals)
Cultural acceptability	Acceptability of the effects of biofuel production

The share of the alternative fuels in the market will very much depend upon the cost of production. The algal biofuels will not be attractive avenue in the market if they are much more expensive than other fuel alternatives. Though, the government subsidies and policies can facilitate the market penetration, however, from sustainability view point, the algal biofuels have to become economically viable to the extent that the industries engaged in algal biodiesel production can flourish without subsidy and sector-specific policies to promote the industry with government sources.

10.9 Resource Utilization for Algal Biodiesel

Algae and cyanobacteria have an adaptability to grow in saline waters or nutrient-rich wastewater that is not suitable for other purposes such as agriculture or human consumption. Since CO_2 enrichment can enhance the growth of algae, CO_2 (flue gases) and other nutrients help maximize photosynthetic algal biomass production on a large scale. Therefore, establishment of algal biomass production sites with stationary industrial CO_2 emission sources like fossil fuel-fired power plants can help in enhancing the productivity of algal cultivation system. By virtue of their ability to utilize the wastewater (sewage or industrial wastewater), algae cultivation systems can be used for achieving dual benefit i.e., algae biomass production in a low-cost medium, and treatment of the wastewater without an isolated treatment plant. An important issue then to assess is the number of potential sites for algae cultivation that are near both a source of CO_2, such as fossil-fired power plants, and

a source of non-potable water (wastewater or saline water). A judicious approach for resource utilization and maintaining the quality of the natural resources are two major requisites for developing algal biofuels.

10.10 Social Well-Being

Although biomass production of algae and cyanobacteria is not likely to compete for high-quality arable land with crops, there could be social concerns about land use that need to be considered in the development of algal biofuels. The use of genetically engineered organisms in production systems could affect social acceptability, and therefore a greater awareness will be required to overcome this hurdle.

10.11 Microalgal Biochar

Biochar is the charcoal of biological origin. It has a demonstrated potential as a tool for carbon sequestration and as a soil ameliorant capable of improving water holding capacity and nutrient status of many soils (Lehmann and Joseph, 2009). The addition of biochar to the soil provides a substrate for enhanced microbial activity (Thies and Rilliz 2009). There is a scarcity of information about algal biochar and their utility for carbon sequestration though the microalgae and macroalgae have shown a remarkable potential for biochar production and therefore, carbon sequestration (Ross et al. 2008; Grierson et al. 2009).

10.12 Major Requirements for Harnessing the Potential of Algae for Atmospheric Carbon Sequestration and Biodiesel Production

Following are the major requisites for tapping the biopotential of indigenous algal species for atmospheric carbon sequestration and biodiesel production:

- Creation of an authentic database for microalgal diversity in diverse habitats (freshwater, marine, and estuarine) of various regions of the country
- Establishment of a National Facility of pure cultures of algal species
- Bioprospecting of isolated algal species for carbon sequestration and biodiesel production
- Development of new technologies for mass cultivation of algae and enhancement of lipid content
- Optimization of transesterification through molecular techniques and development of processes for recycling of chemical by-products of transesterification process and spent medium of algal cultivation

10.13 Conclusions

Light is a major environmental factor regulating the algal growth and hence atmospheric carbon sequestration by algae. Generally, the amount of light converted to metabolic energy through photosynthesis and stored in the cells has a direct relationship with the carbon fixation capacity of the algal species. Therefore, a primary requirement for optimum carbon fixation by algae is the availability of adequate light intensity either by natural (solar) or artificial sources (fluorescent lamps and optical fibers). A common approach to enhance the light availability is increase in surface area and shortening of the light path and layer thickness (Pulz 2001). An increased surface to volume ratio (SVR) of the photobioreactor facilitates higher light utilization efficiency, therefore an improvement in the design of the photobioreactors to achieve higher SVR. Genetic engineering of algal strains for changing the physiology of algal species is another promising area of research that can bring revolutionary changes in algae-based carbon sequestration and subsequent downstream processing of algal biomass for valuable products. Shortening of the size of antenna was suggested by Mussugnug et al. (2007) to enhance the photosynthetic efficiency which may result in a higher light utilization efficiency and lower energy loss. Therefore, an appropriately designed photobioreactor with higher SVR and optimized light supply efficiency is crucial to the enhancement of carbon fixation efficiency of a wild or mutant strain of algae. Apart from this, the light regime is also an important factor in determining the productivity and photosynthetic yield (Jacob-Lopes et al. 2009). Improvement in CO_2 transport efficiency can also bring a remarkable increase in CO_2 fixation efficiency of algae.

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Chapter 11
BioGro: A Plant Growth-Promoting Biofertilizer Validated by 15 Years' Research from Laboratory Selection to Rice Farmer's Fields of the Mekong Delta

Thanh Hien Nguyen, Thi Cong Phan, Abu T.M.A. Choudhury, Michael T. Rose, Rosalind J. Deaker, and Ivan R. Kennedy

Abstract Since their original isolation from rice paddies near Hanoi, the set of microbial strains comprising the biofertilizer BioGro have been subjected to extensive and intensive experimentation in both laboratory and the field. Based on a hypothesis that such strains inoculated onto rice and other plants could significantly reduce the need for chemical fertilizers, this has been successfully tested using numerous procedures, documented in a series of peer-reviewed papers. The BioGro strains have been examined by a range of molecular and biochemical techniques, also providing means of quality control of inoculants. A positive response by rice plants to BioGro strains has been confirmed by proteomics. More than 20 randomized block design field experiments conducted in Vietnam or Australia have confirmed their effectiveness under a range of field conditions, reviewed here. Interactions with different rice cultivars have also been examined. While the response to inoculation is complex, the hypothesis of increased nutrient efficiency has been amply confirmed as consistent with observations. Finally, an extensive participatory research project over 3 years in the Mekong Delta showed reductions in fertilizer needs as high as 52 % as rice farmers learned to apply the technology. This result shows the importance of such adaptive practices for successful application of this biofertilizer technology in field condition.

Keywords PGPR • Pseudomonas • Bacilli • Soil yeast • Rice proteomics

T.H. Nguyen
Biofertilizer Action Research Center, Hanoi, Vietnam

T.C. Phan
Agriculture Green Future, 175/2 Nguyen Thien Thuat, Ho Chi Minh City, Vietnam

A.T.M.A. Choudhury • R.J. Deaker • I.R. Kennedy (✉)
Faculty of Agriculture and Environment, University of Sydney, Sydney, NSW 2015, Australia
e-mail: ivan.kennedy@sydney.edu.au

M.T. Rose
NSW Department of Primary Industries, Wollongbar Primary Industries Institute,
1243 Bruxner Highway, Wollongbar, NSW 2477, Australia

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11.1 Introduction

Nitrogen fertilizer application is essential to maintain growth and yield of rice due to acute N deficiency in the rice soils. However, a substantial portion of the applied fertilizer nitrogen is lost due to ammonia volatilization, denitrification and leaching causing environmental pollution problems (Choudhury and Kennedy 2005). Due to these losses, fertilizer N recovery in rice culture is very low, generally around 30–40 %, in some cases even lower (Choudhury and Khanif 2001, 2009). These losses cannot be completely alleviated. However, the use of bacterial inoculant biofertilizers can lessen the need for the application of fertilizer N by more efficient N uptake by plants (Choudhury and Kennedy 2004; Kennedy et al. 2004, Choudhury et al., 2014).

Seedling growth is important for the successful establishment of rice and other crops. Previous investigations showed that rice seedling growth was enhanced by due to inoculation withof plant growth promoting (PGP) microorganisms, resulting in increased grain and straw yields as well as fertilizer N use efficiency (Biswas et al. 2000a, b; Yanni et al. 1997). Both single- and multi-strain biofertilizers are used to inoculate rice seedlings (Balandreau 2002; Malik et al. 2002). Jacques Balandreau (2002) developed the spermosphere concept in the 1990s suggesting that the role of inoculation was to provide a competitive advantage to beneficial microbes in the high nutrient zone of the rhizosphere. He might equally well have called it the chemosphere, given that carbon and other nutrients excreted by plant roots provide a locally favourable zone for microbial function. Inoculation with significant numbers of beneficial PGP strains is important to ensure that microbes with low benefits to the plant or even antagonists did not preferentially colonize the root zone by being significantly outnumbered. As a result, it is necessary to inoculate with up to 10^{11} viable cells of microbial strains per ha to guarantee an effective result as enhanced plant growth. It is surmised that these microbes predominate forming a biofilm with the surface of the roots.

A multi-strain biofertilizer called BioGro was developed by Nguyen Thanh Hien in Hanoi University of Science using the spermosphere principle during the 1990s (Nguyen et al. 2002). Earlier attempts to assist farmers with limited fertilizer inputs using microbial strains isolated in other countries failed to provide consistent benefits. The strains of this BioGro biofertilizer were isolated from rice rhizosphere near Hanoi and characterized for beneficial traits under laboratory conditions. Then the product was tested in numerous field experiments in Vietnam and Australia under AusAID (Australian Agency for International Development), ACIAR (Australian Centre for International Agricultural Research) and World Bank Development Marketplace funded research projects. This chapter reviews salient findings of those experiments, accumulating the relevant information altogether.

11.2 Isolation and Characterization of Strains of BioGro

Before 2005, BioGro contained three strains of bacteria (1N, 4P and 3C) selected from rice rhizosphere in the Hanoi area of Vietnam. From 2005, a combination of four strains (1N, HY, B9 and E19) has been used. The strains were identified at the University of Sydney using a number of different techniques including morphological, biochemical and genetic methods (Kecskés et al. 2008a). Subsequently, the strain identities were also confirmed at the laboratories of the German Culture Collection of Microbes (DSMZ), Braunschweig, Germany. The strains comprising 'BioGro1I' were identified as *Pseudomonas fluorescens* (1N), *Citrobacter freundii* (3C) and *Klebsiella pneumoniae* (4P), whereas 'BioGro 2' consisted of *P. fluorescens* (1N), *Bacillus subtilis* (B9), *Bacillus amyloliquafaciens* (E19) and a soil yeast, *Candida tropicalis* (HY). Survival of these strains at sufficiently high numbers greater than ca. 107 colony-forming units per g (cfus) in as peat cultures has been evaluated more recently (Rose et al. 2011).

In BioGro 1, strain 4P was selected for its ability to solubilize insoluble PO_4 in an agar medium. Strain 3C was selected for its ability to produce extracellular compounds which inhibited 50 % of a test group of 100 rhizosphere organisms (Nguyen et al. 2002). Each of the three bacteria (1N, 4P and 3C) were grown in separate broth cultures and added to separate bags of carrier formulated by mixing high organic-clay soil 50 %, rice husks 25 %, sugar 1 %, plus water and broth culture 24 %. These separate cultures were mixed in the field immediately before use in the ratio of 10 parts 1N: 10 parts of 4P: 1 part of 3C (Nguyen et al. 2002).

In BioGro 2, each of the four organisms were grown in separate broth cultures, and separately added to the carrier formulated by mixing peat (clay soil) 75 %, plus water and broth culture 24 % and sugar 1 % and incubated for 48 h at 30 °C. These four carriers were mixed before use in equal proportions. (Nguyen 2008). 1N (*Pseudomonas fluorescens*) was selected for its ability to grow on nitrogen-free medium, although it is not an N_2-fixing organism, and to reduce acetylene to ethylene as an indication of potential N_2 fixation. HY (*Candida tropicalis*) is a soil yeast and was initially selected for its ability to solubilize insoluble PO_4 in an agar medium. It has been shown to have a suite of plant growth promoting properties acting to promote robust root systems in rice plants (Amprayn et al. 2012). Two other strains B9 (*Bacillus subtilis*) and E19 (*Bacillus amyloliquefaciens*) were selected for their ability to break down protein, cellulose and starch (Nguyen 2008; Deaker et al. 2011).

Detailed methods for ensuring quality control of the inoculant strains have been published in a widely distributed laboratory monograph (Deaker et al. 2011; Krishnen et al. 2011). This manual includes a range of molecular and physiological methods applicable to many other PGPR microbes (Krishnen et al. 2011).

Table 11.1 Effects of BioGro on grain yield of rice at Dai Moi, Hanoi during 1999 to 2001

Year	Grain yield (t/ha)		Difference	Least significant difference (LSD) at 5 % level
	Without BioGro	With BioGro (111 kg/ha)		
1999	5.7	6.7	+1.0	0.5
2000	6.3	6.0	−0.3	Non-significant
2001	5.5	6.2	+0.7	0.3

Adapted from Nguyen et al. (2002)

Table 11.2 Effect of BioGro on grain yield of rice at farmers' demonstration during 1999–2001

Year	Grain yield (t/ha)		Difference	% increase
	Without BioGro	With BioGro		
1999	5.4	6.1	0.7	12.96
2000	5.3	6.4	1.1	20.75
2001	4.9	5.6	0.7	14.28

Adapted from Nguyen et al. (2002)

11.3 Initial Field Evaluation of BioGro Potential

Field experiments were conducted at Dai Moi near Hanoi under a research project funded by the Australian Agency for International Development (AusAID). In those experiments, BioGro 1 was used. Demonstrations at farmers' fields were also conducted. Details of the experimental procedures and findings are available in Nguyen et al. (2002, 2003). In the field experiments at Dai Moi, BioGro increased grain yield significantly in 1999 and 2001 whereas the effect of BioGro was non-significant in 2000 (Table 11.1). In the demonstrations at farmers' fields, the positive effect of BioGro in increasing grain yield was observed in all the years (Table 11.2). The percent increases in grain yield were 12.96, 20.75 and 14.28 in 1999, 2000 and 2001, respectively. An economic analysis indicated that BioGro application in rice cultivation was economically and environmentally beneficial for the farmers (Barret and Marsh 2002). A similar beneficial effect of BioGro was predicted in Australian conditions (Williams and Kennedy 2002).

Although the positive effect of BioGro 1 was observed, one of strain *Citrobacter freundii* (3C) is a human enteric organism, thus caution regarding health safety was recommended. Development of a newer version of BioGro excluding *Citrobacter freundii* (3C) and *Klebsiella pneumoniae* (4P) was recommended.

11.4 Field Evaluation of BioGro

A fortunate follow-up of the findings of AusAID-funded project led to conduct further research in both Australia and Vietnam under an ACIAR (Australian Centre for International Agricultural Research)-funded project. The findings are summarized

Table 11.3 Effects of BioGro on biomass, yield and N uptake of Jarrah rice, field experiment at Yanco, November 2004 to April 2005

Parameter	Treatment		Difference
	50 kg N/ha	50 kg N/ha + BioGro	
Plant dry biomass (t/ha) at panicle initiation (PI) stage	2.38	2.86	+0.48
Grain yield (t/ha)	6.10	6.42	+0.32
Straw yield (t/ha)	5.69	5.83	+0.14
Total N uptake (kg/ha) by whole plant at maturity	75.3	80.0	+4.7

Adapted from Kecskés et al. (2008b)

in the proceedings of ACIAR (Kennedy et al. 2008). Some of the findings were also published in impact factor journals (Phan et al. 2009, 2011; Nguyen et al. 2014; Kecskés et al. 2016). Some salient findings are presented here.

11.5 Field Experiment at Yanco Agricultural Research Institute Australia

A field experiment was conducted during November 2004 to April 2005 to evaluate the beneficial effect of BioGro 1 and other two biofertilizers on rice yield (Kecskés et al. 2008b). A short-duration rice variety Jarrah was used as the test crop. At panicle initiation (PI) stage, BioGro increased plant biomass by 0.48 t/ha, while at maturity grain yield increase by BioGro was only 0.32 t/ha (Table 11.3). This finding indicates that the positive effects of BioGro inoculation decreased at later growth stages of the rice plant. This might be an effect of the much longer growth duration of the rice plants in Australia compared to that of Vietnam. Reinoculation of bacteria at PI stage, and application of nitrogen fertilizer in at least two splits (2/3 at final land preparation and 1/3 at PI stage) are recommended for the next field experiments. Lower N rates are also recommended.

11.6 Field Experiment at Jerilderie Rice Research Institute

A field experiment was conducted during 2005 to 2006 to evaluate the beneficial effect of BioGro 1 and other two biofertilizers on rice yield (Kecskés et al. 2008b). A long-duration rice variety Amaroo was used as the test crop. At PI stage, BioGro inoculation increased plant biomass and N uptake slightly while this positive effect disappeared at maturity stage (Table 11.4). As Amaroo is a long-duration variety, reinoculation of bacteria at PI stage, and application of nitrogen fertilizer in at least two splits (2/3 at final land preparation and 1/3 at PI stage) are recommended for the next field experiments. Lower N rates are also recommended.

Table 11.4 Effects of BioGro on Biomass, N uptake and yield of Amaroo rice, field experiment at Jerilderie, 2005–2006

Parameter	Treatment		Difference
	50 kg N/ha	50 kg N/ha + BioGro	
Plant dry biomass (t/ha) at PI stage	2.66	2.77	+0.11
N uptake (kg/ha) at PI stage	37.3	44.8	+7.5
Grain yield (t/ha)	7.28	6.27	−1.01
Straw yield (t/ha)	6.33	6.11	−0.22

Adapted from Kecskés et al. (2008b)

Table 11.5 The effect of inoculating rice with BioGro for one, two or three seasons on yield and panicle formation in the third season, spring 2006

BioGro inoculation programme	Grain yield (t/ha)	Number of panicles/hill
Uninoculated	5.99	6.25
Inoculated in season 1 only	5.45	6.40
Inoculated in seasons 1 and 2	6.75	6.70
Inoculated in seasons 1, 2 and 3	7.32	7.90
F probability	0.054	0.013
LSD (0.05)	–	0.95
LSD (0.10)	1.10	0.77

Adapted from Nguyen (2008)

11.7 Field Experiments Conducted in Northern Vietnam

A set of replicated field experiments were conducted in the Hanoi area of Vietnam in 2005, 2006 and 2007 to evaluate the beneficial effect of BioGro 2 on rice cultivation. Three types of experiments (successive application of BioGro, rates of BioGro application and timing of BioGro application) were carried out by Hanoi University of Science. Another experiment on varietal difference on BioGro response was conducted at Thanh Tri, near Hanoi in 2006 by the Vietnam Academy of Agricultural Science, Hanoi.

11.8 Successive Application of BioGro

These field experiments were conducted in the Hanoi area for three successive crops beginning in spring 2005 at the same site to determine whether repeated inoculation with BioGro would further increase grain yield or affect its components. In the third season (spring 2006), application of BioGro in each of the three seasons increased grain yield significantly over both uninoculated rice and rice inoculated in the first season only at 10 % level of probability, confirming its beneficial effect on rice crops (Table 11.5). When inoculated with BioGro for two seasons the yield of rice

Table 11.6 Effect of the amount of BioGro applied on grain yield, spring 2006

Amount of BioGro applied (kg/ha)	Grain yield (t/ha)	Increase over control (t/ha)
0	5.45 b	–
50	6.91 a	1.46
100	6.83 a	1.38
200	6.25 a	0.80

Adapted from Nguyen (2008)
Values followed by a common letter in a column are not significantly different at 5 % level by least significance difference (LSD)

was 0.57 t/ha less than when applied for three seasons, but this difference was not significantly different (P=0.10). However, inoculation in two seasons did increase yield by 1.30 t/ha compared with a single inoculation in the first season. Application of BioGro in all three seasons increased the number of panicles per hill significantly over all other treatments at 5 % level of probability.

11.9 Rates of BioGro Application

This field trial was established in spring 2006 on plots on six farms in the Hanoi area to investigate the effect of the rate of BioGro applied on grain yield and yield components. Four rates of BioGro (0, 50, 100 and 200 kg/ha) were applied at transplanting. Treatment plots of farmers 1 and 2 received 200 kg/ha of BioGro, treatment plots of farmers 3 and 4 received 100 kg/ha of BioGro and treatment plots for farmers 5 and 6 received 50 kg/ha of BioGro. A control (0 kg/ha of BioGro) was included at all the farmers' plots. BioGro applied at 50, 100 or 200 kg/ha increased grain yield significantly compared with uninoculated rice at 5 % probability level (Table 11.6). Thus it was evident that increasing the amount of BioGro from 50 to 100 or 200 kg/ha did not further increase yield.

11.10 Timing of BioGro Application

This field trial was established in spring 2007 to evaluate the effects of higher amounts of BioGro and the timing of the application on yield and yield components of rice. The experiment was conducted on three farms in Dai Mo village near Hanoi. An uninoculated control was also established on each farm. In farm one, a 150 m^2 plot was used for the uninoculated control and another 150 m^2 plot used for the rice seedlings treated with BioGro at 38 kg/ha in the nursery area. In farm two, a 120 m^2 plot was used for the uninoculated control treatment and another 120 m2 plot was used for rice treated at the seedling stage with 38 kg/ha and at transplanting with BioGro at 278 kg/ha. In farm three, a 203 m^2 plot was used for the uninoculated

control treatment and another 203 m^2 plot was used for rice treated at the seedling stage and at transplanting as in Farm 2, plus 139 kg/ha BioGro at 1 month after transplanting.

Details of the experimental findings are available in Nguyen (2008), not presented in this chapter. On the first farm where a single application of BioGro at 38 kg/ha was used in the nursery only, it increased grain yield by 9 %. On the second farm where a total of 316 kg/ha was applied in a split application to the rice paddy, the increased grain yield was 80 %. When three applications of BioGro were made on a third farm, grain yield obtained by two or three applications were similar. However, the variation in % increase over control was due to the variation in the yields in control plots between the farms. This indicates an effect of soil fertility on the effectiveness of biofertilizer application. When averaged over all plots, inoculation increased the number of panicles per m^2 from 240 to 331, and the number of fertile seeds per panicle from 76 to 150. Neither of these parameters appeared to be significantly influenced by the third application of 139 kg/ha at 28 days after transplanting the seedlings.

11.11 Varietal Differences

The experiment was initiated in spring 2006 in Thanh Tri, Hanoi and repeated in the following season (summer 2006) to evaluate the beneficial effects of BioGro2 on six rice varieties (three common varieties: KD18, AYT01, VD8; and three quality varieties: LT2, HT1, BT7). Three treatments used in the experiment were T 1: 100 % NPK + farmyard manure (FYM) as control, T 2: BioGro + 50 % NP +100 % K+ FYM, and T 3: Biogro + 30%NP + 100%K + FYM. N fertilizer rates for spring and summer were 260 kg and 220 kg urea/ha, respectively. In both the seasons, 450 kg triple super phosphate (TSP), 180 kg muriate of potash (KCl), 10 tonnes FYM and 283 kg Biogro/ha were used.

Full amounts of FYM and P was applied at transplanting time in both the seasons. N and K were applied in two splits (50 % at transplanting + 50 % at active tillering stage) in both the seasons. BioGro was applied in two splits (83 kg/ha during seed sowing by mixing with seeds + 200 kg/ha applied on experimental plots during transplanting). The formulation of BioGro was 1N + HY + B9 + E19 at the ratio of 1:1:1:1.

Some salient findings of the Summer 2006 experiment are presented (Table 11.7). Grain yield with BioGro along with 50 % reduced amount of NP fertilizer gave statistically similar amount of grain with 100 % NP in four varieties (LT2, HT1, KD18 and AYT01). In variety VD8 BioGro application increased grain yield significantly while the opposite result was obtained in the other variety BT7. This finding thus indicates that BioGro application should be related to rice variety.

Table 11.7 Grain yields (t/ha) of rice varieties as affected by BioGro in combination with chemical fertilizers, Thanh Tri, Hanoi, summer 2006

Rice variety	Treatment		Difference
	T1 (Control): 100 % NP	T2 (BioGro): 50%NP + BioGro	
LT2	3.79	3.73	−0.06^{ns}
HT1	4.42	4.53	+0.11^{ns}
BT7	3.91	3.57	−0.34*
KD18	5.57	5.53	−0.04^{ns}
VD8	5.60	5.95	+0.35*
AYT01	4.28	4.27	−0.01^{ns}

Adapted from Pham et al. (2008)
ns non-significant
*significant at 5 % level by LSD

Table 11.8 Effects of BioGro on grain and straw yields of Trau Nam rice, Chau Thanh District, Vietnam

Season	Parameter	Without BioGro	With BioGro	Difference
First Rainy Season 2006	Grain yield (t/ha)	2.76	2.86	+0.10*
	Straw yield (t/ha)	2.65	2.79	+0.14*
Second Rainy Season 2006	Grain yield (t/ha)	3.06	3.30	+024**
	Straw yield (t/ha)	2.62	2.79	+0.17*

Adapted from Phan et al. (2009)
*Significant at 10 % level of probability, **Significant at 5 % level of probability

11.12 Field Applications Conducted in Southern Vietnam

The effect of BioGro 2 biofertilizer on nutrient uptake and grain yield of rice was investigated on a grey degraded soil of Thanh Dien village, Chau Thanh district, TayNinh province, Southern Vietnam. Initial soil was loamy sand with pH 5.31, 1.5 % organic matter content, 4.08 cmol/kg cation exchange capacity and 0.11 cmol/kg exchangeable K. Field experiments were carried out in two consecutive seasons (the first and the second rainy seasons). Different application rates of N and P were used to investigate the response of rice crop and the interactions between biofertilizer and nutrients. Trau Nam, a local rice variety with duration of 110 days, was used as the test crop.

Details of the experimental procedures and findings are available (Phan and Tran 2008; Phan et al. 2009, 2011). Some salient findings are presented in Table 11.8 of this chapter. The findings indicate that BioGro increased gain yield significantly at 10 and 5 % levels of probability in the first and second seasons, respectively. This indicates the seasonal impacts on the effectiveness of BioGro in rice crop. The intensity of solar energy, which is a seasonal attribute, might influence the activity of microorganisms, thereby affecting excretion of nutrients into the rhizosphere. The effect of BioGro on significantly increasing yield over a full range of N applications with maximum solar energy (Fig. 11.1). By contrast, BioGro applied in the

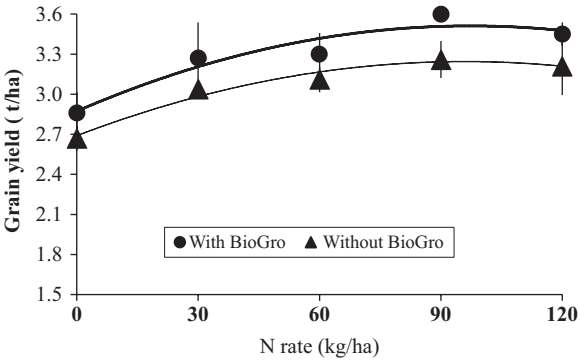

Fig. 11.1 Estimated grain yield response of Trau Nam rice to added N with and without BioGro, Chau Thanh district, Vietnam, second rainy season 2006 (Courtesy of Phan and Tran 2008)

first (heavy) rainy season with less solar insolation gave peak stimulation in grain yield in the range 30–60 kg N per ha, but no stimulation at maximum yield of 120 kg per ha N.

$$y = 2.8726 + 0.0131x - 0.00007x2\left(\text{with BioGro}\right), r2 = 0.911 at\ P < 0.05$$

$$y = 2.6894 + 0.0116x - 0.00006x2\left(\text{without BioGro}\right), r2 = 0.9668 at\ P < 0.05$$

A follow-up study was conducted using the ^{15}N-labelled urea in the Institute of Agricultural Sciences, Ho Chi Minh City, Southern Vietnam. Details of the experimental procedures and findings are available in Phan et al. (2008). A greenhouse experiment was conducted to evaluate the inoculation effect of 1N (*Pseudomonas fluorescens*) bacteria on dry matter production and fertilizer N uptake of rice plants. A grey degraded soil was used for this experiment, which was collected from Chau Thanh district, TayNinh province of Southern Vietnam. Four kilograms of soil were used per pot, and four replicates were used per treatment. Phosphorus and potassium were added to all pots as fused-magnesium phosphate (P_2O_5) and K_2O as muriate of potash (KCl) at a rate equivalent to 60 kg/ha of each fertilizer.

In the first set, N was added as ^{15}N-urea (4.634 % ^{15}N atom excess) at two rates equivalent to 0 and 20 kg N/ha. Twenty five pregerminated seeds, inoculated with 1N bacteria at106 colony-forming units (CFU) per g, were placed in each pot, which is equivalent to 100 kg seed sown per hectare. There were two inoculation treatments—with 1N and without 1N. Seedlings were harvested at 20 days after sowing (DAS) by cutting rice plants at ground level. In the second set, N was added from the same source at three rates equivalent to 0, 20 and 40 kg N/ha. Inoculated pregerminated seeds were placed in the pots and five seedlings were left in each pot. There were two inoculation treatments—with 1N (108 CFU/g) and without 1N. Seedlings were harvested at 45 DAS by cutting rice plants at ground level.

Plant samples were cut at ground level, air and oven dried at 70 °C and ground. Total N contents of the plant samples were determined by the micro-Kjeldahl procedure (Yoshida et al. 1976), and subsequently the ^{15}N abundance was estimated by

Table 11.9 Effects of inoculation of 1N (Pseudomonas fluorescens) bacteria and fertilizer N rate on plant dry weight, total N content and uptake of rice seedlings at 20 DAS

Inoculation	Fertilizer N rate (kg N/ha)		Mean
	0	20	
Dry weight (g/pot)			
Without 1N	8.73	9.02	8.88 b
With	9.02	9.24	9.13 a
Mean	8.88 B	9.13 A	
Total N content (%)			
Without 1N	3.19	3.66	3.43 a
With	3.24	3.57	3.41 a
Mean	3.22 B	3.62 A	
Total N uptake (mg/pot)			
Without 1N	278.4	330.3	304.4 a
With	292.6	330.0	311.3 a
Mean	285.5 B	330.2 A	

Adapted from Phan et al. (2008)
Interaction effect of inoculation and N rate was not significant among the three parameters. Within a parameter, values followed different capital letters in a row or different small letters in a column are significantly (P<0.05) different by least significant difference (LSD)

emission spectrometry (Hauck 1982) at the Nuclear Centre of Ho Chi Minh City using emission spectrometer NOISE-7. The percent ^{15}N atom excess (AE) was calculated by subtracting the natural abundance of ^{15}N (0.3663) from the abundance data of plant samples (Axman and Zapata 1990; Panda et al. 1995).

The calculations for estimating recovery in the plant from ^{15}N-labelled fertilizer were made according to procedures described by Axman and Zapata (1990). The percentage of *N* derived from fertilizer (NdfF) was calculated as follows:

$$\text{NdfF (\%)} = \frac{^{15}\text{N atom \% excess in plant sample}}{^{15}\text{N atom \% excess in labelled fertilizer}} \times 100.$$

The following computations were done as described by Choudhury and Khanif (2001) as follows:

1. Total N uptake (mg/pot) = total N in plant samples (%) × plant dry matter weight (g) × 1000/100
2. Fertilizer N uptake by rice plants = NdfF (%) × total N uptake by rice plants/100
3. Non-fertilizer N uptake = total N uptake − fertilizer N uptake.

At 20 days after sowing (DAS), the interaction effect of inoculation and N rate applied was not significant. However, inoculation individually increased plant dry weight significantly (Table 11.9). On the other hand, ^{15}N AE (%), NdfF (%) and fertilizer N uptake were decreased significantly (P<0.05) as a result of inoculation (Table 11.10). These were attributed to the increase in non-fertilizer N uptake from some source dependent on inoculation. The effect of N rate was significant on dry weight, total N content (%) and total N uptake (Table 11.9). Inoculation decreased

Table 11.10 Effects of inoculation of 1N bacteria on ^{15}N atom excess (AE), %N derived from fertilizer (NdfF), fertilizer N and non-fertilizer N uptakes of rice seedlings at 20 DAS

Inoculation	^{15}N atom excess (%)	NdfF (%)	Fertilizer N uptake (mg/pot)	Non-fertilizer N uptake (mg/pot)
Without	0.84 a	18.01 a	59.2 a	271.0 a
With	0.47 b	10.14 b	33.4 b	296.5 a

Adapted from Phan et al. (2008)
Values followed by different letters in a column are significantly (P<0.05) different by LSD

Table 11.11 Effects of inoculation of 1N bacteria and fertilizer N rates on plant dry weight, total N content and uptake of rice seedlings at 45 DAS

	Fertilizer N rate (kg/ha)			
Inoculation	0	20	40	Mean
Dry weight (g/pot)				
Without	19.24 b C	22.43 a B	24.71 aA	
With	21.79 a B	22.55 a B	24.55 aA	
Total N content (%)				
Without	1.51 aA	1.48 b A	1.51 b A	
With	1.44 b C	1.74 a B	1.95 aA	
Total N uptake (mg/pot)				
Without	290.1	331.3	371.8	331.1 b
With	314.2	392.3	479.2	395.2 a
Mean	302.2 C	361.8 B	425.5 A	

Adapted from Phan et al. (2008)
Interaction effect of inoculation and N rate was significant (P<0.05) on dry weight and total N content while it was not significant on total N uptake. Within a parameter, values followed different capital letters in a row or different small letters in a column are significantly (P<0.05) different by LSD

fertilizer N uptake significantly while the non-fertilizer N uptake increased from inoculation (Table 11.10) although the difference was not significant, but it contributed on the total N uptake which was not affected significantly due to inoculation (Table 11.9). The increase in non-fertilizer N uptake due to inoculation might be due to Biological Nitrogen Fixation (BNF). This can be estimated in future studies by using a non-fixing reference crop. These results indicated the benefit on growth of inoculating 1N bacteria, which significantly increased plant dry matter at 20 DAS.

At 45 DAS, the interaction effect of N rates and inoculation was significant (P<0.05) between plant dry weight and total N content (%) and between weight non-fertilizer N uptake (Tables 11.11 and 11.12). With no N applied inoculation increased dry matter yield significantly while at other two N rates the effect of inoculation was not significant. N fertilization increased dry matter yield significantly with increasing N rates without inoculation while the effect of N rate was significant only at N rates of 40 kg/ha with inoculation. Total N uptake increased significantly with inoculation and with N rates. Nitrogen fertilization increased ^{15}N AE (%), NdfF (%) and fertilizer N uptake significantly. Inoculation increased

Table 11.12 Effects of inoculation of 1N bacteria and fertilizer N rates on ^{15}N AE, NdfF (%), fertilizer N and non-fertilizer N uptakes of rice seedlings at 45 DAS

Inoculation	Fertilizer N rate (kg/ha)		Mean
	20	40	
^{15}N AE (%)			
Without	0.55	1.14	0.85 a
With	0.54	1.09	0.82 a
Mean	0.55 B	1.12 A	
NdfF (%)			
Without	11.86	24.51	18.19 a
With	11.69	23.53	17.61 a
Mean	11.78 B	24.02 A	
Fertilizer N uptake (mg/pot)			
Without	39.3	91.1	65.2 b
With	45.9	112.7	79.3 a
Mean	42.6 B	101.9 A	
Non-fertilizer N uptake (mg/pot)			
Without	292.0 b A	280.7 b A	
With	346.4 a B	366.4 aA	

Adapted from Phan et al. (2008)
Interaction effect of inoculation and N rate was significant only on no-fertilizer N uptake
Within a parameter, values followed different capital letters in a row or different small letters in a column are significantly (P<0.05) different by LSD

fertilizer N uptake significantly, while its effect was not significant on ^{15}N AE (%) and NdfF (%). This was a result of increased fertilizer N uptake in inoculated plants.

The interaction effect of N rate and inoculation was significant on non-fertilizer N uptake. Inoculation increased non-fertilizer N uptake significantly at both N rates while the N fertilizer effect was variable between inoculation treatments. The effect of N rate was not significant on non-fertilizer N uptake without inoculation, while N fertilization increased non-fertilizer N uptake significantly with inoculation. The possible reason might be the increase in root mass due to 1N inoculation which increased rice plants' capacity to absorb more soil N with increase in fertilizer N application rate. The increased root mass of rice seedlings were observed resulting from inoculation in a greenhouse experiment at University of Sydney (Kecskés et al. 2008b). Similar results were reported by Nguyen (2008) in these proceedings.

These results indicate the positive effects of 1N inoculation in increasing plant biomass, and N uptake from both fertilizer and non-fertilizer sources at 45 DAS. Although this experiment was not continued up to maturity stage, evidences from the other field experiments showed the beneficial effect of BioGro inoculation on grain and straw yields as well as N and P uptakes at maturity (Phan and Tran 2008). Increase in N uptake might be due to increase in fertilizer N uptake, BNF or increase in soil N uptake due to thicker and longer root system. The results of this experiment demonstrated the beneficial effect of 1N inoculation in increasing fertilizer N uptake by the rice plants. This study clearly demonstrates the significant effect on the

growth of rice of the plant growth promoting (PGP) organism, the 1N strain of *Pseudomonas fluorescens*, isolated by Professor Nguyen Thanh Hien near Hanoi as a N_2-fixing organism (Nguyen 2008). The use of labelled urea has indicated that the bacterium allows the plant to access significant alternative sources of nitrogen to urea after 3 weeks, more than 40 mg per plant. Once a more extensive root system has developed aided by inoculation as discussed above, the plant's access to fertilizer nitrogen is also enhanced.

11.13 Field Evaluation of BioGro by Participating Rice Farmers

Field experiments were conducted on 20 rice cropping farms in the Mekong Delta, Vietnam with funding of World Bank's Development Marketplace project to evaluate the effectiveness of BioGro2 at farm level with farmers' participatory research. Ten farms were selected from each of two localities: Cai Lay District and Phung Hiep District. All farms had a history of high-yielding rice production for at least 20 years prior to these field experiments. In this area of the Mekong Delta, three crops of rice are regularly grown per year, including a dry season crop planted after the annual flood in September, an early wet season crop, and a late wet season crop. Field experiments were conducted in four successive seasons from summer 2009 to winter 2011, commencing with a late wet season crop prior to the flood. Details of the experimental procedure and findings are available in Rose et al. (2014).

The general experimental design consisted of paired plots at each of the farms. For each farm, a plot of 2000 m^2 was devoted to the trials conducted by farmers. The plot was equally split into two subplots, one for biofertilizer treatment and another one for farmer's normal practice as the control. The subplots were separated with small bunds to ensure isolation from each other and no mobility of water and applied fertilizers. One plot received conventional farmer fertilizer application and the other plot received a biofertilizer application at a rate of100 kg/ha combined with a reduction in chemical fertilizer rate. Fertilizer application rates in the control plots and the percent reduction in the biofertilizer plots differed at each farm depending on individual farmer practice.

The salient findings of these experiments (Rose et al. 2014) are as follows:

- The efficacy of a commercial biofertilizer is strongly dependent on seasonal and site-specific environmental conditions.
- Up to 45 % of the variation in the biofertilizer effect could be ascribed to differences in the timing and magnitude of chemical fertilizers applied simultaneously to the growing crop. Such variation can therefore be managed in order to minimize farmers' risk in adopting the technology.
- The biofertilizer BioGro2 could replace between 23 and 52 % of N fertilizer without loss of yield, but did not appear to be able to replace P or K fertilizer.

- A farmer participatory approach to the application of biofertilizers enabled rapid optimization under field conditions, which in turn increased farmer confidence and the reproducibility of agronomic benefits.

The information will accelerate the practical adoption of biofertilizers into cropping practices by addressing current knowledge gaps that exist between laboratory and field scales. The outcome will be a more sustainable rice production system through a reduced reliance on high inputs of chemical fertilizers.

11.14 Conclusions

The beneficial effect of BioGro in both Australia and Vietnam was observed in field conditions in several experiments conducted under AusAID and ACIAR projects. There are varietal and seasonal differences on the effectiveness of BioGro. Rice crops in Vietnam are grown with short season varieties, allowing up to three crops a year. By contrast, Australian rice crops are long season (Kecskés et al. 2008a, b), with only one crop a year, although the total annual yields in dry matter are similar to those from three crops in Vietnam. A ^{15}N tracer study under greenhouse controlled condition confirmed that the BioGro strain 1N (*Pseudomonas fluorescens*) bacteria is capable of increasing both fertilizer and non-fertilizer N uptake by the rice plant significantly. Apart from reducing the cost of inputs, this technology will also help in reducing environmental pollution, helping satisfy the 'three reductions' sought for inputs of seed, fertilizers and other chemicals (Tran 2008) and safening crop production.

There are still many issues regarding uptake of this beneficial technology by farmers despite its scientific basis being proven. Farmers' participatory research under the World Bank Development Marketplace project showed that application of biofertilizer BioGro is effective in reducing the use of chemical N fertilizer up to 52 % at farm level without decreasing rice yield. Reduced nitrogen inputs can be optimized for BioGro application (Marsh 2008). However, in Vietnam chemical fertilizers are subsidized by government authorities and farmers may be reluctant to take the risk of using biofertilizer products like BioGro when government extension services recommend chemicals. Widespread application of this biofertilizer technology will need financial incentives, perhaps from credits resulting from international policy on climate change.

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Chapter 12
Priming Host Defense Against Biotic Stress by Arbuscular Mycorrhizal Fungi

Supriya Gupta, Pankaj Rautela, Chandan Maharana, and K.P. Singh

Abstract Mycorrhizal symbiosis has an important impact on plant interactions with pathogens and insects. Direct competition has been suggested as mechanism by which arbuscular mycorrhizae (AM) fungi can reduce the abundance of pathogenic fungi in roots. Priming set the plant to an "alert" state in which defenses are not actively expressed but in which the response to an attack occurs faster and/or stronger compared to plants not previously exposed to the priming stimulus, efficiently increasing plant resistance. Thus, priming confers important plant fitness benefit thereby defense priming by AM has a great ecological relevance. With regard to its bioprotective properties, the mycorrhizal symbiosis has become a focal point of research as an alternative to chemical fertilizers and pesticides in sustainable agriculture. In this chapter, we summarize the information available regarding mycorrhiza-induced resistance (MIR) with special emphasis in those involving plant defense responses.

Keywords Mycorrhiza • Host defense • Biotic stress • Priming • Symbiosis

12.1 Introduction

Plants encounter a large and diverse community of microorganisms in their life, within this microbial community, a whole range of beneficial and deleterious organisms can be found, leading to the establishment of mutualistic and pathogenic interactions. Symbionts called mycorrhizal fungi occur in most biomes on earth and are a fundamental reason for plant growth and development on the planet. The most common group of mycorrhizal fungi are the arbuscular mycorrhizal fungi (AMF) which colonize the roots of over 80 % of land plant families. AMF are primarily responsible for nutrient transfer from soil to plant but have other roles such as changes in plant architecture, root exudation, soil aggregation, protection of plant

S. Gupta (✉) • P. Rautela • C. Maharana • K.P. Singh
Department of Plant Pathology, College of Agriculture, G B Pant University of Agriculture and Technology, Pantnagar, Udam Singh Nagar, Uttarakhand, India
e-mail: gupta.supriya15@gmail.com

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against drought stress and soil pathogens, and increasing plant diversity may all be relevant. The establishment of the arbuscular mycorrhizal (AM) symbiosis implies remarkable changes in the physiology of the host plant. The changes span from alterations in the hormonal balance and transcriptional profile to altered primary and secondary metabolism (Hause et al. 2007). This global reprogramming of plant functions has an impact on the plant interaction with the environment, modifying its responses to biotic and abiotic stresses. It should be noted that the impact of the symbiosis in terms of resistance to biotic stresses differs among AM fungal isolates for a given plant–pathogen interaction. Generally, enhanced resistance to soil-borne pathogens has been widely reported in mycorrhizal plants. Furthermore, it is clear that the symbiosis may also impact plant interactions with above ground attackers, the outcome of those interactions ranges from enhanced resistance to increased susceptibility and seems to be depending largely up on the life style of attacker (Pozo and Azcón-Aguilar 2007). With regard to its biofertilizer and bioprotective properties, the bacterial and mycorrhizal symbiosis has become a focal point of research as an alternative to chemical fertilizers and pesticides in sustainable agriculture (Singh et al. 2011a, b, c, 2016a, b; Singh 2013, 2014, 2015a, b; Singh and Singh 2013; Singh and Strong 2016). In this chapter, we summarize the information available regarding mycorrhiza-induced resistance (MIR) with special emphasis in those involving plant defense responses.

12.2 Mycorrhiza

The term mycorrhiza was coined by a German botanist to describe the symbiotic association of plant roots and fungi. Mycorrhiza literally means "fungus root." Mycorrhizal fungi appear to have coevolved with plants for over 400 million years to become part of the root system as evidenced by fossil mycorrhiza found in carbonaceous deposits. These fungi in soil are ubiquitous throughout the world and form symbiotic relationships with the roots of most terrestrial plants. In natural ecosystems, it is exceptional for a plant not to possess a mycorrhizal root system. Therefore, it could be said that mycorrhizal association is very common or almost universal phenomenon in plant kingdom (Bagyaraj 2011). Though there are different kinds of mycorrhiza, the most common mycorrhizal association occurring in crops important in agriculture and horticulture is the arbuscular type.

12.3 Kinds of Mycorrhiza

Mycorrhiza is basically classified into three main categories based on the physical relationship between the fungus and the root cells of plant. These are as follows:

12.3.1 Ectomycorrhizas (Ectotrophic Mycorrhiza)

Ectomycorrhiza grow in between root cortical cells and form a mantle or hyphal sheath around the rootlets and also enter the roots forming an intercellular net of hyphae, called the Hartig net. They are most common among temperate forest tree species in the families Pinaceae, Salicaceae, Betulaceae, Fagaceae, and Tiliaceae, as well as in some members of Rosaceae, Leguminaceae, Myrtaceae, and Juglandaceae. Both the mantle and Hartig net provide protection for the roots from pathogenic root fungi. The ectotrophic mycorrhiza are often differentiated and classified on the basis of their mode of ramification, color, and the structure of their sheath. In *Pinus*, the mycorrhizal rootlets are often forked or dichotomously branched, while in *Fagus* and all other species, the branching is racemose.

Numerous fungi have been identified as forming ectomycorrhiza. Most of them are basidiomycetous fungi belonging to the genera *Boletus, Suillus, Russula, Hebeloma, Tricholoma, Laccaria, Rhizopogon, Scleroderma, Alpova, Pisolithus*, etc. Some ascomycetous fungi also form ectomycorrhiza such as *Tuber, Elaphomyces,* and *Cenococcum*. Thus, they are mostly fungi forming mushrooms, puffballs, or truffles. The mycorrhizal association helps in the uptake of nutrients from soil, protects roots against invasion by pathogens, and also decomposes organic matter.

12.3.2 Endomycorrhiza (Endotrophic Mycorrhiza)

Endomycorrhiza roots externally appear similar to non-mycorrhizal roots in shape and color, but the fungus invades the cortical cells of the feeder roots, with a portion lying outside as a loose mass of hyphae in the soil. These are studied under two groups on the basis of nature of the fungi: (A) those are produced by septate fungi belonging to Basidiomycota, and (B) those are produced by aseptate fungi, belonging to Zygomycota.

12.3.3 Endotrophic Mycorrhiza with Septate Fungi)

These are found in the plants belonging to plant families Ericaceae, Orhidaceae, Gentianaceae, etc.

12.3.4 Ericoid Mycorrhiza

Ericoid mycorrhizal fungi usually colonize plants belonging to the families Ericaceae, Empetraceae, and Epacridaceae, which are commonly referred to as heath plants, e.g., *Azalea* and *Rhododendron*. They form loose network on surface

and hyphal coils inside epidermal cells of hair roots where nutrient exchange is thought to take place. These plants occur in temperate regions of the world. The fungus that forms mycorrhizal association is *Hymenoscyphus ericae,* earlier called as *Pezizellaericeae*. These fungi help in the uptake of both N and P.

12.3.5 Arbutoid Mycorrhiza

The fungi of arbutoid mycorrhizas are basidiomycetes, often the same fungal species that form endomycorrhizal associations. In any given area of forest therefore, it is the particular taxon of plant which determines the type of mycorrhiza formed. Root structures of plants in the *Arbustus* and *Arctostaphylos* genera are differentiated into long and shoot roots, similar to those of forest trees. A fungal sheath or mantle of between 20 and 80 mm covers the root system. Nutrients scavenged by the mycelium and rhizomorphs in the soil have to pass through the sheath and into the short roots.

12.3.6 Orchid Mycorrhiza

Orchids belong to the family Orchidaceae. This family has nearly 30,000 species. Orchid seeds contain very limited reserves in the form of starch or lipid. At the time of germination, seeds absorb water, swell slightly and the seed coat breaks exposing the epidermal hair, and this structure is referred to as the "protocorm." The protocorm has to be infected by the mycorrhizal fungus to develop into a plant. Protocorms wait up to 6 months to be infected by the mycorrhizal fungus. If not infected by the fungus, it dies. Orchids are thus obligatorily dependent on mycorrhizal fungi. After developing in to a plant orchid "pays off" to fungus in the form of carbohydrate, this phase is known as autotrophic phase. Extensive studies have been made in the orchid mycorrhizal fungus *Tulasnellacalospora.* This fungus can be cultured in the laboratory. The symbiosis exemplified by the orchids is called phase separation symbiosis.

12.3.7 Endotrophic Mycorrhiza with Aseptate Fungi (Vesicular–Arbuscular Mycorrhiza)

The vesicular and arbuscular type of mycorrhiza (VAM) is most common among all mycorrhiza. These are ubiquitous and occur in 80 % of terrestrial plant species and fungi from the small phylum Glomeromycota (Schüßler et al. 2001). VAM are obligate biotrophs that require the host plant to complete their life cycle. The fungus colonizes the root cortex and forms intracellular structures called arbuscules (from the Latin "arbusculum," meaning bush or little tree) known as feeding organ where

the exchange of nutrients between the partners takes place and vesicles, i.e., food storing organ. The extracellular hyphal network spreads widely into the surrounding soil, thereby reaching out of the nutrient depletion zone and improving the supply of inorganic nutrients, especially phosphate and nitrate. In return, the heterotrophic fungal partner receives photosynthates from the host plant. There are six genera that form the VAM, and all belong to the order Glomales of class Zygomycetes.

12.3.8 Ectendotrophic Mycorrhizas

It shares characteristics of both ecto- and endotrophic mycorrhiza. The fungus forms a typical mantle and hartig net, as do the ectotrophic mycorrhiza and also establish haustoria and hyphal coils in the epidermal and cortical cells, like the endotrophic mycorrhiza. One of the best studied examples is the *Monotropa indica*, the Indian pipe.

12.3.9 Monotropid Mycorrhiza

The family Monotropaceae consists of ten genera in which all are entirely achlorophyllous. This means they contain no chlorophyll, and hence are unable to photosynthesize and produce carbohydrates. Instead, they use their mycorrhizas not only to obtain minerals and nutrients, but also to tap the carbon supplies of nearby plants via their roots. *Monotropa* grows on forest floors under Fagus, oak, pine, spruce, fir, Salex, and other trees. Since *Monotropa* species are most commonly found in coniferous forests, carbohydrates pass from conifer to *Monotropa* via their common mycorrhizal partner. The tree, already providing energy to the fungus, is probably physiologically "unaware" of the additional loss of carbon, and it is likely that it is the fungus that controls the passage of carbon to *Monotropa*. *Monotropa* may supply a different source of carbon to the fungus, or nothing at all, and whether there is a net carbon gain by *Monotropa* over the course of a season is not yet known. The Monotropa symbiosis is, thus, a three-tier system, involving chlorophyllous and non-chlorophyllous symbionts and a fungal bridge connecting the two.

12.4 Disease Control by AM Fungi

AM symbiosis implies remarkable changes in the plant physiology. As a consequence, the association may impact the plant interaction with other organisms. Many studies have shown the protective effect of colonization by AM fungi against infections by microbial pathogens and other deleterious organisms in different plant systems. Arbuscular mycorrhizal symbiosis control disease by several ways.

12.4.1 Protection Against Soil-Borne Pathogen

Mechanisms that could account for the protective activity of AM fungi include improvement of plant nutrition, root damage compensation, competition for photosynthates or colonization in infection sites, production of anatomical or morphological changes in the root system, changes in mycorrhizosphere microbial populations, and activation of plant defense mechanisms.

12.4.2 Improved Nutrient Status of the Host Plant

It is evident that increased nutrient uptake made possible by the AM symbiosis results in more vigorous plants, the plant itself may become more resistant to or tolerant of pathogen attack. Although in many studies of improved nutrition as a mechanism for disease control, enhanced phosphorus (P) nutrition could account for the higher tolerance of mycorrhizal plants to pathogens (Linderman 1994). For example, P-tolerant AM fungi reduced nematode effects even under high-P conditions, indicating that non-P-mediated mechanisms are involved, probably physiological changes in the roots (Smith 1987).

12.4.3 Damage Compensation

It has been suggested that AM fungi increase host tolerance of pathogen attack by compensating for the loss of root biomass or function caused by pathogens (Linderman 1994), including nematodes (Pinochet et al., 1996) and fungi (Cordier et al. 1996). This represents an indirect contribution to biocontrol through the conservation of root system function, both by fungal hyphae growing out into the soil and increasing the absorbing surface of the roots and by the maintenance of root cell activity through arbuscule formation (Cordier et al. 1996).

12.4.4 Competition for Host Photosynthates

It has been proposed that the growth of both the AM fungi and root pathogens depends on host photosynthates, and they compete for the carbon compounds reaching the root (Linderman 1994). When AM fungi have primary access to photosynthates, the higher carbon demand may inhibit pathogen growth. However, there is little or no evidence that competition for carbon compounds is a generalized mechanism for pathogen biocontrol activity of AM symbiosis. Both localized and nonlocalized mechanisms could exist, probably depending on the pathogen point to a

localized effect. (Cordier et al. 1996) showed that *Phytophthora* development is reduced in AM fungal-colonized and adjacent-uncolonized regions of AM root systems, and the pathogen does not penetrate arbuscule-containing cells. This means that localized competition occurs, and that even in the absence of systemic resistance, resistance was still induced at some distance from the AM-colonized tissue.

12.4.5 Anatomical and Morphological Changes in the Root System

It has been demonstrated that AM colonization induces remarkable changes in root system morphology, as well as in the meristematic and nuclear activities of root cells. This might affect rhizosphere interactions and particularly pathogen infection development. The most frequent consequence of AM colonization is an increase in branching, resulting in a relatively larger proportion of higher order roots in the root system. However, the significance of this finding for plant protection has not yet been sufficiently considered. In most studies on AM fungi and biocontrol, the roots have not been examined for anatomical changes. Thus, more attention needs to be given to root system morphology in the future because it could modify the infection dynamics of the pathogen as well as the pattern of resistance of AM roots to pathogen attack.

12.4.6 Microbial Changes in the Mycorrhizosphere

AM formation induces changes in host physiology that can be decisive for root exudation pattern and, consequently, cause qualitative and quantitative alterations in microbial populations in the rhizosphere. There is evidence that microbial shifts occur in the mycorrhizosphere and that the resulting microbial equilibria could influence the growth and health of plants. Although this effect has not been specifically evaluated as a mechanism for AM-associated biological control, there are indications that such a mechanism does operate (Barea et al. 1996). Changes in soil microorganism populations induced by AM formation may lead to stimulation of certain components of the microbiota, which in turn may be antagonistic to root pathogens. Further studies have corroborated these findings and demonstrated that such an effect is dependent on the AM fungus involved (Linderman 1994). The prophylactic ability of some AM fungi could be exploited in association with other rhizosphere microorganisms known to be antagonistic to root pathogens that are being used as biological control agents (Barea et al. 1996). As previously mentioned, among the microorganisms known to be antagonists of fungal pathogens are fungi such as *Trichoderma*, *Gliocladium,* and rhizobacteria such as *Pseudomonas* and *Bacillus* (Linderman 1994).

12.4.7 Activation of Plant Defense Mechanisms

It is likely that AM associations as agents in biological control will be acting by more than one mechanism. The activation of specific plant defense mechanisms as a response to AM colonization is an obvious basis for the protective capacity of AM fungi. The elicitation by an AM symbiosis of specific plant defense reactions could predispose the plant to an early response to attack by a root pathogen. Current research using molecular biology techniques and immunological and histochemical analyses will probably provide more information about these mechanisms. During their life cycle, plants evolve a number of defense responses elicited by various signals, including those associated with pathogen attack. Among the compounds involved in plant defense to AM formation are phytoalexins, enzymes of the phenylpropanoid pathway, chitinases, β-1, 3-glucanases, peroxidases, pathogenesis-related (PR) proteins, callose, hydroxyproline-rich glycoproteins (HRGP), and phenolics. Electrophoretic analysis of soluble extracts from AM roots has demonstrated that the host plant produces a number of new proteins (endomycorrhizins) in response to AM colonization (Gianinazzi-Pearson et al. 1995). However, this altered pattern of protein synthesis in the plant is not necessarily related to defense reactions. This is a research area deserving further attention.

12.4.8 Systemic Protection Against Leaf Pathogens

Recent findings have shown the ability of certain beneficial soil fungi for controlling shoot pathogens by eliciting a plant-mediated resistance response. The pathogens' lifestyles have been shown to determine the outcome of the interaction with regard to the biocontrol of shoot diseases by the AM symbiosis (Jung et al. 2012). Early studies reported a higher susceptibility of AM plants to viruses, and biotrophic pathogens appear to develop better on AM plants although an increased tolerance was observed in terms of plant productivity. Regarding pathogens with a hemibiotrophic lifestyle, the effect of the symbiosis varies from no effect to a reduction of the disease (Chandanie et al. 2006). In contrast, several studies evidence the positive effect of AM symbiosis on plant resistance against necrotrophic shoot pathogen.

12.4.9 Effect of AM Symbiosis on Phytophagous Insects

Insects may be deleterious to plants by directly damaging them through herbivory or acting as vectors for pathogens such as viruses and phytoplasmas. However, they can also have positive effects on plant health acting as natural enemies of pests or as pollinators (Jung et al. 2012). The outcome of the AM–plant–herbivore interaction

depends on many factors, such as the AM fungus, host plant, insect species involved, and prevailing environmental factors (Pineda et al. 2010). The AM symbiosis can actually influence insect herbivore performance, but the magnitude and direction of the effect depend mainly upon the feeding mode and lifestyle of the insect (Koricheva et al. 2009). Furthermore, the induction of defense mechanisms operating in resistance against microbial pathogens may also impact herbivorous insects. Generalist insects, able to feed on diverse plants and sensible to the plant defense mechanisms, are usually negatively affected by the presence of AM fungi. However, specialist insects, which feed from one or only a small number of host species and show a high degree of adaptation to their hosts' defense responses, usually perform better on AM plants. These insects feed on the leaf tissue and cause massive damage which activates defenses that depend on the plant hormone JA. Remarkably, as discussed later on, JA seems to be a key signal in MIR.

12.4.10 Impact of AM Symbiosis on Root Parasitic Plants

Plants of the genera *Striga* and *Orobanche* can parasitize a number of important crop plants around the world. They attach to the host roots and acquire nutrients and water from them, constituting one of the most damaging agricultural pests. Several studies reported that the attachment and the emergence of *Striga* are reduced in AM plants. It is known that seeds of these weeds germinate upon perception of strigolactones (SLs), a group of carotenoid-derived signaling molecules that are exuded by the roots of the host plant. These signals are produced by the plant under conditions of phosphate starvation and promote AM hyphal branching and thereby facilitate mycorrhiza establishment. Root parasitic plants have intercepted this recruitment system and utilize the signal for the detection of an appropriate host plant. Remarkably, mycorrhization downregulates the level of SLs once a well-established mycorrhiza is achieved, thus reducing the germination rate of weed seeds. This reduction seems to be the underlying reason for the decrease in the incidence of root parasitic plants on mycorrhizal plants. In addition to the root parasitic plants, it has been suggested that certain AM fungi may suppress growth of other aggressive agricultural weeds which cause crop yield losses every year.

12.5 Mechanisms Underlying the Impact of Arbuscular Mycorrhizal Fungi on Plant Protection Against Pathogens

It is evident that AM fungi may alleviate biotic stresses through a combination of different mechanisms ranging from direct interactions as competition with the aggressor to indirect, plant-mediated effects. Direct effects include competition for carbon, nitrogen, and other growth factors and competition for niches or specific

infection sites. Direct competition has been suggested as mechanism by which AM fungi can reduce the abundance of pathogenic fungi in roots (Filion et al. 2003). Presumably, pathogenic and AM fungi exploit common resources within the root, including infection sites, space, and photosynthates. Negative correlations between the abundance of AM fungal structures and pathogenic microorganisms have been found in roots and soil (St Arnaud and Elsen 2005). Full exclusion of the pathogenic oomycete *Phytophthora* from arbusculated cells was also evidenced (Cordier et al. 1998). Root colonization by AM fungi also induces changes in the root system architecture, in morphology, and in root exudates. These changes may alter the dynamics of infection by the pathogen or impact on the microbial community of the mycorrhizosphere favoring components of the microbiota with the capacity to antagonize root pathogens. Changes in root exudation can directly impact microbial pathogens and nematodes (Vos et al. 2011). More recent findings indicate that a primary mechanism of pathogen control occurs through the ability of AM fungi to reprogram plant gene expression (Campos-Soriano et al. 2012). As a consequence, alterations in the primary and secondary metabolism of the plant do occur, many of these changes being related to plant defense. Actually, as other biotrophs, AM fungi are able to trigger plant defense responses at initial stages. Thus, for a successful colonization, the fungus has to cope with these reactions and actively modulate plant defense responses. This modulation may result in preconditioning of the tissues for efficient activation of plant defenses upon a challenger attack, a phenomenon that is called priming (Pozo and Azcón-Aguilar 2007). Priming set the plant is an "alert" state in which defenses are not actively expressed but in which the response to an attack occurs faster and/or stronger compared to plants not previously exposed to the priming stimulus, efficiently increasing plant resistance. Thus, priming confers important plant fitness benefit thereby defense priming by AM has a great ecological relevance.

12.5.1 Modulation of the Host Defense Responses

During interactions with microorganisms plants are able to recognize pathogen-derived molecule and tailor their defense responses according to the type of microorganism countered. Modulation of the host plant's immune system by AM fungi is an integral component of induced systemic resistance. Both mutalistic and pathogenic biotroph fungi are initially recognized as alien organisms, and the plant reacts with the activation of an immune response. As stated before, the AM fungus has to deal with the plant's immune system, contend with the defense mechanisms, and overcome them for a successful colonization of the host (Zamioudis and Pieterse 2012). Once established, the plant has to regulate the level of AM fungal proliferation within the roots to prevent excessive colonization and carbon drainage, thus maintaining the interaction at equilibrium to limit the colonization by the mutualistic symbionts. Actually, plants possess a feedback system to prevent excessive

colonization over a critical threshold, a phenomenon termed autoregulation of the symbiosis (Vierheilig et al. 2008). From pre-symbiotic stages and throughout a well-established AM association, plant defense mechanisms are tightly regulated to control the symbiosis. As a side effect, this regulation of plant defenses in the root may directly impact root pathogens. During the early stages of the interaction, the plant reacts to the presence of AM fungi activating some defense-related responses that are subsequently suppressed (García-Garrido and Ocampo 2002). A quick but transient increase of endogenous salicylic acid (SA) occurs in the roots with a concurrent accumulation of defensive compounds, such as reactive oxygen species, specific isoforms of hydrolytic enzymes, and the activation of the phenylpropanoid pathway. These initial reactions are temporally and spatially limited compared to the reaction during plant–pathogen interactions, suggesting a role in the establishment or control of the symbiosis. To promote successful colonization, AM fungi likely have to evade and manipulate the host innate immune system. Indeed, recent studies support that AM fungi can actively suppress plant defense reactions by secreting effector proteins that interfere with the host's immune system (Kloppholz et al. 2011). However, even in later stages of the interaction, the levels of SA and other defense-related phytohormones, such as jasmonic acid (JA), abscisic acid (ABA), and ethylene (ET), may be altered in mycorrhizal roots. These changes may contribute to control the extension of fungal colonization and the functionality of the symbiosis on mutualistic terms (Hause et al. 2007). Indeed, regulation of JA has been reported to have a central role in the correct functioning of the AM symbiosis (Hause and Schaarschmidt 2009). Mycorrhizal plants are more resistant to necrotrophs and chewing insects, which are targeted by JA-dependent defense responses, while frequently being more susceptible to biotrophs, as these are targeted by SA-regulated defenses. This pattern correlates with an activation of JA-dependent defenses and repression of SA-dependent ones in a well-established mycorrhiza. The antagonistic interaction between SA and JA signaling is a conserved mechanism for plant defense regulation (Thaler et al. 2012).

12.6 Priming for Enhanced Defense by AM Fungi

Priming of the plant's innate immune system is common upon interaction with beneficial microorganisms and has important fitness benefits compared to direct activation of defenses (Conrath, 2009). Several mechanisms have been proposed to mediate the induction of the primed state as a moderate accumulation of defense-related regulatory molecules, such as transcription factors or MAP kinases and chromatin modification. Examples of primed defense responses in AM plants were first observed in root tissues. Mycorrhizal-transformed carrot roots displayed stronger defense reactions at sites challenged by *Fusarium* (Benhamou et al. 1994). In tomato, AM colonization systemically protected roots against *Phytophthora parasitica* infection. Only mycorrhizal plants formed papilla-like structures around the

sites of pathogen infection through deposition of nonesterified pectins and callose, preventing the pathogen from spreading further, and they accumulated significantly more PR-1a and basic β-1,3 glucanases than non-mycorrhizal plants upon *Phytophthora* attack (Pozo et al. 1999). Similarly, mycorrhizal potatoes showed amplified accumulation of the phytoalexinsrishitin and solavetivone upon *Rhizoctonia* infection, whereas AM fungi alone did not affect the levels of these compounds (Yao et al. 2003). Primed accumulation of phenolic compounds in AM date palm trees has been related to protection against *F. oxysporum* (Jaiti et al. 2007), and priming has also been involved in AM induction of resistance against nematodes (Hao et al. 2012). However, the primed response is not restricted to the root system as priming of defenses has also been shown in shoots of AM plants (Pozo et al. 2010). Actually, the AM symbiosis induced systemic resistance in tomato plants against the necrotrophic foliar pathogen *Botrytis cinerea* while the amount of pathogen in leaves of mycorrhizal plants was significantly lower; the expression of some jasmonate-regulated, defense-related genes was higher in those plants (Pozo et al. 2010). A primed response of JA-dependent defenses was confirmed by transcript profiling of leaves after exogenous application of JA, since JA-responsive genes were induced earlier and to a higher extend in AM plants (Pozo et al. 2008). The use of tomato mutants impaired in JA signaling confirmed that JA is required for AM-induced resistance against Botrytis (Jung et al. 2012), corroborating that MIR is similar to the well-studied rhizobacteria-induced systemic resistance (ISR) in *Arabidopsis* and requires a functional JA signaling pathway for the efficient induction of resistance (Pieterse et al. 1998).

12.7 Signal Transduction Between AMF and Plant Upon Pathogen Attack

During plant–microbe interactions, an extensive exchange of molecular messages in the form of signal transduction befalls. These signaling pathways are an integrated system of diverse defense-related compounds. It includes cytosolic calcium (Ca), reactive oxygen species (ROS), fatty acids, jasmonic acid, salicylic acid, and ethylene which get activated through a cascade of reactions. Cytosolic free calcium concentration ([Ca2+]cyt) is an illustrious component of signal transduction pathways involved in plant–pathogen interactions (Sanders et al. 2002). Upon receiving signals from plant, AMF replies through yet an unidentified small molecule known as Myc factor which is responsible for triggering downstream responses including Ca responses, thereby leading to form a symbiotic relationship with the host plant. A Ca–calmodulin-dependent protein kinase (ccaMK) is essential for AMF symbiosis (Mitra et al. 2004). ROS includes superoxide anion and hydrogen peroxide which are associated with normal plant biochemical processes. They are also responsible for the lipid peroxidation with membrane destruction, protein inactivation, DNA mutation (Torres et al. 2006), oxidative burst and probably hypersensitive response

(HR), or systemic acquired resistance (Bolwell 2004) at the pathogen-infected site of plants. Generation of hydrogen peroxide exhibits antimicrobial activity which inhibits spore germination of fungal pathogens and participates in the formation of phenoxyl radicals during phenol polymerization within the plant cell wall.

In addition, lipid peroxidation through ROS generation leads to membrane integrity, tissue necrosis and induction of phytoalexins, which is synthesized by the action of lipoxygenases (LOXs). LOX metabolites possibly exert antimicrobial activity and induce or alter wound/pathogen defense gene expression through an octadecanoid pathway (Hause et al. 2007) which leads to Jasmonate biosynthesis. Jasmonates serve as a putative endogenous signal in mycorrhiza-induced systemic resistance (El- Khallal 2007), which still requires elucidation. Furthermore, several antioxidant enzymes such as peroxidase (POX), superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) take part in the ROS metabolism during the pathogen attack (El- Khallal 2007). Augmentation in SOD activity has been implicated in inducing pathogen-related HR development in plants; however, catalase activity gets reduced during microorganism-induced HRs (Delledonne et al. 2002). In another experiment, it was observed that *G. intraradices* regulates the catalase and peroxidase in bean and wheat. The induced cytosolic calcium ($[Ca^{2+}]$ cyt) elevation also induces mitogen-activated protein kinase (MAPK) and alterations in G-protein after or parallel with ROS generation. MAPKs and G-protein modifications regulate activity of several enzymes responsible for defense mechanisms through phosphorylation or dephosphorylation external stimuli to the cell's machinery, which leads to bringing about a response against phytopathogens.

12.8 Conclusions

Mycorrhizal symbiosis has an important impact on plant interactions with pathogens and insects. The association generally leads to reduction of damage caused by soil-borne pathogens, but effects on shoot-targeting organisms depend greatly on the pathogen lifestyle. Mycorrhiza-induced resistance (MIR) in aboveground tissues seems effective against necrotrophic pathogens and generalist chewing insects, but not against biotrophs. Experimental evidence confirms that this protection is based not only on improved nutrition or local changes within the roots and rhizosphere, but that priming of plant immunity plays a major role in MIR. Although the molecular basis for the regulation of plant defenses and the priming of the plant immune system during mycorrhization remains mostly unknown, a prominent role of jasmonate signaling has been confirmed. The great majority of land plants form arbuscular mycorrhiza, thus, unveiling the principles behind a successful symbiosis and the functional interplay between plant and fungus is of major interest. There is growing awareness about the importance of soil microbiota in natural and man-made ecosystems. Indeed, progresses in basic knowledge of plant interactions with mycorrhizae fungi are still required.

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Chapter 13
Role of Phosphate-Solubilising Microorganisms in Sustainable Agricultural Development

Rajesh Kumar and Beenu Shastri

Abstract Phosphorous (P) is an essential macronutrient required for plant growth and development and comes next to Nitrogen (N). The quantity of phosphorous present in soil is huge but is unavailable to the plants due to its fixation with the other elements in soil necessitating the application of chemical phosphatic fertilisers to the soil for plant growth and development. Injudicious use of phosphatic fertiliser though has resulted in enhancement of crop yield but had left an adverse effect on the ecosystem. In the present scenario, to manage the nutritional security and the environment, sustainable agriculture holds the key which uses phosphate solubilising microorganisms (PSM's) as an important alternative, which can solubilise soil phosphate and supply it to the plants in a more eco-friendly and sustainable manner. PSM's are diversified in nature and are abundant in normal to stressed environments. They include bacteria, fungi, algae, actinomycetes and mycorrhizae which solubilises soil phosphate by different mechanisms including production of organic acids and enzymes, thus making phosphorous available to the plants for their growth and development. Molecular biotechnology brings out a better technique that could help researchers to understand the mechanisms responsible for solubilisation and also improve the performance of PSM's by manipulating the genes responsible for phosphorous solubilisation for the betterment of crops and also in managing a sustainable environment system.

Keywords Sustainable agriculture • Phosphate-solubilising microorganisms • Bioinoculant • Ecosystem • Genetic engineering

R. Kumar (✉) • B. Shastri
Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar (Central) University, Raibarelly Road, Lucknow, Uttar Pradesh 226025, India
e-mail: Rajesh_dem@bbau.ac.in; rajesh_skumar@yahoo.co.in

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13.1 Introduction

Steady increase in urbanisation and industrialisation has resulted in shrinking of land in last few decades, subsequently leading to food crisis all over the world (Abbasdokht and Gholami 2010; Krishnaraj and Dahale 2014). Additionally, alarming increase in the human population has further aggravated the world's food security concern. The pressure of increasing population on earth will increase the demand for agricultural land as well as agricultural products in order to supply the food in future. Thus, food crisis would be the major challenge to be faced by human community. Therefore, there is an urgent need for enhancing food production by around 50 % in order to sustain the population pressure (Vasil 1998; Leisinger 1999). Earlier, traditional agricultural practices were practised which were limited to only cultivation of food grains, vegetables and rearing of domestic animals along with using organic matter in soil in order to achieve a high yielding variety (Krishnaraj and Dahale 2014). With the advent of technological development and modernisation in the agronomical practices, uses of agrochemicals (pesticides and fertilisers) sharply increased for obtaining the high yield of crops.

Fertilisers usually emphasise on the nutritional aspects of crop required for healthy plant life. Various macro- and micronutrients are supplied by the chemical fertilisers. Phosphorus is considered as a key element in the nutrition of plants after nitrogen (N), whose deficiency restricts the yield of crops (Sharma et al. 2013). Photosynthesis, respiration, energy transfer, signal transduction, macromolecular biosynthesis and nitrogen fixation in legumes are all the major metabolic processes occurring in plant in the presence of phosphorus (Saber et al. 2005; Khan et al. 2010). On an average, most of the mineral nutrients are present in millimolar amounts in soil solution but phosphorus is present only in micromolar or lesser quantities (Ozanne 1980). Though organic and inorganic forms of phosphorus are abundant in soil, but are major limiting factor for plant growth because of their fixation into an unavailable form (Sharma et al. 2013). So, in order to ensure continuous phosphate availability to the crops, chemical phosphate fertilisers are added in recommended doses, as excess use may harm the environment (Khan et al. 2007).

The dependence on chemical fertiliser clearly indicates that a conventional agricultural practice fails to support the healthy crop system for too long. This shift from traditional methods to chemical one in agriculture occurred due to the acceptance of the high yielding hybrids, which subsequently reduced the organic matter addition to soil. Since green revolution, consumption of chemical fertilisers has increased over the years without considering their deteriorating effect to our environment (Vance 2001; Krishnaraj and Dahale 2014). Over-exploitation and continuous uses of resources and unmanageable application of inorganic phosphorus fertiliser and pesticide products has resulted in environment quality reduction.

Therefore, other than chemical fertilisers, researchers, environmentalists and policy makers are interested to find alternative strategies to solubilise phosphorus in the soil that can ensure competitive yields in addition to protecting the health of the soil. This new approach to farming, often referred to as sustainable agriculture, requires agricultural practices that are eco-friendlier to the environment and that maintain the long-term ecological balance of the soil ecosystem (Khan et al. 2007). In reference to this context, the use of microorganisms has been cited to play a significant role in the enhancement and sustenance of crop yields and productivity with improvement in soil health (Krishnaraj and Dahale 2014). The microbial populations are instrumental to fundamental processes that drive stability and productivity of agroecosystems (Singh et al. 2011). Therefore, use of microbial inoculants possessing P-solubilising activities generally referred to as Phosphate-solubilising microorganisms (PSM's) in agricultural soils is considered as an eco-friendly and sustainable approach as compared to chemical phosphate fertilisers (Sharma et al. 2013). Therefore, it is important to use bioinoculants, which are biofriendly, economically feasible and replaceable sources of inorganic phosphorus (Pi), before the world consumption rate reaches point of no return in coming future (Yasin et al. 2012).

13.2 Phosphorus Status and Availability in the Soil

Phosphorus is one of the master elements which are known for multifunction and metabolism in plants like photosynthesis, energy transfer, signal transduction, macromolecular biosynthesis and respiration (Khan et al. 2010) and nitrogen fixation in legumes (Saber et al. 2005). Poor availability causes browning of leaves followed by small leaves, weak stem and slow development which ultimately results in low yield of crops (Mahantesh and Patil 2011). Hence, with increasing demand for agricultural production, phosphorus (P) is receiving more attention as a non-renewable resource. Although, P is abundant in soil, but low availability and high fixation are the unique characteristics of phosphorus in soils, due to which it is unavailable to plants. Thus, in both natural ecosystems and agricultural systems, phosphorus availability in soils is one of the major limiting factors. Therefore, for long-term availability to plant and for higher sustained agricultural productivity, P is required (Scervino et al. 2011). Phosphorus is generally present at levels of 400–1200 mg/kg of soil (Begon et al. 1990), but only 0.1 % of the total P exists in available form for plant uptake (Zhou et al. 1992). Large reserves of phosphorus are present in most of the agricultural soils of which considerable part has been accumulated as a consequence of regular applications of P fertilisers (Richardson 1994; Rodríguez and Fraga 1999). By the application of chemical fertilisers, efficiency of P-solubilisation rarely exceeds 10–20 % (Kuhad et al. 2011). Soluble inorganic phosphate as phosphate fertilisers is applied to soil and is rapidly immobilised soon after application and becomes unavailable to plants (Dey 1988; Rodríguez and Fraga 1999). The phenomenon for immobilisation of phosphorus is highly dependent on particular

pH of soil (Mahantesh and Patil 2011). For instance, in acidic soils (low pH), phosphorus is associated with aluminium (Al) and iron (Fe) compounds (Norrish and Rosser 1983), whereas calcium phosphate is the predominant form of inorganic phosphate in calcareous/alkaline soils (high pH) (Lindsay et al. 1989).

Generally, soil phosphorus is mainly present in two forms i.e. inorganic and organic phosphorus (Richardson and Simpson 2011). Inorganic phosphorus (Pi) usually accounts for 35–70 % and organic phosphorus (Po) accounts for just 15–80 % of the total phosphorus in soils. Inorganic P mainly includes Ca-P, Fe-P and Al-P. Organic P in soil mainly exists in stabilising forms as they are synthesised by microorganisms and plants in the form of inositol phosphates (soil phytate), phosphonates, and active forms as orthophosphate diesters (phospholipids and nucleic acid), labile orthophosphate monoesters, and organic polyphosphates (Condron et al. 2005). Orthophosphate is a soluble form of phosphate which is taken by the plants (Ranjan et al. 2013).

13.3 Constraints in Using Phosphatic Fertilisers

To avail the unavailable form of P from the soil solution, different phosphatic fertilisers are manufactured from mining phosphate rocks. The production of chemical phosphatic fertilisers is a highly energy-intensive and costly process which requires energy worth US $ 4 billion per annum in order to meet the global needs (Goldstein et al. 1993). At present, mining rate is about 7100 million tonnes/annum. If this rate of mining continues in future, reserve will soon be depleted in about 500–600 years (Sharma et al. 2013). Besides, mining of phosphate minerals and using it as chemical P fertilisers on the soil surface is neither eco-friendly nor economically feasible and sustainable as it possesses following constraints (Sharma et al. 2013).

1. Disposal of gypsum
2. Emission of the fluorine (F) as a highly volatile and poisonous HF gas
3. Accumulation of cadmium (Cd) and other heavy metals in soil and crops as a result of repetitive use of P fertilisers

Some of the countries (including India), however, import these fertilisers, which are often in limited supply and also represent a major disbursement for resource-poor farmers. In India, reservoir of enriched phosphatic rocks is limited and hence it imports two million tons of rock phosphate annually. However, using phosphatic fertiliser is further complexed by the fact that almost 75–90 % of phosphorus is precipitated by metal cation complexes (Stevenson 1986). The repeated and injudicious application of chemical phosphate fertilisers leads to the loss of soil fertility (Gyaneshwar et al. 2002) by disturbing microbial diversity, and consequently reducing yield of crops (Sharma et al. 2013). High doses of chemical phosphorus lead to eutrophication of surface waters, a major phenomenon that leads to algal blooms because of high levels of P in the environment (Schindler et al. 2008). Inhibition of substrate-induced respiration by streptomycin sulphate (on fungal activity) and

actidione (on bacterial activity) and microbial biomass carbon (C) has been viewed as the long-term effect of different sources of P fertilisers on microbial activities (Bolan et al. 1996). Theoretically, it has been estimated that no further increase in crop yield is possible when chemical fertiliser is reaching the beyond maximum (Ahmed 1995). Thus, dependency of fertiliser production on a fossil energy source and the prospects of the diminishing availability of costly input of fertiliser production in years to come have obviously brought the subject of mineral phosphate solubilisation (mps) to the forefront (Khan et al. 2007). About 98 % of cropland in India is deficient in available forms of soil phosphorus and only 1–9 % has high phosphorus status. Hence, it becomes imperative to explore alternative phosphatic sources.

13.4 Urgent Need of Phosphate-Solubilising Microorganisms in Plant Phosphate Nutrition

It has been estimated that the world's known reserve of high quality rock P may be depleted within the coming years on the basis of its current usage (Cordell et al. 2009). Beyond this time, processing of lower grade rock at significantly higher cost will be done for the production of P-based fertilisers (Isherwood 2000). After realisation of all these potential problems associated with high cost of chemical phosphate fertilisers in manufacturing, as well as deteriorating the quality of environment, alternative strategies (economically and ecologically) has been searched out for management of phosphates. Goldstein et al. (1993) also suggested that in agricultural soils, phosphates are accumulated in such a sufficient concentration that can sustain maximum crop yields worldwide for about 100 years. Improving the phosphorus status in soil has been the urgent priority for agronomist in order to meet the global demand for food. Therefore, new options are needed for bioavailability of phosphorus from the soil in order to ensure the sustainability of agroecosystem. Selection of efficient microorganisms comes as an alternative strategy for the better management of soil by optimising bioavailable phosphorus, meanwhile ameliorating the adverse effect of chemical P fertilisers (Dhankhar et al. 2013). Thus, microbial-mediated P management is an eco-friendly and cost-effective approach for sustainable development of agricultural crop (Sharma et al. 2013). Microorganisms are an integral component of the soil and also play an important role in the transfer of phosphate between different pools of soil phosphorous. This led to the discovery of Phosphate-Solubilising Microorganisms (PSM's), which through various mechanisms of solubilisation and mineralisation are able to convert inorganic and organic soil phosphate respectively (Khan et al. 2009) into the bioavailable form, making it available for the plants. In the diverse soil and agroclimatic conditions, the phosphate-solubilising microorganisms have proved their ability economically against more expensive superphosphates and also possess a greater agronomic utility. The phosphate-solubilising microorganisms not only increase the availability of soluble phosphate but also enhance plant growth by increasing the efficiency of biological nitrogen fixation or enhancing the

availability of other trace elements such as iron and zinc, and by production of plant growth-promoting regulators (Sattar and Gaur 1987; Kucey et al. 1989; Ponmurugan and Gopi 2006).

13.5 Phosphate Solubilising Microorganisms (PSM's)

It is very well known that microorganisms play a significant role in improving the productivity of soil by their beneficial or detrimental activities which directly and indirectly influence the health of the soil. Microorganisms localised in rhizospheric zone are responsible for regulating various soil processes like mobilisation and mineralisation, decomposition, release of nutrients and water, nitrogen fixation and denitrification. Beside this, rhizospheric microorganisms have also been known for mineral phosphate solubilisation since 1903. Since then, extensive studies have been done on mineral phosphate solubilisation by naturally abundant rhizospheric microorganisms. Therefore, microbial inoculants from the rhizosphere zone have been considered as components of integrated nutrient management systems along with their ability to increase the availability of phosphate for crops (Kucey et al. 1989; Bowen and Rovira 1999; Jakobsen et al. 2005; Adesemoye and Kloepper 2009; Harvey et al. 2009; Khan et al. 2010). The population of highly metabolically active PSM's is more concentrated in the rhizospheric zone than non-rhizospheric zones (Vazquez et al. 2000). Bacteria, fungi, actinomycetes and even algae are some of the microorganisms involved in the solubilisation of insoluble phosphorus (Khan et al. 2007; Wani et al. 2007; Chun-qiao et al. 2009; Santos et al. 2013; Sharma et al. 2013). Phosphate-solubilising microorganisms are omnipresent in rhizosphere, but their numbers vary from soil to soil. Of the total population of PSM's in soil, percentage of phosphate-solubilising bacteria and fungi are 1–50 % and 0.5–0.1 %, respectively. Generally, numbers of the phosphate-solubilising bacteria are 2–150 times more than phosphate-solubilising fungi (Kucey 1983). *Bacillus and Pseudomonas* are important genera of bacteria as well as *Aspergillus* and *Penicillium* are important genera of fungi for the mineral phosphate solubilisation (Illmer and Schinner 1992; Motsara et al. 1995). Literature surveyed shows that PSM's not only increased the yield of crop but also showed reduction in the application of phosphate fertiliser by approximately 50 % after using Phosphate-Solubilising Microorganisms (PSM's) (Jilani et al. 2007; Yazdani et al. 2009). Other than bioavailability of phosphates to plants and increasing their yield, PSM's may also be useful in the bioremediation of soil polluted with heavy metals (Ahemad 2015; Monica and Harshada 2015) or for bioleaching of rare elements for mined ores (Shin et al. 2015). Most of the stress (salt, pH and temperature) tolerant phosphate-solubilising bacteria have also been investigated to be maximum in the rhizoplane region followed by the rhizosphere and root-free soil in alkaline soils (Johri et al. 1999). Due to survival in these stressed conditions, PSM's strains serve as an excellent model for studying the physiological, biochemical and molecular mechanisms of phosphate solubilisation under stressed ecosystems (Khan et al. 2007).

Phosphate-solubilising (PS) activity of both bacterial and fungal strains are visually detected by the formation of clear halo zone (a sign of solubilisation) around their colonies on an agar plate media having inorganic phosphate (mainly calcium phosphate) as a sole source of phosphorous. In 1948, Pikovskaya media was proposed for detecting phosphate solubilisation on agar plate (Pikovskaya 1948); since then, numerous protocol and media, such as bromophenol blue dye method (Gupta et al. 1994) and National Botanical Research Institute P (NBRIP) medium (Nautiyal 1999), have been proposed for the isolation of PSM's. Protocol for the isolation of PSM's has been described in Fig. 13.1.

By various studies, it is known that phosphate-solubilising bacteria lose the phosphate-solubilising activity upon repeated sub-culturing; howsoever, phosphate-solubilising fungi do not lose such activity (Kucey 1983). Therefore, PSM's are repeatedly sub-cultured to test the persistence of their phosphate-solubilising activity. An additional quantitative test to assay phosphate dissolution should be performed for selection of PSM's besides plate assay test on Phosphate solibilising (PS) media. This is followed by further testing of strain for abundant production of organic acids. In general, acids are largely produced by phosphate-solubilising fungi (PSF) than bacteria in both liquid and solid media. Thus, higher the amount of acid production, greater is the phosphate-solubilising activity of microorganisms (Venkateswarlu et al. 1984). Nitrogen source used in the media also plays a significant role in testing the phosphate-solubilising ability of PSM's. For example, it has been observed that when ammonium salts are used as a source of N in the media, solubilisation rate is greater as compared to nitrate, as a nitrogen source. This has been attributed to the extrusion of protons to compensate for ammonium uptake, leading to a decreased extracellular pH (Roos and Luckner 1984). In some cases, however, ammonium can lead to a decrease in phosphorus solubilisation (Reyes et al. 1999; Khan et al. 2007).

13.6 Biodiversity and Occurrence of PSM's

Biodiversity of microbial species exhibiting phosphate solubilisation ability includes bacteria, fungi, actinomycetes and even algae (Table 13.1).

13.6.1 Bacteria

The populations of phosphate-solubilising bacteria are mainly concentrated in soil and in plant rhizospheric zone (Sperber 1958; Katznelson et al. 1962; Raghu and MacRae 1966; Alexander 1977). Both aerobic and anaerobic strains of bacteria are prevalent with greater number of aerobic strains in submerged soils (Raghu and MacRae 1966; Rodríguez and Fraga 1999). Bacterial species have been reported to solubilise different insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock phosphate (Goldstein 1986; Rodríguez and Fraga 1999). *Pseudomonas,*

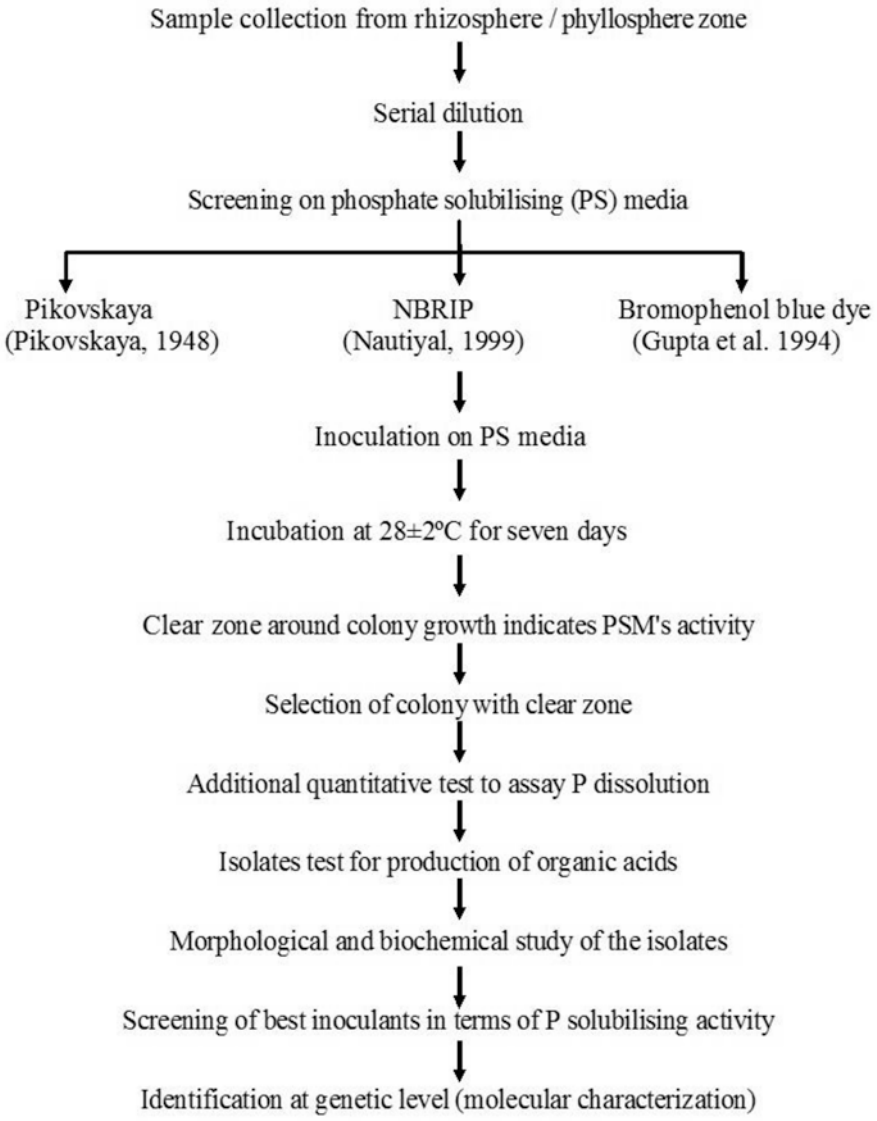

Fig. 13.1 Protocol for the isolation and characterisation of PSM's

Bacillus, Burkholderia, Rhizobium Achromobacter, Agrobacterium, Aerobacter, Micrococcus, Flavobacterium and *Erwinia* are listed as P-solubilising bacterial genera (Rodríguez and Fraga 1999). In addition to these bacteria, other bacteria have also been reported as P-solubilisers which include *Rhodococcus, Arthrobacter, Serratia, Enterobacter, Chryseobacterium, Gordonia, Phyllobacterium, Delftia* sp., *Azotobacter, Xanthomonas, Klebsiella, Pantoea, Vibrio proteolyticus* and *Xanthobacter agilis* (De Freitas et al. 1997; Vazquez et al. 2000; Kumar et al. 2001;

Table 13.1 Biodiversity of PSM's

Organisms	References
Bacteria	
Pseudomonas fluorescens, P. putida, P. mendocina, P. striata, P. aeruginosa, P. cepacia	Taha et al. (1969), Gaur (1990), Illmer and Schinner (1992), Bar-Yosef et al. (1999), Vazquez et al. (2000), Ponraj et al. (2013) and Muleta et al. (2013)
Bacillus amyloliquefaciens, B. licheniformis, B. atrophaeus, B. firmus, B. polymyxa, B. licheniformis, B. subtilis, Bacillus sp.	Banik and Dey (1982), Banik and Dey (1983), Gaur (1990), Gupta et al. (1994) and Vazquez et al. (2000)
Enterobacter aerogenes, E. taylorae, E.asburiae, E. intermedium	Vazquez et al. (2000) and Hoon et al. (2003)
Acetobacter sp.	Galar and Boiardi (1995)
Burkholderia cepacia, Burkholderia sp.	Lin et al. (2006)
Serratia marcescens	Krishnaraj and Goldstein (2001)
Rhizobium leguminosarum, Rhizobium meliloti, Bradyrhizobium sp., Sinorhizobium meliloti	Halder et al. (1990), Gaur and Gaind (1999) and Sharma et al. (2013)
Escherichia freundii, E. intermedia	Sperber (1958) and Sharma et al. (2013)
Micrococcus sp.	Banik and Dey (1982)
Fungi	
Aspergillus flavus, A. niger, A. awamori, A. foetidus, A. terricola, A. amstelodemi, A. tamari , A. japonicus, A. foetidus, A. fumigatus, A. candidus, A. tereus, A. wentii	Banik and Dey(1982), Venkateswarlu et al. (1984), Gaur (1990), Gupta et al. (1994), Singal et al. (1994), Illmer et al. (1995), Vazquez et al. (2000), Maliha et al. (2004) and Sharma et al. (2013)
Penicillium canescens, P. rugulosum, P. radicum, P.variable, P. bilaji, P. simplicissimum, P. digitatum	Parks et al. (1990), Cunningham and Kuiack (1992), Reyes et al. (1999), Whitelaw et al. (1999), Vassilev et al. (2006) and Maliha et al. (2004)
Actinomycetes and Streptomycetes	
Actinomyces, Streptomyces	Banik and Dey (1982) and Sharma et al. (2013)
Mycorrhizae	
Glomus manihotis, Glomus fasciculatum and Entrophospora colombiana	Sharma et al. (2013)
Cyanobacteria	
Anabaena sp., Calothrix braunii, Nostoc sp., Scytonema sp.	Sharma et al. (2013)

Chung et al. 2005; Wani et al. 2005; Chen et al. 2006). Symbiotic nitrogenous rhizobia, which fix atmospheric nitrogen into ammonia and export the fixed nitrogen to the host plants, have also shown phosphate solubilisation activity (Zaidi et al. 2009). There are only a few reports on phosphate solubilisation by nodule symbiotic bacteria like *Rhizobium* (Halder et al. 1991; Abd-Alla 1994; Chabot et al. 1996a) and the non-symbiotic nitrogen fixer, *Azotobacter* (Kumar et al. 2001). For instance, *Rhizobium leguminosarum* bv. *trifolii* (Abril et al. 2007) and *Rhizobium* species nodulating *Crotalaria* species (Sridevi et al. 2007) improved plant P-nutrition by mobilising inorganic and organic phosphate. Phosphate-solubilising bacteria (PSB) have also been isolated from stressed environments, for example, the halophilic bacteria *Kushneria sinocarni* isolated from the sediment of Daqiao saltern on the eastern coast of China, which may be useful in salt-affected agricultural soils (Zhu et al. 2011). Thus, PSB can play an important role in plant nutrition through an increase in phosphorus uptake by plants (Rodriguez et al. 2006). These bacteria are vital for sustainable agriculture in order to promote the growth and yield of plants and circulation of plant nutrients, thereby reducing the need for chemical fertilisers. Hence these bacteria could be a promising source for plant growth-promoting agent in agriculture and can be used to ameliorate the soil health.

13.6.2 Fungi

Various studies have been undertaken on fungi having phosphate-solubilising ability and wider diversity of phosphate-solubilising filamentous fungi is described. Generally, the ability of fungus to solubilise phosphate is greater than bacteria due to the larger production of acids (Venkateswarlu et al. 1984). Among the fungi, *Aspergillus* and *Penicillium* are the potent genera that solubilise phosphate (Fenice et al. 2000; Khan and Khan 2002; Reyes et al. 1999, 2002), whereas strains of *Trichoderma* and *Rhizoctonia solani* (Altomare et al. 1999; Jacobs et al. 2002) have also been reported as phosphate solubilisers. Study of *Arthrobotrys oligospora*, a nematofungus, also exhibits the ability to solubilise phosphate in vivo as well as in vitro (Duponnois et al. 2006). Fungi are able to travel over long distances in soil more easily than bacteria, therefore they may be more important for phosphate solubilisation in soil (Kucey 1983). Among the yeasts, *Torula thermophila, Saccharomyces cerevisae, Rhodotorula minuta, Yarrowia lipolytica Schizosaccharomyces pombe* and *Pichia fermentans* can solubilise the inorganic phosphate (Varsha-Narsian et al. 1994; Vassilev et al. 2001).

13.6.3 Actinomycetes and Streptomycetes

In recent years, P-solubilising actinomycetes and streptomycetes have attracted interest because of its survival in extreme environments (e.g. drought, fire.) but also possessing other beneficial activities (e.g. production of antibiotics and

phytohormone-like compounds) that could simultaneously benefit plant growth (Fabre et al. 1988; Hamdali et al. 2008a, b; Shrivastava and Kumar 2015; Shrivastava et al. 2015, 2016). A study by Hamdali et al. (2008a) has indicated that approximately 20 % of actinomycetes can solubilise phosphate, including those in the common genera *Streptomyces* and *Micromonospora* (Sharma et al. 2013).

Algae (cyanobacteria and actinomycetes) and mycorrhizae have also been reported to show phosphate solubilisation activity other than bacteria and fungi (Sharma et al. 2013).

13.6.4 Mycorrhizae

Arbuscular mycorrhizal fungi (AMF) have been found to be an essential component of sustainable soil-plant systems (Schreiner et al. 2003). AMF not only help in increasing the uptake of phosphate, nitrogen and soil aggregation but also act as an antagonist against some plant pathogens (Barea et al. 1991; Bolan 1991; Burkert and Robson 1994; Tisdall 1994; Duponnois et al. 2005). Moreover, it has been demonstrated that plants inoculated with arbuscular mycorrhizal fungi utilise more soluble phosphate from rock phosphate than noninoculated plants (Antunes and Cardoso 1991; Guissou et al. 2001). Formation of extramatrical mycelium by mycorrhiza which increases the root phosphate absorbing sites is considered as a major reason for P-solubilisation (Bolan 1991). AMF possess the following functions:

1. Modification of root functions (i.e. root exuadation) (Marshner et al. 1997)
2. Changes the carbohydrate metabolism of the host plant (Shachar-Hill et al. 1995)
3. Influences rhizospheric populations (Hobbie 1992)
4. Increases the nutrient and water uptake by the external hyphae (Khan et al. 2007)
5. Enhances the phosphate nutrition of plants by scavenging the available phosphorus due to the large surface area of their hyphae, and by their high-affinity phosphate uptake mechanisms (Hayman 1983)

The 350 million years old symbiotic relationship between AMF and plants involved numerous interactions at the physiological, ecological and molecular levels between these organisms, thus making this association fundamentally important in all ecosystems (Trappe 1987; Remy et al. 1994). AMF played an important role in improving plant phosphate nutrition along with their co-interaction with other soil microorganisms but the mechanism of their action is still unknown. Similar to bacteria and fungi, organic acids are also produced by AMF to solubilise the insoluble mineral phosphate (Lapeyrie 1988). Amongst the mycorrhizal fungi, ectomycorrhizal fungi possess phosphate-solubilising and phosphatase activity and are capable of utilising phosphate from inositol phosphates and the release of phosphate from soil organic matter, respectively (Lapeyrie et al. 1991; Koide and Shreinner 1992). In addition to phosphate solubilisation, AMF also make available iron phosphates to the crops (Bolan et al. 1987). Synergistic interaction between AMF and PSB has also proved to be a better mechanism for better utilisation of poorly available phosphorus. The interactive study of arbuscular mycorrhizal fungi (AMF) and

rhizobacteria on the growth and nutrients uptake of plant *Sorghum bicolor* in acidic soil has been well established. Thus, co-inoculation of AMF (*Glomus manihotis* and *Entrophospora colombiana*) and *Pseudomonas* sp., serve as potential biofertilisers in acid soil. This interactive and beneficial dual inoculation of different PSM's needs to be further evaluated under different crop and agroclimatic conditions, particularly in the field (Widada et al. 2007). Hence the studies have shown that the diversity of the PSM's is highly varied in different ecological niches and there is ample scope to identify many new potent isolates from varied environments in coming times (Sharma et al. 2013).

13.7 Mechanism of Phosphate Solubilisation by PSM's

Soil microbial interaction plays a significant role in mediating the distribution of phosphate between the available pool in soil solution and the total soil phosphorous through solubilisation, mineralisation and immobilisation reaction of sparingly available forms of inorganic and organic soil phosphate. Dissolution–precipitation, sorption–desorption and mineralisation–immobilisation are the major processes of the soil phosphorous cycle that affects concentration of soil solution phosphate (Sims and Pierzynski 2005). Soil microorganisms mainly solubilise insoluble phosphate by the following mechanism:

1. First, by releasing mineral dissolving compounds such as organic acid, siderophores, protons, hydroxyl ions (H^+) and CO_2.
2. Second, by liberation of extracellular enzymes such as phosphatases.
3. At last, phosphate is released during substrate degradation (biological phosphate mineralisation) (McGill and Cole 1981).

Therefore, microorganisms play an important role in all three major components of the soil phosphorous cycle. PSM's becomes a direct source of phosphate to plants by rapidly immobilising phosphate even in soils with low concentration of phosphorous. Generally, immobilised phosphate is released by PSM's when microbial cells die due to sudden changes in the environmental conditions such as drying–rewetting or freezing–thawing (Butterly et al. 2009).

The mechanism of inorganic and organic phosphate solubilisation that occurs through solubilisation and mineralisation process, respectively, is as shown below:

13.7.1 Inorganic Phosphate Solubilisation

It is generally called as solubilisation. It occurs mainly through organic acid production, either by:

a) lowering the pH or
b) enhancing chelation of the cations bound to P or
c) competing with phosphate for adsorption sites in the soil

13.7.1.1 Organic Acid

Production of organic acids by the microorganisms is the major mechanism responsible for P-solubilisation (Duff and Webley 1959; Sundara Rao and Sinha 1963; Banik and Dey 1982; Leyval and Berthelin 1989; Salih et al. 1989; Halder et al. 1990; Whitelaw 2000; Maliha et al. 2004; Zaidi et al. 2009). Bacterial species with secretion of organic acid have been known to solubilise insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite and rock phosphate (Goldstein 1986). The amount and type of organic acid produced by microorganisms varies from organism to organism (Krishnaraj and Dahale 2014). The amount of soluble phosphate depends on the strength and type of acid (Krishnaraj and Dahale 2014). Production of organic acid results in acidification of the microbial cell and its surroundings, thereby lowering the pH of the medium. Oxalic, citric, lactic, gluconic, isovaleric, isobutyric, acetic, glycolic, tartaric, aspartic, malonic, 2-ketogluconic and succinic acid are the organic acids secreted by PSM's (Banik and Dey 1982; Venkateswarlu et al. 1984; Illmer and Schinner 1992). These acids are the product of the microbial metabolism, mostly by oxidative respiration or by fermentation of organic carbon sources (e.g. glucose) (Atlas and Bartha 1997; Trolove et al. 2003; Sharma et al. 2013). Among them, gluconic acid and 2-ketogluconic acid are reported to be the most frequent agent of mineral phosphate solubilisation (Rodríguez and Fraga 1999; Krishnaraj and Dahale 2014). Release of organic acid results in the acidification of the microbial cell's surrounding, which consequently releases phosphate from apatite by proton substitution/excretion of H^+ (accompanying greater absorption of cations than anions) (Goldstein 1994; Illmer and Schinner 1995; Villegas and Fortin 2002). Consequently, inorganic phosphate may be released from a mineral phosphate by proton substitution for Fe^{+3}, Al^{+3} and Ca^{+2} (Goldstein 1994).

Chelation generally involves the formation of two or more coordinate bonds between an anionic or polar molecule and a cation, resulting in a ringed structure complex (Whitelaw 2000). The phosphate-solubilising activity has been attributed both to chelation and to reduction processes. Organic acids can either directly dissolve the mineral phosphate as a result of anion exchange of phosphate (PO_4^{2-}) by acid anion or can chelate iron (Fe), aluminium (Al) and calcium (Ca) cations bound to phosphate (Omar 1998; Khan et al. 2007). *Pseudomonas sp., Erwinia herbicola, Pseudomonas cepacia, Burkholderia cepacia, Rhizobium leguminosarum, Rhizobium meliloti, Achromobacter, Agrobacterium, Micrococcus, Aerobacter, Flavobacterium, Bacillus firmus, Bacillus licheniformis* and *Bacillus amyloliquefaciens* are reported as the principal organic acid producer PSM's (Banik and Dey 1982; Halder et al. 1990; Illmer and Schinner 1992; Liu et al. 1992; Halder and Chakrabartty 1993; Goldstein 1995; Rodriguez et al. 2006). Paper chromatography or thin layer chromatography or high performance liquid chromatography and enzymatic methods are the various techniques used for detecting organic acid produced from microorganisms (Parks et al. 1990; Whitelaw 2000; Khan et al. 2007).

13.7.1.2 Inorganic Acid

Although organic acid has been suggested as the principal source of phosphate solubilisation, other inorganic acids such as sulphuric, nitric and carbonic have also been reported to play a role in solubilisation of phosphorous. Hydrochloric acid (HCl) as an inorganic acid exhibits the property of phosphate solubilisation from hydroxyapatite at the same pH at which the organic acids solubilise phosphate; but it has been investigated that it is less efficient than organic acid (Kim et al. 1997). *Nitrosomonas* and *Thiobacillus* species are reported to dissolve phosphate compounds by producing nitric and sulphuric acids (Azam and Memon 1996).

Other than organic acid, inorganic acid and by chelation, solubilisation of phosphorus has been hypothesised due to the release of proton accompanying respiration by ATPase activity or ammonium assimilation and H_2S production.

13.7.1.3 NH_4^+ Assimilation

Parks et al. (1990) proposed that proton (H^+) excreting from NH_4^+ assimilation is an alternative mechanism of phosphate solubilisation. In reference to this context, Illmer and Schinner (1995) while studying the HPLC analysis of the culture solution of *Pseudomonas* sp. found no sign of organic acid but still the solubilisation of phosphate occurred. The author (Illmer and Schinner 1995) further reported that release of protons accompanying respiration or NH_4^+ assimilation would be the possible reason for this solubilisation without acid production. Krishnaraj et al. (1998) have proposed a model highlighting the importance of protons that are pumped out of the cell to be the major factor responsible for P-solubilisation (Sharma et al. 2013). Hence, direct role of solubilisation by organic or inorganic acids has been ruled out. Thus, it is evident that NH_4^+-driven proton release seems to be the sole mechanism to promote P-solubilisation in some microorganisms.

The involvement of the H^+ pump mechanism in the solubilisation of small amounts of phosphate in *Penicillium rugulosum* has been reported (Reyes et al. 1999). Thus, release of H^+ to the outer surface in exchange for cation uptake or with the help of H^+ translocation ATPase could constitute alternative ways for solubilisation of mineral phosphate (Sharma et al. 2013).

13.7.1.4 H_2S Production

Swaby and Sperber (1958) postulated that H_2S production could also be another mechanism for phosphate solubilisation. They suggested that H_2S when reacts with ferric phosphate yields ferrous sulphate with concomitant release of phosphate.

Despite, many mechanisms for phosphate solubilisation, including production of organic acids, H_2S, CO_2, iron chelating substances (siderophore); biologically active substances like indole acetic acid (Chaiharn and Lumyong, 2011), gibberellins and cytokinins (Kucey, 1988) have also been correlated with phosphate solubilisation as

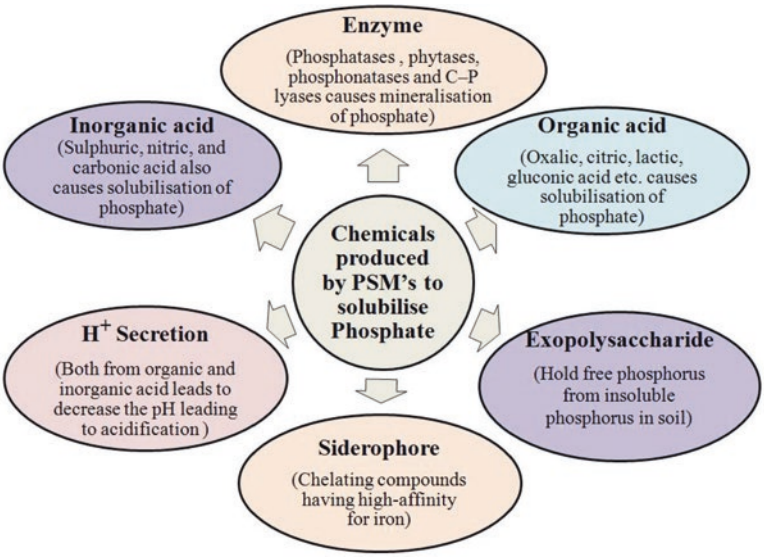

Fig. 13.2 Various chemicals released by PSM's during phosphate solubilisation

shown in Fig. 13.2. Bianco and Defez (2010) reported that RD64 strain, a *Sinorhizobium meliloti* 1021 strain, was engineered to overproduce indole-3-acetic acid (IAA), and displayed improved nitrogen fixation ability compared to the wild-type strain 1021. It also exhibited high effectiveness in mobilising phosphate from insoluble sources, such as phosphate rock (Krishnaraj and Dahale 2014).

13.7.2 Organic Phosphate Solubilisation

It generally occurs through mineralisation process. Organic phosphorus may constitute 4–90 % of the total soil phosphorous (Khan et al. 2009). Such phosphorous can be released from organic compounds in soil by the following enzymes (Sharma et al. 2013):

- Non-specific acid phosphatases (NSAPs)
- Phytases
- Phosphonatases and C–P lyases

13.7.2.1 Non-specific Acid Phosphatases (NSAPs)

These enzymes help in dephosphorylation of phospho-ester or phosphoanhydride bonds of organic matter. Phosphomonoesterases (also called phosphatases) are the most abundant and studied class of phosphatase enzyme released by PSM's (Nannipieri et al. 2011). These enzymes are classified into acid and alkaline

phosphomonoesterases depending on their optimum pH, and both can be produced by PSM's depending upon the external conditions (Kim et al. 1998; Jorquera et al. 2008). Generally, acid phosphatases dominate in acidic soils, whereas alkaline phosphatases are abundantly present in alkaline and neutral soils (Eivazi and Tabatabai 1977; Juma and Tabatabai 1977, 1998; Renella et al. 2006). It has been investigated that plant roots are able to produce more acid phosphatases than alkaline phosphatases, suggesting the suitable habitat for PSM's (Juma and Tabatabai 1998; Criquet et al. 2004). There is difficulty in differentiating phosphatase enzyme produced from plant roots and PSM's (Richardson et al. 2009a, b) but literature suggests that phosphatase enzyme released from microbe possess a greater affinity for organic phosphorus (Po) compounds than those obtained from plant roots (Tarafdar et al. 2001; Sharma et al. 2013). The relationship between PSM's (present in the soil), phosphatase activity and the subsequent mineralisation of organic phosphorus (Po) still remains poorly understood (Chen et al. 2003; Sharma et al. 2013).

13.7.2.2 Phytases

These are another group of enzymes which significantly release phosphorous from phytate degradation. In its general form, phytate is a primary source of inositol and the major stored form of P in plant seeds and pollen, thus contributing major component of organic phosphorus in soil (Richardson 1994; Sharma et al. 2013). Although the ability of plants to obtain phosphorous directly from phytate is very limited, yet the growth and P-nutrition get significantly improved when the plant is genetically transformed with phytase gene (phyA) obtained from *Aspergillus niger* (Richardson et al. 2001; Sharma et al. 2013). This led to an increase in P-nutrition to such an extent that the growth and P-content of the plant were equivalent to control plants supplied with inorganic phosphate. Hence, microorganisms play a key role in regulating the mineralisation of phytate in soil and their presence within the rhizosphere may compensate for a plant's inability to otherwise acquire phosphorous directly from phytate (Richardson and Simpson 2011; Sharma et al. 2013).

13.7.2.3 Phosphonatases

Phosphonatases and C–P lyases cleave the C–P bond of organophosphonates, thus releasing the available phosphorus (Rodriguez et al. 2006). It is therefore clear that different mechanisms are considerable for phosphate solubilisation by PSM's due to considerable variation amongst the organisms. Therefore, it is difficult to pinpoint a single mechanism, but, however, production of organic acids and consequent acidification (lowering of pH) appears to be of great importance for solubilisation of phosphorous. Thus, phosphate solubilisation by PSM's has been a major subject of analysis and research for a long time as the research is still in an infancy (Sharma et al. 2013).

13.8 Role of Siderophore in Phosphate Solubilisation

Siderophores (iron carrier) are small, chelating compounds secreted by microorganisms that have high affinity for iron under low iron stress conditions. Siderophores are amongst the strongest soluble ferric (Fe^{3+}) binding agents known. At present, approximately 500 siderophores are known which are being produced and used by microorganisms and plants (Crowley 2007, Sharma et al. 2013). Siderophore production has been demonstrated to be released by various PSM's but it has not been widely implicated as a phosphate solubilisation mechanism (Vassilev et al. 2006; Caballero-Mellado et al. 2007; Hamdali et al. 2008a). Considering the dominancy in mineral dissolution by organic acid anions over ligand exchange as a P-solubilising mechanism (Parker et al. 2005), the potential role of siderophores in enhancing P availability should be obvious (Sharma et al. 2013).

13.9 Role of EPS in Phosphate Solubilisation

Exopolysaccharides (EPS) are mainly polymers of carbohydrates which are excreted by microorganisms (bacteria and fungi) onto the outside of their cell walls. The composition and structures of EPS are varied. The nature of EPS may be homo- or heteropolysaccharides; in addition they may also contain different organic and inorganic substituent (Sutherland 2001). Yi et al. (2008) reported that polysaccharides excreted by microorganisms play a key role in the solubilisation of phosphate. In their study, four bacterial strains were reported i.e. *Enterobacter* sp. (EnHy-401), *Enterobacter* sp. (EnHy-402), *Arthrobacter* sp. (ArHy-505) and *Azotobacter* sp. (AzHy-510) which possess the capability to solubilise TCP (tricalcium phosphate). These PSB produced a significant amount of EPS and thus possessed a strong ability for P-solubilisation. Therefore, further studies need to be done for understanding the relationship between EPS production and phosphate solubilisation (Sharma et al. 2013).

13.10 Plant Growth-Promoting Attributes of PSM's and Its Role in Crop Production

Besides making soluble phosphorous accessible to the plants, PSM's have been also known for plant growth promotion (Gaur and Ostwal 1972). Production of phytohormones, antibiotics, siderophores and other bacterial metabolites are the several attributes of PSM's which are helpful in growth promotion and yield stimulation of plants. Various PSM's have been shown to promote the growth of many crops. Mohammadi (2012) reported that by using PSM's, crop yield increases up to 70 %. Various field experiments also revealed that phosphate-solubilising bacteria (PSB)

not only improved the growth, yield and quality of crops but also drastically reduced the usage of chemical or organic fertilisers, thus protecting our environment from hazardous effects of chemical fertiliser (Yasmin and Bano 2011). Sharma et al. (2011) also demonstrated that the inoculation of PSB with other PGPRs could enhance the crop yield and reduce the usage of P fertiliser by 50 %. Generally, phosphate-solubilising Microorganisms (PSM's) are present in soil, but their concentrations are low enough to compete with other microbes present in the rhizosphere. Thus, the amount of phosphorous liberated by PSM's is not sufficient for substantial increment in plant growth. Therefore, inoculation of PSM's at a much higher concentration in soil is necessary for simultaneous increment in phosphorous uptake by the plant and crop yield.

Beneficial effects of the inoculation with PSM's to many crop plants including legume and cereals have been described by various authors (Tomar et al. 1996; Antoun et al. 1998; Pal 1998; Peix et al. 2001). Dry matter production, phosphorous uptake and phosphorous content were significantly enhanced by the application of PSM's in many legume plants even under temperate conditions, where low temperature can restrain the microbial growth (Singh et al. 2005; Chand and Singh 2006). 12–15 % increment occurs in plant yield by the use of PSM's and replacement of 25–28 % of phosphate fertilisers was observed in cereals, legumes, potatoes and other crops on the addition of rock phosphate and inoculation with PSM's (Arun 2007). PSB as inoculants avail other trace elements such as iron and zinc to the plant that enhance plant growth and also increase the efficacy of nitrogen fixation biologically (Ponmurugan and Gopi 2006). Growth and grain yield of various plants including maize and wheat have been improved by the use of PSB (Afzal et al. 2005; Yasin et al. 2012). PSM's also exerts positive effect on growth and biosynthesis of specific drugs from medicinal plants (Gupta et al. 2012).

Pseudomonas sp. is among the best PSM's which are known for its beneficial effect on plant. Evidences proved that *Pseudomonas* not only enhanced the nutrient availability and uptake in soybean crop but also increased the number and dry weight of nodules, yield components, grain yield (Yazdani et al. 2009). Phosphate-solubilising bacteria enhance the seedling length of *Cicer arietinum* (Sharma et al. 2007). Yield of sugarcane plant is also enhanced by 12.6 % after inoculation with PSB (Sundara et al. 2002). Another strain of *Pseudomonas putida* stimulates the root and shoot growth and increases radioactive-labelled phosphate (32P) uptake in canola plant (Lifshitz et al. 1987). *Bacillus* spp. (*Bacillus firmus*, *Bacillus polymyxa*, *Bacillus cereus*) also exhibit simultaneous increases in phosphorous uptake and crop yields (Gaur and Ostwal 1972; Datta et al. 1982; Fernández et al. 1984). Simultaneous growth promotion and increase in phosphorous uptake by plants as a result of phosphate-solubilising bacterial inoculations have been reported and postulated that the phosphate solubilisation effect of rhizobia and other mineral phosphate-solubilising microorganisms seems to be the most important mechanism of plant growth promotion in moderately fertile and very fertile soils (Chabot et al. 1996a). Inoculation with two strains of *Rhizobium leguminosarum* selected for their P-solubilisation ability has been shown to improve root colonisation and growth pro-

motion and to increase significantly the phosphorous concentration in lettuce and maize (Chabot et al. 1996a, b; Rodríguez and Fraga 1999). Inoculation of rice seeds with *Azospirillum lipoferum* strain 34H increased the phosphate ion content and ultimately resulted in significant improvement of root length and fresh and dry shoot weights (Murty and Ladha 1988). *Penicillium* sp., *Mucor* sp. and *Aspergillus* sp. have been shown to increase plant growth by 5–20 % after inoculation in agricultural soils (Gunes et al. 2009).

Mixed cultures or co-inoculation with other microorganisms is also an alternative approach for using the phosphate-solubilising bacteria as microbial inoculants. Combined inoculation of phosphate-solubilising bacteria and *Azotobacter* exhibited beneficial effect on yield, as well as on nitrogen (N) and phosphorous storage in different crops (Kundu and Gaur 1984; Monib et al. 1984). Another study investigated that co-inoculation of *Pseudomonas striata* and *Bacillus polymyxa* strains, with a strain of *Azospirillum brasilense*, resulted in a significant improvement of grain and dry matter yields, with a concomitant increase in N and P uptake, compared with separate inoculations with each strain (Alagawadi and Gaur 1992). When phosphate-solubilising strain *Agrobacterium radiobacter* was combined with nitrogen-fixing strain *Azospirillum lipoferum*, it further produced improved grain yield of barley against the single inoculations in vitro as well as in vivo condition (Belimov et al. 1995). These studies suggested that mixed inoculants provided more balanced nutrition for the plants than single inoculation by improving the N and P uptake by the plant.

On the other hand, it has been postulated that some phosphate-solubilising bacteria exhibit synergistic relationship with mycorrhiza (Garbaye 1994; Frey-Klett et al. 1997; Rodríguez and Fraga 1999). Studies in this regard have shown that phosphate-solubilising bacteria interact with vesicular arbuscular mycorrhizae (VAM) for better exploitation of poorly soluble phosphate sources released by PSB (Ray et al. 1981; Azcón-Aguilar et al. 1986; Piccini and Azcón 1987, Rodríguez and Fraga 1999). Mycorrhizae assist in uptake of phosphorous solubilised by PSB by making a bridge between roots and surrounding soil that allows nutrient movement from soil to plants (Jeffries and Barea 1994). Toro et al. (1997) demonstrated that phosphate-solubilising bacteria associated with VAM improved mineral (N and P) accumulation in plant tissues by using radioactive 32P labelling. Single bacteria inoculation increased the biological yield, but the maximum grain weight is achieved by the application of the same bacteria along with mycorrhizae (Mehrvarz et al. 2008). In barley, chlorophyll content of leaf is increased by application of mycorrhiza along with *Pseudomonas putida* (Bartholdy et al. 2001). Combined inoculation of arbuscular mycorrhiza and PSB give better uptake of both endemic phosphorous from the soil and phosphorous coming from the phosphatic rock and enhance plant growth by solubilising phosphate from different fractions of soil (Ahmed et al. 2008). The inoculation of PSB (*Bacillus megaterium*) along with potential N-fixer (*Azotobacter* sp.) was found to induce resistance/tolerance against harmful effects of salinity (ranging from 3000 to 9000 ppm) besides significantly improving growth and yield attributing parameters in wheat (Xuan et al. 2011; Krishnaraj and Dahale 2014).

Commercially, few biofertilisers of mixed bacterial cultures have been developed which are claimed to undergo phosphate solubilisation and also increase the yield of crop. Phylazonit-M (permission at No. 9961, 1992, by the Ministry of Agriculture of Hungary) is a commercially available biofertiliser containing *Bacillus megaterium* and *Azotobacter chroococcum* strain which allows an increase in N and P supply to the plant. KYUSEI EM (EM Technologies, Inc.) is another product containing mixed inoculums of lactic acid bacteria, the lactic acid being the agent for mineral phosphate solubilisation. These evidences support the fact that phosphate-solubilising bacteria play a specific role in phosphate solubilisation and in the enhancement of plant growth. However, not all laboratory or field trials have offered positive results. For example, *Bacillus megaterium* var. *phosphoricum* when applied as inoculants in the former Soviet Union and India, successful result were obtained, but it failed to show the same efficiency in soils of the United States (Smith et al. 1962). Undoubtedly, the efficiency of the inoculation varies with the soil type, specific cultivar, and other parameters. The phosphorous content of the soil is probably one of the crucial factors in determining the effectiveness of the product (Rodríguez and Fraga 1999).

Thus, several investigations revealed that phosphate-solubilising bacteria could increase growth and yield in several crops (Krishnaraj and Dahale 2014) like walnut (Xuan et al. 2011), apple (Aslantas et al. 2007), maize (Hameeda et al. 2008), soybean (Fernandez et al. 2007), sugar beet (Şahin et al. 2004), chickpea (Akhtar and Siddiqui 2009; Verma et al. 2013), peanut (Taurian et al. 2010), rice, tomato (Charana 2012) and wheat (Shah et al. 2001).

13.11 Genetic Engineering of Phosphate –Solubilising Microorganisms

Although, knowledge of the genetics of phosphate solubilisation continues to be scanty, and the studies at the molecular stage to understand how precisely the PSM's brings out the solubilisation of insoluble phosphorous are inconclusive (Rodriguez et al. 2006; Sharma et al. 2013). But however, some genes concerned with mineral and organic phosphate solubilisation have been isolated and characterised (Sharma et al. 2013). Preliminary achievements in the manipulation of these genes through genetic engineering and molecular biotechnology accompanied via their expression in selected rhizobacterial strains open a promising angle for obtaining PSM's strains with improved phosphate-solubilising ability and therefore a greater powerful use of these microbes as agricultural inoculants (Sharma et al. 2013). In this manner Goldstein and Liu (1987) were the first to achieve cloning of phosphate solubilisation gene from the Gram-negative bacteria *Erwinia herbicola*. In addition, the napA phosphatase gene from the soil bacterium *Morganella morganii* was transferred into *Burkholderia cepacia* IS-16, a strain used as a biofertiliser, using the broad-host range vector pRK293 (Fraga et al. 2001; Sharma et al. 2013). Extracellular

phosphatase activity is enhanced in the recombinant strain. Introduction or overexpression of genes concerned in soil phosphate solubilisation (both organic and inorganic) in natural rhizosphere bacteria is a totally appealing technique for enhancing the capability of microorganisms to work as inoculants.

A better understanding of the genetic basis of the release of organic acids could pave the way for transferring the mineral phosphate-solubilising (mps) ability to various bacteria that are competent of colonising a particular rhizosphere (Khan et al. 2007). In organic acid production mechanism, gluconic acid (GA) accounts for the real system of phosphate solubilisation by Gram-negative microscopic organisms (Goldstein et al. 1993; Kim et al. 1998). Gluconic acid is produced by the oxidative digestion system of glucose by glucose dehydrogenase (GDH), which requires pyrroloquinoline quinine (PQQ) as a cofactor. Along these lines, the genes included in the biosynthesis/transport of PQQ can be cloned from different microorganisms and exchanged to other microorganisms (Babu-Khan et al. 1995). For example, the rhizosphere competent bacteria (RCB) e.g. Rhizobium have apo-GDH; it is fascinating to exchange the genes involved in PQQ biosynthesis to Rhizobium to improve the status of PSM's and make them successful bioinoculant (Khan et al. 2007). The subsequent *Rhizobium* strains will therefore have phosphate-solubilising (PS) action along with their original characteristic of nitrogen-fixing capacity. Another important rhizosphere competent bacterium (*Pseudomonas* sp.) can form gluconic acid through the oxidative glucose metabolism system and overexpression of PQQ biosynthesis and GDH genes could likewise improve it as a PSM's. The other methodology is to screen the mineral phosphate-solubilising (mps) gene directly in the target microorganisms by over/underexpression of genes, followed by the selection of transformants with mineral phosphate-solubilising capacity. Such a methodology has been utilised to acquire mineral phosphate-solubilising genes from *Synechocystis* PCC 6803 in *E. coli* (Gyaneshwar et al. 1998). However, there is a doubt that this will remain effective in other bacteria also (Khan et al. 2007). Genetic engineering could also help in increasing the survival of the inoculant strains by incorporating the abilities to utilise certain nutrients better than the rest of the microbial populations (Glick and Bashan 1997; Khan et al. 2007). Also, genes for utilisation of salicylate were transferred to a growth-promoting bacteria and the recombinant bacterium was able to survive and enhance plant growth better than the wild type (Colbert et al. 1993).

Thus, insertion of phosphate-solubilising genes into microorganisms (that don't have this functionality) may also avoid the current need of blending two populations of bacteria, when used as inoculants (nitrogen fixers and phosphate solubilisers) (Bashan et al. 2000; Sharma et al. 2013). There are several advantages of growing genetically transformed PSM's over transgenic plants for enhancing plant overall performance (Sharma et al. 2013) as given:

1. With current technologies, it is far easier to modify a bacterium than complex higher organisms.
2. Several plant growth-promoting traits can be combined in a single organism.

3. Instead of engineering crop by crop, a single, engineered inoculant may be used for several vegetations, especially when using a non-specific genus like *Azospirillum* (Rodriguez et al. 2006).

Dissimilarity of metabolic machinery and specific regulating mechanism between the donor and the recipient strains are some of the obstacles which must be first conquered to achieve a successful gene insertion. In spite of the diffculties, enormous development has been made in acquiring genetically engineered microorganisms for agricultural use (Armarger 2002). Overall, further studies on this aspect of PSM's will provide crucial information in future for better use of these PSM's in numerous environmental situations.

13.12 Conclusion and Future Prospects

An agricultural system is sustainable if it maintains and improves the health of ecosystem, human, animals and plants; as well it produces large amount of food to sustain the overgrowing population. Thus, issues related to population increase, land shrinkage, soil improvement, and food shortage are the major problems which are in critical stages and need to be solved. The high costs of chemical phosphatic fertilisers in general are not afforded by the poor farmers of the developing nations, thus restricting the growth of crop. Additionally, continuous uses of these chemical fertilisers are harnessing the health of our ecosystem. Microbiologists and researchers thus found the PSM's as an alternative biological means to solve these problems which are also cost-effective as well as eco-friendly. PSM's are an integral component of soil microbial community and play an important role in regulating the phosphorous cycle and also make the phosphorous available to the plants. PSM's not only make phosphorous available to the plants but also promote the growth of plants directly or indirectly. Despite their completely different ecological niches and multiple useful properties, P-solubilising microorganisms have yet to meet certain requirement in the field of agriculture. Current developments in our understanding of the functional diversity, rhizosphere colonising ability, mode of action, and judicious application are likely to facilitate their use as reliable component in the management of sustainable agricultural systems. Though important studies associated with PSM's and their role in sustainable agriculture have been taken up over the previous couple of decades, the specified technique remains in its infancy. The use of molecular techniques has enhanced our capacity to understand the workings of PSM's but still more research is to be carried out for the better performance of PSM's in the field and make them commercially available as bioinoculant. Genetic enhancement of PSM's would explore the various plant growth-promoting traits among microorganisms and also help to investigate the stability and their performance under different environmental condition.

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