Dhananjaya Pratap Singh
Harikesh Bahadur Singh
Ratna Prabha *Editors*

Microbial Inoculants in Sustainable Agricultural Productivity

Vol. 2: Functional Applications

Springer

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ISBN 978-81-322-2642-0 ISBN 978-81-322-2644-4 (eBook)
DOI 10.1007/978-81-322-2644-4

Library of Congress Control Number: 2015960842

Springer New Delhi Heidelberg New York Dordrecht London

Printed on acid-free paper

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

Foreword

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सचिव एवं महानिदेशक
Dr. S. AYYAPPAN
SECRETARY & DIRECTOR GENERAL

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Application of microbial inoculants has been realized as an alternative option due to their promising role in substantially reducing excessive use of chemical fertilizers, nutrients and pesticides. Their promising functions in plant growth promotion, protection against diseases and pests and soil fertility can be witnessed as biofertilizers (nitrogen-fixers, phosphate-solubilizers, siderophore producers etc.) that improve availability of minerals for plants and enhance uptake of nutrients (*Rhizobia*, *Azotobacter,* etc.) as phytostimulators to produce phytohormones and directly promote plant growth (*Azospirilium,* cyanobacterial strains etc.) and as biocontrol agents *(Trichoderma, Pseudomonas* and *Bacillus,* etc.) that protect plants against phytopathogenic organisms and enhance tolerance against abiotic stresses. There exist diverse group of microbes (bacteria and cyanobacteria, actinomycetes, methylotrophs, fungus, mycorrhizal fungi and endophytes) that have been developed as microbial inoculants with diverse functions at different levels and many have touched commercial production for applications at the field level to benefit farmers. Recently, plant growth promoting rhizobacteria (PGPRs) have gained attention for their indispensible role in sustainable agriculture. Moreover, selection of efficient strains with well defined mechanisms helped development of biofertilizer/biopesticide inoculants for achieving consistent and reproducible results under field conditions. PGPR-based biofertilizers can be used as effective alternatives to chemical fertilizers to reduce chemical use in the fields by many folds. Similarly, biocontrol agents that still represent a little portion of the total chemical pesticides use for controlling phytopathogens are again gaining prominent contribution as eco-friendly disease management alternatives with different safer mode of action than the chemical pesticides. Looking into the vast potential applicability of microbial inoculants, recent research is focused on identifying microbes or their consortia for their physiological and biochemical traits that make them prominent

biofertilizers or biocontrol agents, use of biorational screening processes to identify biocontrol/biofertilization processes, testing under semi-commercial and commercial production conditions, emphasis on consortium development of potential strains, finding out viable and long-lasting delivery modes. Newly emerging omic's based technologies are further adding towards a better understanding of the microbial functions at molecular level, molecular plant-microbe interactions and less-explored delivery mechanisms.

The editors of this volume, '*Microbial Inoculants in Sustainable Agricultural Productivity Vol. II – Functional Applications*' deserve appreciation for their efforts to compile diverse aspects of microbial functions and linking them with their potential applications for the benefit of crop production and soil health.

New Delhi
27th July 2015

(S. Ayyappan)

Preface

Over the past three decades, microbial biotechnology has benefited greatly from the extraordinary advances in molecular biology and nanotechnology. This has helped not only in the identification of microbial communities but their functional and metabolic diversity too and has resulted in the identification of potential microbial gene pool, proteins, or metabolites. There have been tremendous research advances in the study of plant-microbe (beneficial) and plant-pathogen interactions, pathogen recognition, induced systemic resistance and innate immunity mechanisms in plants, root rhizosphere biology, mechanisms of plant growth promotion and antagonism within microbial communities, and impact assessment of inoculated microbes on soil, plant, and other beneficial microorganisms to establish proof of concept behind microbial inoculation in soils or plants. Dynamic interactions between root exudates, microbial activity, genetic exchange, nutrient transformation, and gradient diffusion are the most likely factors shaping the belowground activities where microbial inoculants need to survive to produce beneficial impacts. Consequently, there remained an increasing demand to understand belowground functioning to effectively manage ecosystem and harness potential benefits. Manipulation of the rhizosphere with microbial inoculation is now being considered as a key mechanism for solving critical issues for agricultural sustainability, food quality management, mitigation of climate change, and conservation of biodiversity. Plants interact with groups of soil microbial communities at different trophic levels for alleviation of biotic and abiotic stresses which involve positive and negative feedbacks between soil microbes, plants, and their chemical environment. These issues have been worked out critically in different plant-microbe systems and led to a broad, yet clearer, understanding of the mechanisms of inoculation of plants with microbes. With this background, the demand for totally novel microbial products creates pressure on microbial biotechnologists to search for more potent and ecologically robust organisms and their specific interactive targets within the plants for developing potential microbial inoculants. Mass-scale inoculation comprises the supply of high-density viable and efficient microbial formulations in the field for a rapid colonization of the host rhizosphere. Prior to registration and commercialization of microbial inoculants, there remain a number of steps to consider. From laboratory to the industrial scale-up, this requires process scaling and mass production of the defined organism

under commercial fermentation conditions while maintaining quality, stability, and efficacy of the product.

The book *Microbial Inoculants in Sustainable Agricultural Productivity Vol. II Functional Applications* addresses the field usage of microbial inoculants (biofertilizers, biostimulants, biopesticides) that need several stages to undergo. Authors contributing to this volume have presented detailed account of mass production of microbial inoculants that involves scaling-up of production process of an efficient microbe from laboratory to industrial level, development of efficient production technology, quality control, commercial aspects, intellectual property rights involved, cross-boundary registration methods, biosafety and biosecurity concerns, and their legal sanctity. It also discusses formulation development that needs to consider factors such as base material, shelf life, compatibility with existing agricultural practices and materials (chemicals, other organisms), cost, and ease of applications. Biosafety and biosecurity considerations were also presented at length as per territorial guidelines to address such issues as nontarget effects on microbes and other organisms, toxigenicity, allergenicity and pathogenicity, persistence in the environment and potential for horizontal gene transfer, etc. Capitalization costs, techno-commercial issues, and potential markets were considered as key issues for making decisions to commercialize microbial inoculants. We are thankful to all the contributing authors for putting their efforts to complete this volume.

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Varanasi, Uttar Pradesh, India — Harikesh Bahadur Singh
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Harikesh Bahadur Singh is presently Professor and Head at the Department of Mycology and Plant Pathology in the Institute of Agricultural Sciences, Banaras Hindu University. He served the State Agriculture University, Central University, and CSIR Institute in teaching, research, and extension roles. His major research focus is on bioinoculants, biological control of plant pathogens, and nano-biotechnology. In recognition of Prof. Singh's scientific contributions and leadership in the field of plant pathology, he is honored with several prestigious awards, notable being CSIR Technology Prize for Biological Sciences; Vigyan Bharti Award; Prof. V.P. Bhide Memorial Award; Society for Plant Research, Scientist of Excellence Awards; BRSI Industrial Medal Award; Jyoti Sangam Award; Akshyavat Samman; Distinguish Scientist Award by the Society for Research Development in Agriculture; Prof. Panchanan Maheshwari Medal by the Indian Botanical Society; Rashtriya Gaurav Award by IIFS; Plant Pathology Leader Award by IPS; CSIR Award for S&T Innovation for Rural Development (CAIRD); Environment Conservation Award; and Vigyan Ratna by the UP Council of Science and Technology. Dr. Singh has been a fellow of the National Academy of Agricultural Sciences. Professor Singh has written two books, several training modules/manuals, and more than 275 publications and has more than 18 US patents and 3 PCTs to his credit.

Ratna Prabha obtained her Master's degree in Bioinformatics from Banasthali Vidyapeeth, Rajasthan, and Ph.D. in Biotechnology from Mewar University, India. She is presently associated with ICAR-National Bureau of Agriculturally Important Microorganisms, India in the Network Project on Agricultural Bioinformatics and Computational Biology of Indian Council of Agricultural Research. Dr. Prabha has been engaged in developing various bioinformatics tools, digital databases on plants and microbes and genomic and metagenomics data analysis and published many research papers in journals of international repute along with book chapters. Her current research interest lies in microbe-mediated stress management in plants, database development, comparative microbial genome analysis, phylogenomics, and pangenome analysis. She is also engaged in developing various online interactive demonstration tools/kits for researchers and students on bioinformatics and computational biology.

Soil Microbes: The Invisible Managers of Soil Fertility

1

Arumugam Sathya, Rajendran Vijayabharathi, and Subramaniam Gopalakrishnan

Abstract

Soil health is represented by its continuous capacity to function as a vital living system. Since soil health is the major driving factor for sustainable agriculture, it has to be preserved. Microorganisms are an essential and integral part of living soil influencing various biogeochemical cycles on major nutrients such as carbon, nitrogen, sulphur, phosphorous and other minerals and play superior role in maintaining soil health than other biological component of soil. They also have the capacity to suppress soil borne pathogens and indirectly help in agricultural productivity. Besides contribution of specific microbes to soil health by participating on nutrient cycles, certain other microbes directly/indirectly promote plant growth through the production of phytohormones, enzymes and by suppressing phytopathogens and insects. The vast functional and genetic diversity of microbial groups including bacteria, fungi and actinomycetes supports in all the above ways for soil health. This book chapter gives an outline of such microbes and their contribution in promoting soil health and its role as soil health indicators.

Keywords

Soil health • Microorganisms • N fixation • Nutrient cycling • Climate change

1.1 Introduction

Soil, a finite and non-renewable resource, supports numerous terrestrial life forms through its critical functions. Soil health is defined as 'the continued capacity of soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity,

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_1

promote the quality of air and water environments and maintain plant, animal and human health' (Doran and Safley 1997). In the context of sustainable agriculture, soil health is meant for crop productivity and protection via the functions such as N_2 fixation and phosphorus (P) solubilization, homeostasis of biogeochemical cycles, maintenance of soil structure, detoxification of pollutants and suppression of plant pathogens. In the absence/inefficiency of these functions, the soil is regarded as an inanimate entity with minerals and chemicals. In addition, soil regeneration through chemical and biological process/weathering of underlying rock requires geological time (Huber et al. 2001; Buscot and Varma 2005). So, maintenance of soil health is crucial for sustainable productivity.

The following 14 nutrients are vital for a proper plant growth and development – macronutrients, which are further divided into (1) structural nutrients: C, H, O; (2) primary nutrients: N, P, K and (3) secondary nutrients: S, Ca, Mg; and micronutrients: Fe, B, Cu, Cl, Mn, Mo, Zn, Ni. Besides the structural nutrients (which are obtained from air and water), the remaining 11 nutrients are obtained through soil and absorb only in some specific/available forms as follows: N – NH_4^+ (ammonium) and NO_3^- (nitrate); P – $H_2PO_4^-$ and HPO_4^{-2} (orthophosphate); K – K^+; S – SO_4^{-2} (sulphate); Ca – Ca^{+2}; Mg – Mg^{+2}; Fe – Fe^{+2} (ferrous) and Fe^{+3} (ferric); Zn – Zn^{+2}, Mn – Mn^{+2}; Mo – MoO_4^{-2} (molybdate); Cu – Cu^{+2}, Cl – Cl^-; B – H_3BO_3 (boric acid) and $H_2BO_3^-$ (borate). Though many of the soil flora and fauna are responsible for bringing these nutrients, microorganisms are the drivers behind various biogeochemical cycles and making the organic and inorganic nutrients in their available form to the plants (Lucas and Davis 1961; Mengel and Kirkby 2001).

Microbes are the largest population that exists in soil with a high diversity index. However, the microbial groups vary in their number *vs.* biomass. The number (number/g soil) and biomass (g/m^2) of various microbial groups are, bacteria: 10^8—10^9 *vs.* 40—500; actinomycetes: 10^7—10^8 *vs.* 40—500; fungi: 10^5—10^6 *vs.* 100—1,500; algae: 10^4—10^5 *vs.* 1—50; protozoa: 10^3—10^4 *vs.* varies (Hoorman and Islam 2010). Typical soil samples have about thousands of individual taxa (also known as operational taxonomic units, OTUs) of bacteria, archaea and fungi. It is understood from some estimates that there can be >106 individual species-level OTUs in a single soil sample (Fierer et al. 2007). During the analysis on genome size of microbial community among soil samples by re-association of community DNA, it is known that, the microbial community genome size equals the size of 6,000—10,000 *Escherichia coli* genomes in unperturbed organic soils, and 350—1,500 genomes in arable or heavy metal polluted soils. Still, the rare and unrecovered microorganisms may not be included in the analysis. In contrast, the genomic complexity recovered by culturing methods was less than 40 genomes. This complexity in microbial community genome size denotes the diversity in terms of genetic information present in the soil and also the overall functional variability (Torsvik et al. 1998; Øvreås 2000).

Among the microbial groups, fungus have higher tolerance and surviving capacity against fluctuating soil disturbances, untilled or no-till soils than bacteria and actinomycetes; though the latter groups also have the tolerance (Hoorman and Islam 2010; Meliani et al. 2012). Besides the smaller voluminous nature, soil microbes are the key drivers of biogeochemical cycles on major nutrients such as C, N, S, P and other mineral cycles (Bloem et al. 1997). They also suppress the soil pathogens via various antibiosis compounds and helps in plant disease protection (Haas and Défago 2005). This book chapter deals with role of microbes in improving soil fertility and also the available techniques for indicating soil microbial activity.

1.2 Carbon Cycle

Carbon (C) in the atmosphere is transferred to soil by photosynthetic plants and photo/chemo autotropic microorganisms for the synthesis of organic materials. Hence, the largest carbon pool on the earth's surface (2,157—2,293 Pg) is/becomes soil. The reverse process, i.e.,

decomposition of organic material built in plants and microbes was carried out by organic C utilizing heterotropic microorganisms as a substrate for their metabolism and energy source. The remaining C is liberated as metabolites or CO_2 to the atmosphere (Prentice et al. 2001). The decomposition product termed as soil organic carbon (SOC) is the largest pool within the terrestrial C cycle with an annual turnover of about 60 Gt (Schlesinger 1997). During the SOC formation, the organic materials were either mineralized to CO_2 or humified. Since the SOC affects plant growth by serving as energy source and by influencing nutrient availability through mineralization, it is one of the most important constituents of the soil.

It is understood that microbes transfer the C primarily for their survival. Under oxic conditions, i.e., in surface of soil and oxic layers of wetland systems, aerobic methane-oxidizing bacteria play the role (Chistoserdova et al. 2005; Gupta et al. 2013), whereas under waterlogged anoxic soils, CO_2 is reduced by hydrogenotropic archaea and methoanogenic bacteria (Lu and Conrad 2005; Trumbore 2006). Typically microbial C accounts for a minimum of 100─1,000 μg g^{-1} in arable soils and a maximum of 500─10,000 μg g^{-1} in forest soils with the intermittent values in other ecosystems such as grasslands and semi-arid regions (Kandeler et al. 2005). Besides the considerable variations, microbial biomass C generally accounts for about 0.9─6 % of total organic C with an indirect relationship for increasing soil depth.

Formation of soil organic matter (SOM), a major fraction containing SOC is aided by the decomposition process through various lytic enzymes including, amylase, glucosidase, proteases, cellulase, chitinase and phenol oxidase. These enzymes convert the complex macromolecules into low molecular weight compound for the ready assimilation of microbial components or for their transformation into CO_2 for energy (Burns and Dick 2002). Though the enzymes were released from plants/animals/microorganisms, the latter are major contributors (Tabatabai 1994). Among the microbial groups, fungi are reported to have higher enzyme activity than bacteria (Baldrian et al. 2010). Role of these lytic enzymes in maintaining soil health is previously reviewed by Das and Varma (2011) and hence a brief note on some essential enzymes is described here.

Amylase: Starch hydrolyzing enzyme breaks the complex polysaccharides and releases low molecular weight simple sugars which acts as an energy source for microbes (Rahmansyah and Sudiana 2010) and it is confirmed by the positive correlation between as enzyme activity and SOM (Kujur et al. 2012).

Cellulase: Cellulose in plant debris is degraded by a group of enzymes called cellulases into glucose, cellobiose and high molecular weight oligosaccharides. Soil fungus is the major contributors of this enzyme activity. Report of Arinze and Yubedee (2000) supports this by documenting negative correlation between increasing fungicide concentration in agricultural soils and cellulase activity. Previous studies by Vincent and Sisler (1968) and Atlas et al. (1978) also documented the same effects.

Chitinase: Chitin is a major component of fungal cell wall, exoskeleton of insects and many arthropods. As already quoted, the higher fungal biomass present in soils will be degraded by the chitinases after the cell death with the release of simple organic molecules. Besides contributing for nutrient cycling, it serves majorly for the control of soil borne fungal phytopathogens such as *Sclerotium rolfsii* and *Rhizoctonia solani*. This indirectly helps in increasing plant growth and yield (El-Tarabily et al. 2000; Sindhu and Dadarwal 2001).

Oxidase: In contrast to the hydrolytic enzymes, oxidases were produced for a variety of functions including ontogeny, defence and the acquisition of C and N by microorganisms (Sinsabaugh 2010). Representative of these enzymes include fungal laccases and prokaryotic laccase-like enzymes (Baldrian 2006; Hoegger et al. 2006).

Dehydrogenase: It is related during microbial respiration, where it oxidizes soil organic matter by transferring protons and electrons from substrates to acceptors and the activity

depends on soil type and soil air–water conditions (Wolińska and Stępniewska 2012; Kumar et al. 2013).

Sequential changes in climatic conditions and related ecosystem factors in the current situation affect all of the nutrient cycles. Hence, the research trend has been directed towards (1) effect on climate change including seasonal variations, elevated CO_2 and long-term climate change disturbances (Durán et al. 2014; Haugwitz et al. 2014); (2) effect of fertilizers (Strauss et al. 2014), soil amendments (Anderson et al. 2011) on long-term (Tyree et al. 2006) and short-term scales (Tyree et al. 2009) and (3) effect of SOM (Schmidt et al. 2011) etc. It is understood that, though the importance of soil microorganisms for global C cycling is well known; only few research attempts have been made to evaluate the chemical and microbiological views of C cycling (Kandeler et al. 2005).

1.3 Nitrogen Cycle

Nitrogen (N), an essential element for the synthesis of amino acids and nucleotides is required by all forms of life in large quantities. It is also involved in several respiratory energy metabolisms in which N compounds may serve as either oxidant or reductant. Atmosphere is the largest reservoir of N (78 %) in the form of triple bonded N_2 gas, though it is not freely available to most living organisms. It is accessible only by N_2 fixing bacteria and archaea which pave the way for other organisms to use the fixed N for its incorporation into their biomass. This fixed N constitutes less than 0.1 % of the N_2 pool and is able to limit the primary production in both terrestrial and marine ecosystems. Within the organisms, N exist in most reduced forms and during the cell lysis it is nitrified to nitrate which in turn denitrified to N_2 gas. So, a balanced N cycle requires the dual action of assimilatory (N fixation and incorporation into biomass) and dissimilatory (recycling of fixed nitrogen to N_2) transformations (Vitousek and Howarth 1991; Canfield et al. 2010).

The first step in N cycle, assimilation, i.e., N fixation (also known as biological nitrogen fixation, BNF) is aided by a group of bacteria called diazotrophs including cyanobacteria, green sulphur bacteria, Azotobacteraceae, rhizobia and *Frankia* at various ecosystems in which the former three occurs by/through non-symbiotic process and the latter two through symbiotic process. BNF occurs through a cascade of reactions involving complex enzymes systems and accounts for about 65 % of N currently used in agriculture (Thamdrup 2012; Peoples et al. 1995). Major quantity of N fixed under the control of legume–rhizobia is harvested as grains. The left out N in the soil, roots and shoot residues supports the succeeding crops for N supply. Hence legume–rhizobial symbiosis substantially reduces the N requirement from external sources (Bhattacharyya and Jha 2012). Crops like wheat, rice, sugarcane and woody species also have the capacity to fix atmospheric N using free living or associative diazotrophs. However, the contribution of legume–rhizobia symbiosis (13–360 kg N ha^{-1}) is far greater than the non-symbiotic systems (10–160 kg N ha^{-1}) (Bohlool et al. 1992). Review of Herridge et al. (2008) on global N_2 fixation estimated from FAO databases and other experimental reports also indicates the higher contribution of legume–rhizobia than other systems in N fixation (Table 1.1). However, N fixation efficiency of legumes depends on the host genotype, rhizobial efficiency, soil conditions, and climatic factors (Belnap 2003). Difference in N fixation efficiency of various legumes is shown in Table 1.2.

BNF is an energy demanding process through which atmospheric N is converted to plant usable organic N and plays an important role in the N cycle. This can be understood by the complexity of the enzyme nitrogenase, a major enzyme involved in the nitrogen fixation, which has two components – dinitrogenase reductase, the iron protein and dinitrogenase (metal cofactor). The iron protein provides the electrons with a high reducing power to dinitrogenase which in turn reduces N_2 to NH_3. Based on the availability of metal cofactor, three types of N fixing systems viz. Mo-nitrogenase, V-nitrogenase and

Table 1.1 Comparison of symbiotic and non-symbiotic N fixation in agricultural systems

Agent	Agricultural system	Area (Mha)	Crop N fixed (Tg/year)
Legume–rhizobia	Crop (pulse and oilseed) legumes	186	21
	Pasture and fodder legumes	110	12–25
Azolla-cyanobacteria	Rice	150	5
Endophytic, associative & free-living bacteria	Sugar cane	20	0.5
	Crop lands other than used for legumes and rice	800	<4
	Extensive, tropical savannas primarily used for grazing	1390	<14

Source: Herridge et al. (2008)

Table 1.2 Comparison data for N fixation efficiency of various legumes

Legume	Shoot N (Tg)a	Crop N (Tg)b	%Ndfa	Crop N fixed (Tg)c
Common bean	1.03	1.45	40	0.58
Cowpea	0.27	0.37	63	0.23
Chickpea	0.48	0.96	63	0.60
Pea	0.65	0.90	63	0.57
Lentil	0.24	0.33	63	0.21
Faba bean	0.27	0.38	75	0.29
Groundnut	2.16	3.03	68	2.06
Soybean	16.11	24.17	68	16.44

Source: Herridge et al. (2008). %Ndfa – Percentage of plant N derived from N_2 fixation
[a]Using %N shoots of 3.0 % for soybean, 2.3 % for groundnut, 2.2 % for fababean and 2.0 % for the remainder
[b]Multiplying shoot N by 2.0 (chickpea), 1.5 (soybean) and 1.4 (remainder) to account for below-ground N
[c]Crop N × %Ndfa

Fe-nitrogenase were documented. Complexity of nitrogen fixation can be further understood by participation of multiple gene clusters as follows: (1) nodulation (including nodA-acyltransferase, *nodB*-chitooligosaccharide deacetylase, *nodC*-N-acetylglucosaminyltransferase, *nodD*-transcriptional regulator of common nod genes, *nodPQ*, *nodX*, *nofEF*, *nodIJ*-Nod factors transport, *NOE*-synthesis of Nod factors substituents, *nol* genes-several functions in synthesis of Nod factors substituents and secretion); (2) nitrogen fixation (including *nifA, nifHDK*-nitrogenase, *fixLJ, nifBEN*-biosynthesis of the Fe-Mo cofactor, *fixK*-transcriptional regulator, *fixABCX*-electron transport chain to nitrogenase, *fixGHIS*-copper uptake and metabolism, fdxN-ferredoxin and *fix-NOPQ*-cytochrome oxidase) and (3) other essential elements (including *hup*-hydrogen uptake, *exo*-exopolyssacharide production, *gln*-glutamine synthase, *nfe*-nodulation efficiency and competitiveness, *dct*-dicarboxylate transport, *ndv-β*-1,2 glucan synthesis, *pls*-lipopolysaccharide production) (Laranjo et al. 2014).

It is a well-known fact that rhizobia belong to the families Rhizobiaceae (excluding the *Frankia* sp.), Bradirhizobiaceae and Phyllobacteriaceae. Rhizobia have a unique association with root nodules of leguminous plants and induce plant growth in many ways. They also have capacity to induce plant growth of non-leguminous plants (Mehboob et al. 2012). The number of species reported in Rhizobiaceae family increased considerably from 8 in 1980 to 53 in 2006. This drastic increase was mainly due to dispersion of leguminous plants to new geographical locations. The other possible reasons could be: (1) only 57 % of 650 genera of leguminous plants have been studied for nodulation and nitrogen fixation, and (2) recent advancements in the taxonomic research with the aid of specific molecular tools (Willems 2006). Besides its role in efficient N fixation, they have multiple plant growth promoting traits such as mineral enhancing capacity, phytohormone production and alleviating biotic and abiotic stress (Gopalakrishnan et al. 2014a). All these help in developing formulation of rhizobial inoculants to achieve substantial increases in legume nodulation, grain and biomass yield, nitrogen fixation and post-crop soil nitrate levels for succeeding crops (GRDC 2013). It is already reported that, inoculation of soybean with rhizobial inoculants showed substantial increases

in nodulation, grain and biomass yield and N fixation (Thuita et al. 2012).

Besides the rhizobia, the associative and free-living nitrogen fixing bacteria were also formulated and commercialized as biofertilizers. The genus *Azospirillum*, an associative N fixing bacteria comprises nearly 15 species: *A. lipoferum, A. brasilense, A. amazonense, A. halopraeferans, A. irakense, A. largimobile, A. dobereinerae, A. oryzae, A. melinis, A. canadense, A. zeae, A. rugosum, A. palatum, A. picis* and *A. thiophilum*. Reis et al. (2011) also reported for its multiple plant growth promoting traits. The next important genus is *Azotobacter*, a free-living nitrogen fixer which comprises of seven species: *A. chroococcum, A. vinelandii, A. beijerinckii, A. paspali, A. armeniacus, A. nigricans* and *A. salinestri* (Jiménez et al. 2011). Besides the N fixing capacity, this genus has the history of more than 35 years in promoting plant growth through multiple phytohormone production, enzymes, enhanced membrane activity, proliferation of the root system, enhanced water and mineral uptake, mobilization of minerals, mitigation of environmental stress factors, and direct and indirect biocontrol against numerous phytopathogens (Bashan and de-Bashan 2010).

The N fixed in the form of ammonium during assimilation process, is further dissimilated by two-step microbial process, i.e., nitrification (the aerobic oxidation of ammonium to nitrite and then to nitrate) and denitrification (the respiratory anaerobic reduction of nitrate via nitrite, nitric oxide, and nitrous oxide to N_2, coupled with the oxidation of organic matter, hydrogen, or reduced iron or sulphur species) (Simon 2002). Nitrification is further carried out by two sets of microbial groups: (1) ammonia oxidizers (nitrosifyers) which convert ammonia to nitrite by the activity of ammonia monooxygenase, e.g. *Nitrosomonas, Nitrosospira* and *Nitrosococcus*; and (2) nitrite oxidizers (the true nitrying bacteria) which convert nitrite to nitrate by the activity of nitrite oxidoreductase, e.g. *Nitrobacter* and *Nitrococcus* (Vaccari et al. 2006).

Though the physiology of nitrogen fixation process is reasonably well characterized, still research studies on the phylogenetic diversity of rhizobial species in the context of common core symbiotic genes (Masson-Boivinemail et al. 2009) and invasive mechanisms behind the symbiotic process (Kiers et al. 2003) are going on. However, the understanding of ecological controls on N fixation is sparse (Vitousek et al. 2002) and it is essential for developing a commercial microbial inoculants. Current research trend is looking over the effect of various environmental factors that limit N fixation, such as soil moisture deficiency, osmotic stress, extremes of temperature, soil salinity, soil acidity, alkalinity, nutrient deficiency, overdoses of fertilizers, pesticides and contaminants (Vance 2001; Galloway et al. 2004; Mohammadi et al. 2012; Peoples et al. 2012).

1.4 Sulphur Cycle

The sulphur (S) present in soil (>95 % of total S) is in the bound form with organic molecules, and it is not directly available to the plants, i.e., inorganic S which constitutes about only 5 %. This minimal part of available S in agricultural soils leads to S deficiency symptoms in plants (Schnug and Haneklaus 1998). Besides the contribution of plant and animal-derived organic S, *in situ* synthesis is also observed, which is mainly mediated by microbial process via immobilization of inorganic S to organic S, interconversion of various organic S forms, mineralization of inorganic sulphur in order to support plant growth. Rhizospheric microbes are the major players in allowing plants to access soil organosulphur (Kertesz 1999). Besides the mineralization and immobilization, oxidation and reduction reactions also influence S cycling. Oxidation of elemental S and inorganic S compounds to sulphate is carried out by chemoautotrophic (*Thiobacillus* sp., *T. ferrooxidans* and *T. thiooxidans*) and photosynthetic (Green and purple bacteria, *Chlorobium* and *Chromatium*.) bacteria. Besides this, heterotrophic bacteria such as *Bacillus, Pseudomonas*, and *Arthrobacter*, fungi such as *Aspergillus* and *Penicillium* and some actinomycetes are also reported to oxidize sulphur compounds. The process of sulphate/sulphuric acid formation has the following advantages: (i) it is the anion of strong mineral acid (H_2SO_4)

which can render alkali soils fit for cultivation by correcting soil pH; and (ii) solubilize inorganic salts containing plant nutrients and thereby increase the level of soluble P, K, Ca, Mg, etc. for plant nutrition. Dissimilatory sulphate reduction also occurs in order to balance the contents, where sulphate-reducing bacteria such as *Desulfovibrio, Desulfatomaculum* and *Desulfomonas* play the key roles through the enzyme activity of desulfurases/bisulphate reductase. Among them, *Desulfovibrio desulfuricans* can reduce sulphates at rapid rate in waterlogged/flooded soils, while *Desulfatomaculum* – a thermophilic obligate anaerobes – can reduce sulphates in dry land soils (Tang et al. 2009). Though many studies have been conducted to evaluate the role of microbes in S cycle, now the research focus has been moved in to deal with enzymes, organisms, pathways, comparative approaches, symbiosis, and environments factors related to the S nutrition (Klotz et al. 2011).

1.5 Phosphorous Cycle

Phosphorous (P) is a key component of nucleic acids, energy molecule ATP and membrane component phospholipids. P accounts for about 0.2—0.8 % of the plant dry weight, but only 0.1 % of this P is available for plants from soil (Zhou et al. 1992). The P content of agricultural soil solutions are typically in the range of 0.01—3.0 mg P L^{-1} representing a small portion of plant requirements. The remaining must be obtained through intervention of biotic and abiotic processes where the phosphate solubilizing activity of the microbes has a role to play (Sharma et al. 2013). Soil microbes help in P release to the plants that absorb only the soluble P like monobasic ($H_2PO_4^-$) and dibasic ($H_2PO_4^{2-}$) forms (Bhattacharyya and Jha 2012). Many bacteria (*Pseudomonas* and *Bacillus*) (Rodriguez and Fraga 1999), fungi (*Aspergillus, Penicillium* and *Trichoderma*) (Mittal et al. 2008) and actinomycetes (*Streptomyces* and *Nocardia)* (Tallapragada and Seshachala 2012) are noticed for P solubilizing capacity and enhancement of plant growth. This is aided by the synthesis of protons and organic acids, the significant contributors for solubilization of metal compounds though the excretion of other metabolites, siderophore also contribute to the solubilization process (Sayer et al. 1995). Low molecular organic acid – 2-ketogluconic acid – with a P-solubilizing ability has been identified in *R. leguminosarum* (Halder et al. 1990) and *R. meliloti* (Halder and Chakrabarty 1993). Mineralization of organic P takes place by several enzymes of microbial origin, such as acid phosphatases (Abd-Alla 1994), phosphohydrolases (Gügi et al. 1991), phytase (Glick 2012), phosphonoacetate hydrolase (McGrath et al. 1998), D-α-glycerophosphatase (Skrary and Cameron 1998) and C-P lyase (Ohtake et al. 1996). Other mineral elements also turn into their available form by any of the above mechanism.

1.6 Suppression of Soil Borne and Other Phytopathogens

Soil health is not only based on abundance and diversity of total soil microbes but also on high population of beneficial organisms. Incidence and severity of root diseases is an indirect assessment of soil health (Abawi and Widmer 2000). Certain rhizospheric microorganisms are known to have antagonistic activities against soil borne and other phytopathogens. This may be achieved by lytic enzymes cellulase, chitinase, protease and β-1, 3-glucanase which either induces direct suppression of plant pathogens or indirectly by enhancing the host plant resistance. Some oligosaccharides derived from fungal cell wall breakdown contribute to indirect mechanism (Pliego et al. 2011; Kilic-Ekici and Yuen 2003). Role of the genus *Pseudomonas* in disease suppression is reviewed by Haas and Défago (2005) in the context of antifungal antibiotic production, induction of systemic resistance in the host plant or interference on fungal pathogenicity factors. Mycorrhizal associations are one among them which are found in all ecological situations including normal cropping systems and in natural ecosystems. Among them arbuscular mycorrhizas (AM) are the most common (Harley and Smith 1983; Gianinazzi and Schüepp 1994), but

the excellency depends on its pre-establishment and extensive development on plant roots before the pathogen attack. Still, AM's broad-spectrum inhibition was noticed against pathogens such as *Aphanomyces, Chalara, Fusarium, Gaeumannomyces, Phytophthora, Pythium, Rhizoctonia, Sclerotium* and *Verticillium* (Azcón-Aguilar and Barea 1996). Another soil fungus *Trichoderma*, a well-known avirulent plant symbiont, characterized as biocontrol agent against broad range of phytopathogens works via competition, mycoparasitism, induced resistance, antibiotic and enzyme production. Beside this, it acts as plant growth promoting agents (Howell 2003; Harman et al. 2004). Others such as *Bacillus, Paenibacillus* and *Streptomyces* were also found to have inhibitory activity against soil borne and other phytopathogens (Cao et al. 2011; Köberl et al. 2013). A list of available commercial formulations of these microbes has been summarized by Junaid et al. (2013).

1.7 Indicators of Soil Health

It is understood from the literature that soil health is the result of continuous conservation and degradation processes in an ecosystem with the unique balance of chemical, physical and biological (including microbial) components. So, evaluation of soil health requires indicators of all these components. Since microbes quickly respond to changes in the soil ecosystem and vice versa, they are the excellent indicators of soil health. Changes in microbial populations or activity can precede detectable changes in soil physical and chemical properties, thereby providing an early sign of either soil improvement or an early warning of soil degradation (NERI 2002). The techniques were improved on the basis of the continuous identification and documentation of microbial processes. Some of the analytical and molecular techniques available are summarized in Table 1.3.

Table 1.3 Biological, physical and chemical indicators used for determining soil health

Indicator	Analytical techniques	Molecular techniques
Microbial biomass	Direct microscopic counts	Fluorescence microscopy
	Chloroform fumigation	Computerized image analysis
	SIR	Soil DNA estimation
	CO_2 production	FISH
	Microbial quotient	
	Fungal estimation	
	PLFA	
Microbial activity	Bacterial DNA synthesis	RNA measurements using RT-PCR
	Bacterial protein synthesis	
	CO_2 production	FISH
Carbon cycling	Soil respiration	SIP
	Metabolic quotient (qCO_2)	FISH
	Decomposition of organic matter	
	Soil enzyme activity	
Nitrogen cycling	N-mineralization	SIP
	Nitrification	FISH
	Denitrification	
	N-fixation	
Biodiversity and microbial resilience	Direct counts	–
	Selective isolation plating	
	Carbon and nitrogen utilization patterns	
	Extracellular enzyme patterns	
	PLFA	

(continued)

Table 1.3 (continued)

Indicator	Analytical techniques	Molecular techniques
Genetic and functional biodiversity	–	DGGE
		TGGE
		T-RFLP
		mRNA diversity using RT-PCR
		BIOLOGTM assay
Microbial resilience	–	Equitability (J) index
Bioavailability of contaminants	Plasmid-containing bacteria	RNA measurements
	Antibiotic-resistant bacteria	Geochemical indicators
Physical and chemical properties	Bulk density	–
	Soil physical observations and estimations	
	pH	
	EC	
	CEC	
	Aggregate stability and soil slaking	
	Water holding capacity	
	Water infiltration rate	
	Macro/micronutrient analysis	
SOM lipid analysis	–	PLFA(GC-MS)
SOM humic substances analysis	–	Non-destructive techniques:
		15 N-NMR, 13C NMR
		UV/Vis and IR spectroscopy
		Destructive techniques:
		Pyrolysis-GC-MS
		Chemolysis-GC-MS

Source: Arias et al. (2005)

1.8 Work at ICRISAT

Microbes play positive roles in plant growth promotion in addition to its direct or indirect participation in the nutrient cycles. These are called plant growth promoting (PGP) microbes which reside in rhizosphere/rhizoplane and promotes plant growth: (1) by using their own metabolism (solubilizing phosphates, producing hormones or fixing nitrogen) or directly affecting the plant metabolism (increasing the uptake of water and minerals), enhancing root development, increasing the enzymatic activity of the plant or helping other beneficial microorganisms to enhance their action on the plants; and (2) by suppressing plant pathogens (Pérez-Montano et al. 2014). Representative genera are *Bacillus, Pseudomonas, Trichoderma, Rhizobium, Bradyrhizobium, Sinorhizobium, Mesorhizobium* and *Streptomyces* (Vessey 2003). Many reviews were periodically available on these PGP microbes (Loon 2007; Bloemberg and Lugtenberg 2001; Saharan and Nehra 2011; Bhattacharyya and Jha 2012). So this book chapter just gives a glimpse on those and the related studies conducted by our research group.

ICRISAT has identified over 1,500 microbes including bacteria and actinomycetes, isolated from various composts and rhizospheric soil, in which at least, one out of six has documented either single or multiple agriculturally favourable traits. Our research group has a collection of 59 PGP bacteria and actinomycetes isolated from various herbal vermi-composts and organically cultivated fields with documented PGP traits *in vitro* and also at field conditions (Gopalakrishnan et al. 2014b). PGP bacteria such as *Pseudomonas plecoglossicida, P. monteilii, Brevibacterium*

antiquum, B. altitudinis, Enterobacter ludwigii and *Acinetobacter tandoii* isolated from rhizospheric soil of system of rice intensification (SRI) fields has documented *in vitro* PGP traits and also under field conditions on rice. Enhanced growth performance was observed via increased tiller numbers, panicle numbers, filled grain numbers and weight, stover yield, grain yield, total dry matter, root length, root volume and root dry weight (Gopalakrishnan et al. 2012). Similar type of enhanced growth performance on rice by actinomycetes such as *Streptomyces* sp., *S. caviscabies, S. globisporus subsp. caucasicus, S. griseorubens* is also recorded. In addition, upregulation of PGP genes such as indole acetic acid and siderophore producing genes were documented (Gopalakrishnan et al. 2014c). A PGP bacterium *Pseudomonas geniculata* IC-76 showed its capacity on chickpea under field conditions by enhanced plant growth performance and also agronomic performance via increased nodule number, nodule weight, pod number, pod weight, seed number and seed weight (Gopalakrishnan et al. 2014d).

Besides increasing plant growth, they significantly enhanced rhizospheric total nitrogen (8—82 %), available phosphorous (13—44 %) and organic carbon (17—39 %). Production of lytic enzymes such as cellulase, chitinase, lipase and protease by these microbes (Table 1.4) is an additional evidence for the enhanced soil organic carbon and nitrogen contents (Gopalakrishnan et al. 2014b, 2014c). Analysis of soil health microbial indicators recorded enhanced microbial biomass carbon (23—48 %), microbial biomass nitrogen (7—321 %) and dehydrogenase activity (14—278 %) on experimental plots over the uninoculated control during our field studies on crops such as rice (Gopalakrishnan et al. 2012; 2013; 2014c), chickpea (Gopalakrishnan et al. 2014d) and sorghum (unpublished results). Figures 1.1, 1.2, and 1.3 illustrate the combined results of our published reports on rhizospheric PGP microbes on increasing soil health during the field trials.

Apart from the PGP traits, they also have the capacity to act as biocontrol agents by suppressing soil pathogens, one of the keystone logic for healthy soil. Our PGP bacteria such as *P. plecoglossicida,*

Table 1.4 Extracellular enzyme profile identified for PGP bacteria and actinomycetes

Isolates	Cellulase	Chitinase	Lipase	Protease
PGP bacteria				
SRI-156	+	+	+	+
SRI-158	+	+	+	+
SRI-178	+	+	+	+
SRI-211	+	+	+	+
SRI-229	+	+	+	+
SRI-305	+	+	+	+
SRI-360	+	+	+	+
SBI-23	+	-	-	+
SBI-27	+	-	-	+
PGP actinomycetes				
KAI-26	+	+	+	+
KAI-27	+	+	+	+
KAI-32	+	+	+	+
KAI-90	+	+	+	+
KAI-180	+	+	+	+
SAI-13	+	+	-	+
SAI-25	+	+	+	+
SAI-29	+	+	-	+

Source: Gopalakrishnan et al. (2014b)

B. antiquum, B. altitudinis, E. ludwigii, A. tandoii and *P. monteilii*, and actinomycetes *Streptomyces* sp., *S. tsusimaensis, S. caviscabies, S. setonii* and *S. africanus* were found to have inhibitory activity against soil borne pathogens such as *Fusarium oxysporum* f. sp. *ciceri*, and *Macrophomina phaseolina* under greenhouse conditions. Antagonistic activity of these PGP actinomycetes on *Fusarium* wilt-sick fields has also been demonstrated (Gopalakrishnan et al. 2011a, b). These strains are already reported for lytic enzymes in the context of biocontrol such as chitinase and β-1,3 glucanse (Gopalakrishnan et al. 2014b).

1.9 Future Outlook

Microbes have multiple functions and features in influencing soil health and also in promoting plant growth and controlling diseases. Hence maintenance of beneficial microbial load will help in replacing inorganic fertilizer, pesticides and artificial plant growth regulators which have numerous side effects to sustainable agriculture. Beside this,

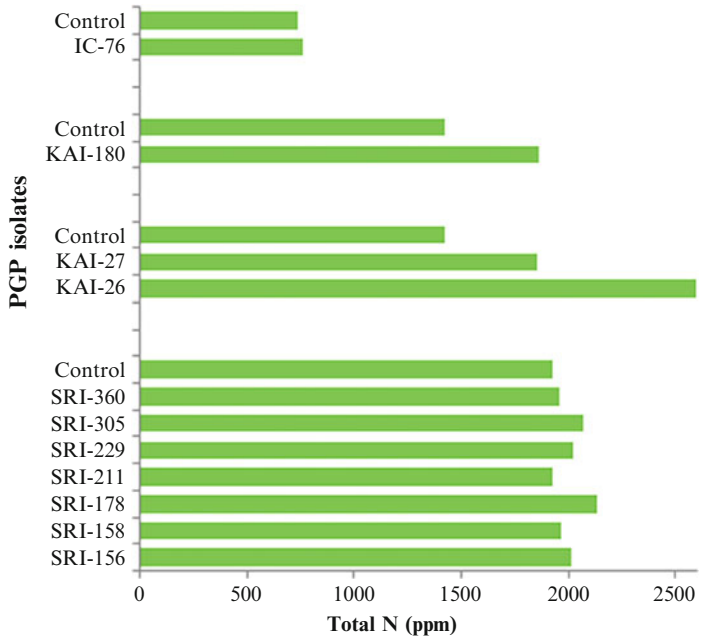

Fig. 1.1 Effect of PGP bacteria and actinomycetes on soil total N under field conditions of chickpea and rice cultivation (Note: Control indicates the treatment groups without any PGP bacterial inoculation, Gopalakrishnan et al. 2012, 2013, 2014c, d)

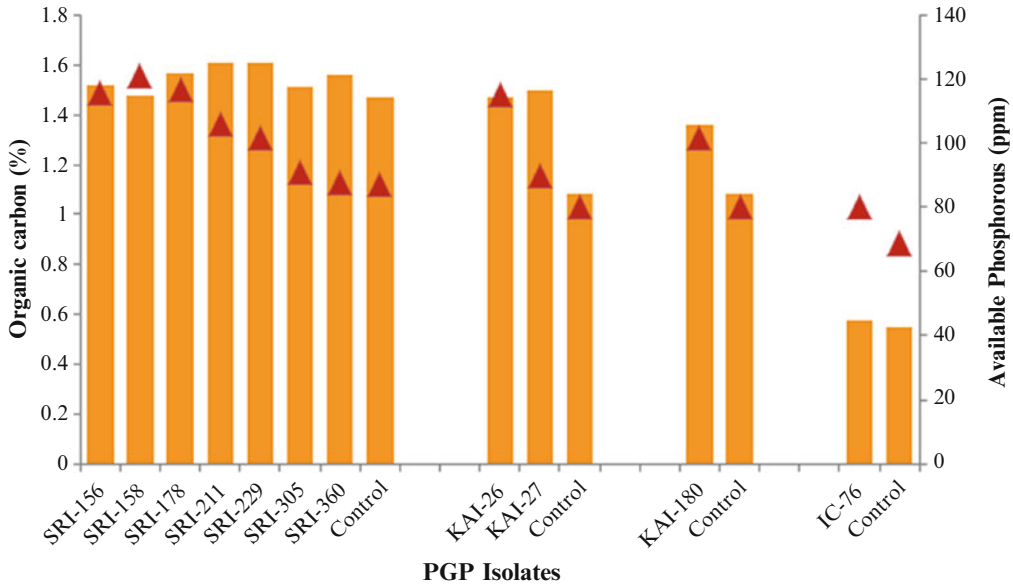

Fig. 1.2 Effect of PGP bacteria and actinomycetes on soil organic carbon and available phosphorous under field conditions of chickpea and rice cultivation
Solid bars (■) are the % organic carbon on the left axis and solid triangles (▲) are the available phosphorous (ppm) on right axis. Control indicates the treatment groups without any PGP bacterial inoculation (Gopalakrishnan et al. 2012, 2013, 2014c, d)

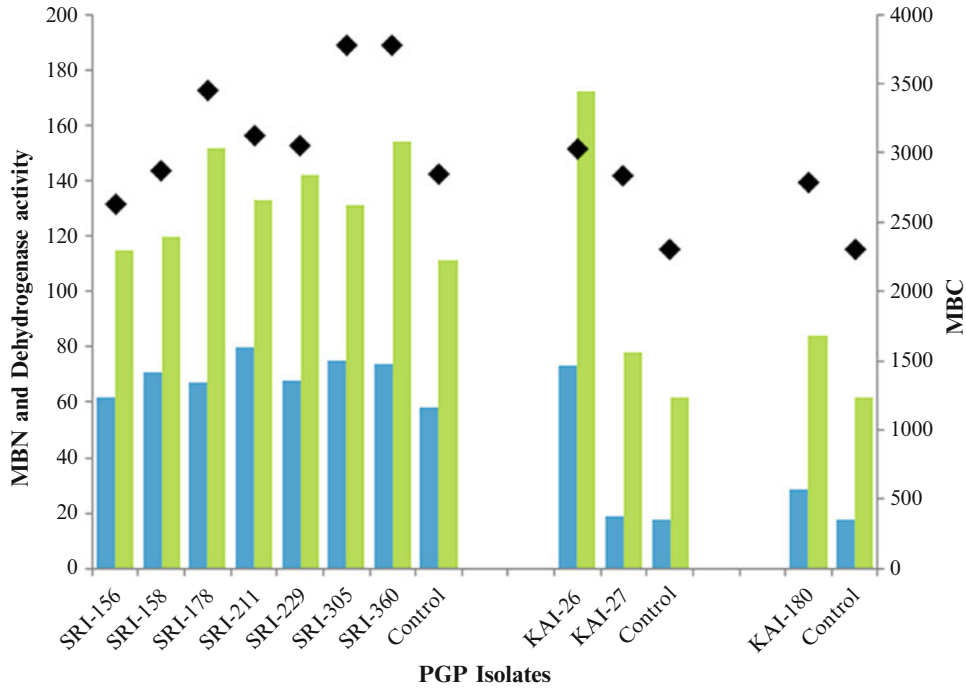

Fig. 1.3 Effect of PGP bacteria and actinomycetes on soil health indicators during field trials of rice cultivation Solid bars (■;■) are the microbial biomass nitrogen (μg g−1 soil) and dehydrogenase activity (μg TPF g−1 soil 24 h−) on left axis and solid diamond (◆) is the microbial biomass carbon (μg g−1 soil) on right axis. Control indicates the treatment groups without any PGP bacterial inoculation (Gopalakrishnan et al. 2012, 2013, 2014c)

understanding the responses of terrestrial ecosystems to global climatic changes and modern agricultural practices remains a major challenge, since soil has a mixed interaction with physical, chemical and biological component along with the influence of water, air/atmosphere, soil amendments etc. So research in each of this context individually and also in combination at various ecosystem levels is necessary.

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Efficacy of Biofertilizers: Challenges to Improve Crop Production

2

E. Malusà, F. Pinzari, and L. Canfora

Abstract

Different kinds of soil microorganisms belonging to several taxa of the bacteria, fungi, and possibly, protozoa kingdoms, colonizing the rhizosphere or the plant tissues and promoting plant growth (PGPM), can be utilized for the production of microbial-based fertilizers (biofertilizers). However, their application in agricultural practice is still hindered by several factors. The main reasons derive from the unpredictability of results, problems to identify and track inoculated strains in the field, the poor understanding of the interrelationships between microorganisms and plants, and the technology of production. After describing in brief which microorganisms have been utilized up until now to improve plant productivity through enhanced nutrition, we mention for possible exploitation of new groups of microorganisms (e.g. non-mycorrhizal fungi). Furthermore, we review the factors affecting the efficacy of biofertilizers on crop productivity, from the point of view of the farmers, who appraise their application on the base of their efficacy. In particular, we consider the factors related to the production process (including quality and marketing standards), the persistence and traceability of inoculants, the relations between plant, soil conditions and microorganisms, as well as the effect of farmers' practices (fertilization, soil management practices, application method). In conclusion, it emerges that biofertilizers could allow obtaining a crop productivity similar to that obtained with mineral fertilizers, but with a significant reduction of their use. Therefore, biofertilizers can play a key role to develop an integrated nutrient management system, sustaining agricultural productivity with low environmental impact.

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_2

Keywords
Biofertilizers • Crop production • Microorganisms • Mycorrhizal fungi • Inoculants • Farming practices

2.1 Introduction

Exploiting microbial-based fertilizers can be traced back to ancient times—already Classical Greek and Roman writings (namely Virgil's *Georgics* or Pliny the Elder's *Naturalis Historiae*) described agricultural practices for improving yield, that we can today link to microbiological activity (e.g. rotation with legumes or use of animal manure); the ancient Maya were managing water in the Mexico wetlands to support a complex mixture of algae, cyanobacteria, and other microorganisms also with the purpose of increasing the content of nutrients in the soil (Morrison and Cozatl-Manzano 2003). However, a conscious use of microorganisms for soil fertilization started in the late nineteenth century, when patenting and marketing of microorganisms for fertilization purposes began (Kilian et al. 2000; Nobbe and Hiltner 1896, cited in Bashan 1998). Since then, particularly in the last couple of decades, the development and use of microbial-based fertilizers has gained significance in the effort of reducing the negative environmental effects generated by the excessive and/or improper application of chemical fertilizers. However, despite the huge amount of studies and findings of beneficial strains, the application of microbial-based fertilizers in agricultural practice is still hindered by several factors. The main reasons derive from the unpredictability of results, problems to identify and track inoculated strains in the field, the poor understanding of the interrelationships between microorganisms and plants, and the technology of production (Bashan et al. 2014; Lucy et al. 2004; Owen et al. 2015). In this chapter, we aim to describe which factors we consider as mostly affecting a widespread exploitation of microbial fertilizers. Moreover, we want to foster actions by the different stakeholders interested in this sector that could promote a wider practical application of these products. We also briefly describe which microorganisms have been utilized up until now to improve plant productivity through enhanced nutrition, also providing information about new groups of microorganisms not widely exploited yet.

2.2 Microorganisms for Biofertilizers

Different kinds of soil microorganisms belonging to several taxa of the bacteria, fungi, and possibly, protozoa kingdoms, colonizing the rhizosphere or the plant tissues and promoting plant growth (PGPM), can be utilized for the production of biofertilizers (Lucy et al. 2004; Smith and Read 2008; Vessey 2003). Their contribution to plant nutrition can be limited to a single nutrient element, as in the case of N-fixing bacteria, or to a variety of elements, such as for arbuscular mycorrhizal fungi (AMF) (Bardi and Malusà 2012, and references therein). However, they can have a remarkable impact on the yield and quality of plants, increasing the nutrient uptake capacity and the use efficiency of applied chemical or organic fertilizers. Rhizobia, the best known N_2-fixing bacteria symbionts of legume plants, are able to provide up to 90 % of the N requirements of the host through atmospheric N_2 fixation (Franche et al. 2009), but they can also behave as plant growth promoting rhizobacteria (PGPR) with non-legumes such as maize, wheat, rice, and canola (Hayat et al. 2010; Yanni et al. 2001). Non-symbiotic free-living N-fixing bacteria species have been proved to enhance N uptake of plants (Bardi and Malusà 2012; Lucy et al. 2004; Okon and Labandera-Gonzalez 1994), which can derive nitrogen from biological nitrogen fixation in 7–58 % range in cereals (Baldani et al. 2000; Malik et al. 2002) and up to 60–80 % in sugarcane (Boddey et al. 1991). Cyanobacteria

(*Anabaena*, *Aulosira* and *Nostoc*), as free-living or in symbiosis with Azolla, a small free floating fresh water fern, were found to fix N and to release it for rice uptake in the range of 30–40 up to 70–110 kg N ha^{-1} (Wagner 1997). Arbuscular mycorrhizal fungi (AMF) may supply more than 50 % of plant N requirements (Govindarajulu et al. 2005; Leigh et al. 2009), which is particularly important under arid and semi-arid conditions, where water availability limits uptake of mobile nutrients such as inorganic N (Subramanian and Charest 1999). AM fungi can take up nitrogen both as inorganic (either ammonium or nitrate) and organic (Hawkins et al. 2000).

Arbuscular mycorrhizal fungi form the major group of microorganisms contributing to plant phosphorus (P) uptake (Smith and Read 2008) by increased exploitation of the soil (Cavagnaro et al. 2005), the solubilization of inorganic P forms (Tawaraya et al. 2006), and the hydrolization of organic P (Richardson et al. 2009). Several PGPR are very effective in solubilizing P from the highly insoluble tricalcium phosphate, hydroxyl apatite and rock phosphate (Rodríguez and Fraga 1999; Owen et al. 2015).

A wide array of bacterial genera (e.g. *Pseudomonas*, *Burkholderia*, *Acidothiobacillus*, *Bacillus* and *Paenibacillus*) are able to release potassium from minerals such as mica, illite, muscovite, biotite and orthoclases (Bennett et al. 1998, 2001; Liu et al. 2012), increasing K availability up to 15 % (Supanjani et al. 2006).

The search of new strains of microorganisms showing beneficial effects for plant nutrition has fostered studies on species that were less considered in the past. Following, we present an overview of results and potentialities which could derive from the introduction of non-mycorrhizal fungi into biofertilizers.

2.2.1 Potentialities of Non-Mycorrhizal Fungi as Inoculants for Biofertilizers

Fungi are ubiquitous in soil, and can be dominant components of the microbiota in many soil types (Gadd 2004; Burford and Gadd 2003). For example, fertile soil may contain a fungal network up to 10,000 km/m^2 (Burford et al. 2003). By adapting their metabolism to the availability of varying nutritive compounds in the soil environment, fungi produce a wide range of oxidative and hydrolytic enzymes that allow them to efficiently break down organic matter like ligno-cellulosic materials but also other natural or human-derived compounds, like in the field of xenobiotic and organic pollutant degradation (Harms et al. 2011).

The plasticity of fungi biology and the plethora of functions that can be attributed to fungal metabolism suggest that there are several potential uses and forms of exploitation of non-mycorrhizal fungi for the production of biofertilizers. The ability of some fungal groups or species in the dissolution or leaching of minerals and elements' chelation and translocation has been very little evaluated and even less exploited as a potential for the production of innovative soil amendments.

The biological activity of fungi can cause the enrichment of C, N, and S in the soil, making these as well as other nutrients available to plants. Moreover, fungi are capable of transporting substances in their hyphae that act as pipes connecting microenvironments with different concentration of nutrients and can actually transport ions against a chemical osmotic gradient (Banitz et al. 2011, 2013). Translocation across distant parts of the mycelium enables fungi to colonize places with low initial resource availability and to actively change the microenvironment and the availability of nutrients in the substrates, turning the colonizing mycelium from a resource sink into a source (Banitz et al. 2011, 2013). Jongmans et al. (1997) proposed that tunnels formed inside weatherable mineral grains were likely to have been formed by fungal hyphae and coined the term "rock-eating fungi" to describe such microscopic tunnels within feldspar and hornblende grains in the eluviated horizon of podzol soils. Within soils, a vertical distribution can be distinguished regarding fungal type in terms of their ecology (Pinzari et al. 2001). Organic layers are mostly colonized by

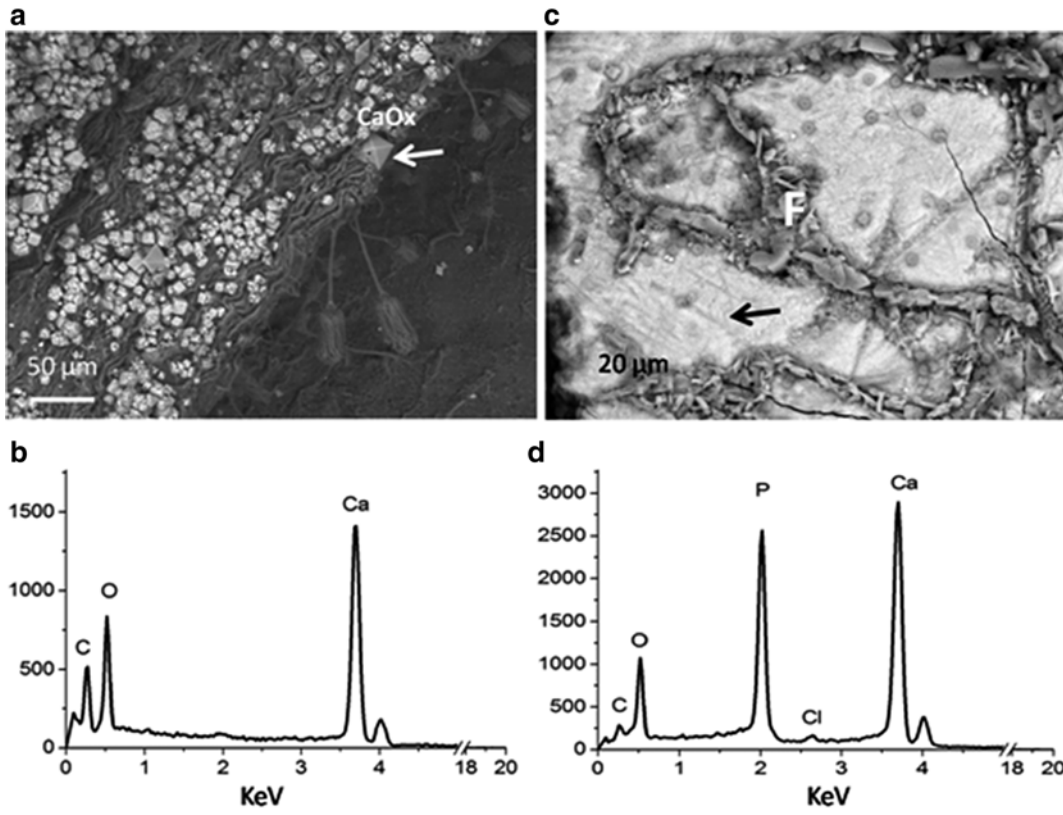

Fig. 2.1 Precipitation of calcium oxalates by filamentous fungi and solubilization of P-containing minerals observed with a Zeiss EVO 50 variable pressure scanning electron microscope (VP-SEM) operating at an accelerating voltage of 20 kV equipped with a detector for backscattered electrons (BSE) (Pinzari et al. 2012): (**a**) bipyramidal structures of calcium oxalate produced in vitro by an *Aspergillus* (*A.terreus*); (**b**) energy dispersive spectroscopy (EDS) spectra with chemical characterization of the crystals mainly containing Ca; (**c**) solubilization of apatite (P-containing minerals) by fungal hyphae (*Aspergillus niger*): tracks of dissolution of the mineral material are caused by fungal growth. In the tracks, around fungal threads other biogenic crystals are deposited. (**d**) X-ray area scan of the apatite that contains P and Ca. The y-axis on the spectra represents the EDS counts in arbitrary units

saprophytic fungi, whereas mineral layers are colonized by mycorrhizal fungi (Van Schöll et al. 2008).

Fungi can dissolve rocks and leach minerals by different mechanisms that involve the excretion of H+, or the production of primary and secondary metabolites with mineral solubilization or metal-chelating properties like siderophores, phenolic compounds, carboxylic acids, and amino acids. The potential of some fungal species in the breakdown of mineral phosphates could be very high, as shown in some recent papers (Pinzari et al. 2012) (Fig. 2.1). Fungi are more efficient than bacteria in P solubilisation, on both solid agar and in liquid cultures (Saxena et al. 2013). According to some authors, subculturing of most of the P-solubilizing bacteria results in the loss of the phosphate solubilizing activity (Halder et al. 1990) while fungi maintain their ability to leach P-containing rocks even after prolonged culturing (Kucey 1983). Such features could be important in the industrial manufacturing of biofertilizers for P nutrition.

P mobilization, particularly from Fe and Al phosphates, has been shown to be performed also by non-symbiotic fungi from different species of genera such as *Penicillium, Aspergillus*, *Trichoderma*, *Mucor*, *Candida*, *Discosia*, *Eupenicillium*, and *Gliocladium* (Ahmed and Shahab 2009; Jain et al. 2012; Saxena et al. 2013; Wakelin et al. 2007; Whitelaw 2000). The solubilizing ability of P minerals by the different organic acids produced by fungi also allows the mobilization of minerals other than phosphates (Achal et al. 2007; Ahmed and Shahab 2009; Asea et al. 1988). Esterase type enzymes released by fungi are known to be involved in liberating phosphorus from organic P compounds (Ahmed and Shahab 2009).

Fungal dissolution mechanisms can release also other cations like Si^{4+}, Fe^{3+}, Al^{3+}, and Ca^{2+} (Boberg et al. 2009). In general, fungi are strong solubilizing agents of K containing minerals such as feldspar, biotite, and phyllosilicates by organic acid release (Ahmed and Shahab 2009; Gadd 1999; Sayer et al. 1995; Singhal et al. 1994). *Piloderma* was able to extract potassium and/or magnesium from biotite, microcline, and chlorite to satisfy plant nutritional requirements (Glowa et al. 2003).

Fungal cells can also represent elective sites for biogenic mineral precipitation. This is the case of calcite or metal oxalates precipitation, which would also influence the availability of phosphates for plants that have been widely documented as coupled to fungal growth in near-surface limestones (calcretes), calcic and petrocalcic horizons in soils (Gadd 2007). Reduced forms of metals (such as Ca, Cd, Co, Cu, Va, Mn, Zn, Ag, Ni, and Pb) can be precipitated by many fungi within and around fungal cells (Gadd 2007). Mechanisms of fungal mineral weathering, translocation or bio-precipitation are still little known, but could represent useful tools especially in the perspective of using fungi in the formulation of biofertilizers aimed at improving soils' chemical and structural properties as well as plant nutrition (Table 2.1).

Table 2.1 Fungal properties that can be further explored for new biofertilizers

Fungal property	Potential applications for improving crop production	Examples or reference studies
Hyphae highly suited to growth across soil physical structure (surfaces, pores, and air gaps)	Fungi as highways or pipelines for nutrients translocation	Wick et al. (2010), Furuno et al. (2012), Banitz et al. (2011, 2013)
Ability to develop in patchy environments	Improvement of soil fertility and treatment of extreme heterogeneous soils (i.e. saline soils)	Green et al. (2008), Bashan and de-Bashan (2010)
Translocation and redistribution of biogenic elements	Improvement of soil fertility	Boberg et al. (2009)
Growth in low nutrient habitats	Widening the possibility of crop production in sites with low resource availability	Green et al. (2008)
Dissolve rocks, leach minerals, precipitation of calcium oxalate	P solubilization and availability	Sudhakara et al. (2002), Kucey (1983), Chuang et al. (2007)
	Si solubilization	Meena et al. (2014), Pradhan and Sukla (2005)
Chitin as elective sites for biogenic mineral precipitation	Ca insolubilization	Gadd (2000), Burford et al. (2003)
	Toxic metals precipitation	
Precipitation of reduced forms of metals (like Ca, Cd, Co, Cu, Va, Mn, Zn, Ag, Ni, and Pb) within and around fungal cells	The precipitation of metal oxalates may provide mechanisms that allow fungi tolerating high concentrations of toxic elements	Gadd (2007)
		Gadd (2008)
		Harms et al. (2011), Fomina et al. (2003, 2004, 2005), Daghino et al. (2010)
Degradation of organic compounds	Compost stabilization	Harms et al. (2011), Gadd (2008), Wick et al. (2010)
	Organic pollutants decomposition	

2.2.2 Fungal Inocula for Micronutrients Mobilization: The Case of Silicon

Lack of trace elements in soil is not uncommon (Bell and Dell 2008). However, the limitation of vital micronutrients can be attributed to some factors that reduce their availability for crops such as low organic matter content, high amounts of sand (soils with coarse textures), use of chemical fertilizers that change the equilibrium between soil fungi and bacteria as well as between the mineral substrates and microorganisms, or to other menaces that alter soil functions and fertility (compaction, desertification, etc.) (Brevik and Burgess 2012). Although these elements could be abundant in rocks, they are not always available to plants, as in the case of silicon (Si). Silicon is present in plants, and several studies have shown beneficial effects of silicon fertilization for agricultural crops (Belanger et al. 1995; Savant et al. 1997, 1999; Meena et al. 2014). The beneficial effects seem mainly associated with Si deposition in plant tissues, which enhances their rigidity and resistance to mechanical stress. This increased strength improves the light-receiving posture of the plant, benefiting photosynthesis, and enhances the resistance to biotic and abiotic stresses (Gascho 2001). Plants absorb silicon from the soil solution in the form of monosilicic acid, also called orthosilicic acid (H_4SiO_4) (Meena et al. 2014). Typical silicon absorbers and accumulator crops are rice, wheat, millet, and sugarcane, which require a relative large amount of silicon. However, inorganic materials such as quartz, clays, micas, and feldspars, although rich in Si, are poor sources because of their low solubility (Meena et al. 2014).

Fungi and bacteria can solubilize insoluble silicates (Wainwright et al. 1997; De Mico et al. 2004). Fungi, while degrading silica-based rocks, can release other mineral nutrients (e.g. potassium, iron and magnesium) (Daghino et al. 2010). The solubilization process occurs mainly via the production of organic and inorganic acids and complexing agents (Gadd 2008) and it is faster than that of bacteria (Castro et al. 2000; Gadd 2008; Daghino et al. 2010). The ability of *Aspergillus niger* in weathering olivine, serpentine, feldspar and other minerals, of *Penicillium simplicissimum* disgregating basalt, of *Penicillium expansum* and *Scopulariopsis brevicaulis* solubilizing alumino-silicates has been demonstrated (Daghino et al. 2010). The solubilization of silica by fungi (and bacteria) is considered as a source of supply for several crops such as cotton, maize, wheat, potato and tomatoes (Meena et al. 2014). Therefore, to fully exploit this capacity and benefit of Si nutrition by plants, further studies on application of bioinoculants for improving Si availability are needed.

From this general brief overview it appears that the availability of several beneficial strains from the different groups of microorganisms is not hindering the possibility of formulating an efficient biofertilizer.

2.3 Factors Affecting the Efficacy of Biofertilizers

The various mechanisms involved in plant promotion may be host plant-specific and strain-specific. Furthermore, once introduced into the soil, plant growth promoting microorganisms (PGPM) face competitive conditions that may severely reduce their beneficial effects (Bashan 1998). Therefore, the beneficial effects deriving from the application of a specific biofertilizer may differ greatly under different agro-environmental conditions and this has resulted in contesting the efficacy of microbial-based products (Cummings 2009; Owen et al. 2015). However, to overcome such perception and improve the propensity of farmers in using biofertilizers, it is useful to consider which factors affect the efficacy of biofertilizers on crop productivity trying to meet the point of view of the farmers, who appraise the application of a biofertilizer as for any other technical mean, on the base of its efficacy. For practical purposes, we have grouped the factors that could mainly be considered as those mostly affecting biofertilizers efficacy with relation to the plant, the soil, the farmers and the products themselves (Fig. 2.2).

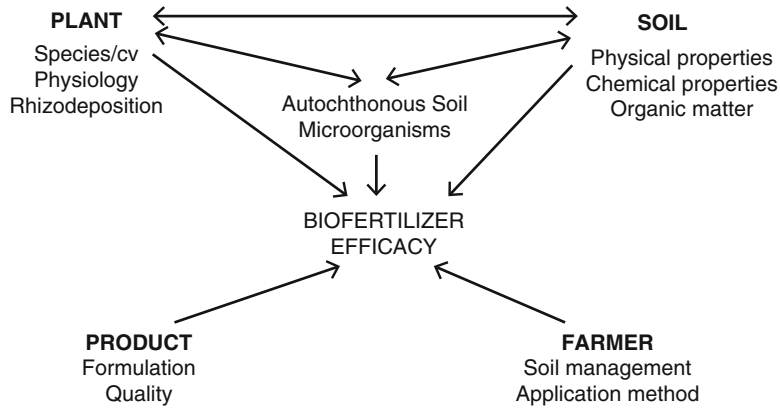

Fig. 2.2 Major factors affecting the efficacy of biofertilizers in improving crop nutrition, growth and yield

2.3.1 Factors Related to the Product

2.3.1.1 Production Process

The production process of the inoculum is key to a final high-quality product (Bashan et al. 2014), since there is a direct relationship between the population density of mother culture and the quality of the final products (Stephens and Rask 2000). Commonly, the inoculum is formed of one strain. However, the understanding of the complex relationships among the microorganisms interacting in the rhizosphere has fostered the study on inocula composed of more than one microorganism which have showed promising results both in legumes and non-legume plants. Successful examples, in case of legumes, comprised the co-inoculation of rhizobia with arbuscular mycorrhizal fungi (AMF) (Wang et al. 2011), dual inoculation of *Rhizobium* and phosphate solubilizing bacteria (Alagawadi and Gaur 1988), an inoculum formed of *Rhizobium* together with a plant growth promoting rhizobacteria (PGPR) and a phosphorous solubilizing bacteria (PSB) (Prasad and Chandra 2003). In non-legumes, nutrients uptake comparable to chemically fertilized plants have been reported with dual inoculations involving AMF and free-living N-fixing bacteria (Adesemoye et al. 2008; Barea et al. 2002; Lisette et al. 2003; Wang et al. 2011), also under dry conditions (Aseri et al. 2008). Consortia of AMF and different PGPR were beneficial for different annual and horticultural crops (Malusà et al. 2007; Wu et al. 2005), leading to a reduction of fertilizer application by up to 50 % (Singh and Adholeya 2003). Better nutrient efficacy was reported also in the case of PSB and KSB mixture inocula (Han and Lee 2005; Vassilev et al. 2006a).

When designing a consortium for a biofertilizer, it should be considered that certain bacterial groups appear to associate more frequently with AM fungi or to be inhibited by them by several mechanisms (Filion et al. 1999; Mansfeld-Giese et al. 2002; Sood 2003; Toljander et al. 2006; Vestergård et al. 2008; Wamberg et al. 2003), including the fungal release of stimulatory or inhibitory compounds (Johansson et al. 2004), which could result in a higher or limited colonization of the roots, respectively. Also the species specificity of the strains, even in case of AMF, or the differences in adaptation to environmental condition should be considered when selecting strains to formulate a biofertilizer (Antunes et al. 2011; Zoppellari et al. 2014; Malusà et al. 2012).

A PGPM consortium could be more efficient due to the different mechanisms of action of the various microorganisms present, sometimes overlapping also plant protection mechanisms (e.g. Vassilev et al. 2001, 2006b), which tend to match the requirements of both farmers, in using "multifunctional" products, and manufacturers, preferring to market a product for several purposes. A potential example of such kind of product could be that patented in the USA by Reddy and Janarthanam (2014) or already marketed in Europe under the brand name Micosat (CCS Aosta). However, not always the efficacy of consortia has proven to be consistent, and also their

production and commercialization raises some technical problems (Herridge 2008; Stephens and Rask 2000). This could derive from the observation that a key aspect determining the relationship of microorganisms with plants is not their taxonomic diversity, but rather their functional diversity (Nannipieri et al. 2003; Maherali and Klironomos 2007).

A sufficiently long shelf life of the inoculant (up to at least one season), maintaining its biological traits at an adequate level, is key for assuring the efficacy of the biofertilizer, though being a major challenge for any kind of formulated product (Bashan et al. 2014). Therefore, the formulation of the inocula, i.e. a multistep process which results in mixing one or more strains of microorganisms (inoculum) with a particular carrier, with or without additives (e.g. sticking agents or other additives), plays an important role in assuring the efficacy of the biofertilizer. It allows the protection of the cells during storage and transport, possibly enhancing the persistence of the inocula in soil, in order to obtain the maximal benefits after inoculation of the host plants (Manikandan et al. 2010; Schoebitz et al. 2012).

Different carriers can be used in the formulation process, and each of them presents specific positive qualities and drawbacks, affecting thus the overall quality and efficacy of the biofertilizers (Bashan et al. 2014; Herrmann and Lesueur 2013; Herridge 2008; Malusà et al. 2012). Nevertheless, granular inoculants are showing better results under harsh soil conditions (Clayton et al. 2004; Lupwayi et al. 2006; Rice et al. 2000). Liquid inoculants, though easier to distribute, have shorter shelf life (Bashan et al. 2014; Date 2001; Stephens and Rask 2000). Encapsulation into polymers, though theoretically allowing very diverse compositions and structures (Vassilev et al. 2005), has been mainly limited in practice to formulations based on alginate, which still presents some limitations for industrial production (Bashan et al. 2014; John et al. 2011).

Besides the various additives used to improve the shelf life of the product (Bashan et al. 2014; Malusà et al. 2012; Herrmann and Lesueur 2013), specific compounds can be introduced into the formulation to enhance the efficacy of biofertilizers. Legume biofertilizers containing elicitors of nodulation are already marketed (Mabood et al. 2006; Skorupska et al. 2010; Smith and Smith 2012), but other rhizobial metabolites related to the nodulation process (Nod factors) were successful in enhancing the performance of N-fixing bacteria inoculants on soybean and maize (Marks et al. 2013). Strigolactones, also in the form of synthetic analogous, could be used to foster the establishment of the mycorrhizal symbiosis (Ruyter-Spira et al. 2011; Xie et al. 2010).

2.3.1.2 Marketing of Biofertilizers and Quality Standards

The development of a new kind of products based on microorganisms is requiring a general agreement on the definition of the terminology or name used. Frequently, in the scientific literature the term ‘biofertilizer’ has been used to describe a simple microorganism showing plant growth promotion effects (Bardi and Malusà 2012, and references therein). However, as mentioned above, to be used within agronomical practices, any beneficial microorganism (inoculum) requires to be formulated to allow the effective delivery to the soil or plant. Along with the increased understanding of the mechanisms of action of the different kinds of beneficial microorganisms, the term biofertilizer has been defined in different ways (Okon and Labandera-Gonzalez 1994; Vessey 2003; Fuentes-Ramirez and Caballero-Mellado 2005), sometimes associating also a confusing terminology that does not take into consideration the legal definitions in place for other kinds of fertilizers or amendments (Owen et al. 2015). Recently, in an effort to propose a definition that could be useful also for regulatory purposes, Malusà and Vassilev (2014) have proposed to define biofertilizers, in analogy to the mineral or organic fertilizers, as “the formulated product containing one or more microorganisms that enhance the nutrient status (the growth and yield) of the plants by either replacing soil nutrients, and/or by making nutrients more available to plants and/or by increasing plant access to nutrients”. An agreed, legally binding, definition of these products, as well as the establishment of minimum legal standards for registration and marketing of biofertilizers are

also important to assure a minimum quality standard, which is another factor affecting the efficacy of biofertilizers' field performance. Indeed, it seems that the quality of biofertilizers has not been improving in the last few decades. Surveys carried out in the 1990s on products containing rhizobia showed a high level of contamination, with alien bacteria outnumbering the rhizobia in the great majority of products (Olsen et al. 1994, 1996). A similar situation emerged from a recent survey where 40 % of 65 tested commercial bacterial products (formulating also PSB and free-living N-fixing strains) did not contain the claimed strain, but only contaminants (Herrmann and Lesueur 2013). The situation does not appear more promising in case of AMF-based biofertilizers: surveys of products showed a very low quantity of viable propagules and reduced colonization potentials (Corkidi et al. 2004; Faye et al. 2013; Rowe et al. 2007; Tarbell and Koske 2007). Such frauds, together with insufficient label information, are probably the major reason for inconsistency of outcomes in field use of biofertilizers and are thus causing a lack of confidence in this kind of products which is affecting their market potential (Bhattacharyya and Jha 2012; Gemell et al. 2005; Husen et al. 2007). Marketing of biofertilizers should thus be regulated assuring a minimum quality standard of the final product (Herrmann and Lesueur 2013; Malusà and Vassilev 2014).

The distribution chain can also further affect the overall quality of biofertilizers. Indeed, several studies have demonstrated the decline of microbial populations in inoculants over time, particularly under non-optimal storage conditions, resulting in lower inoculation efficiency (Biederbeck and Geissler 1993; Maurice et al. 2001) and reduced quality (Hartley et al. 2005).

2.3.1.3 Persistence and Inoculant Traceability in Soil: Need for a Standard Method

The assessment of the persistence and traceability in soil of the strains applied with biofertilizers can be very difficult to investigate due to the complex web of microorganisms present in the soil and the rhizosphere, which can exceed hundred million units (Torsvik and Ovreas 2002), and the high variability of the microbial communities which reflects ecological, environmental and structural soil characteristics, as well as the large variety of agricultural management systems (see headings below). Therefore, no single qualitative and quantitative approach of traceability can capture the persistence of a bioinoculant in soil because of the variety of organisms marketed as biofertilizers. This raises questions about which methods should be considered suitable to monitor the persistence of the different inoculated strains. Such information is fundamental to evaluate the success of inoculation, thus helping to fine tune its application strategy. There is a perceived need for accurate and standard methods that can identify and trace the inoculants in soils.

During the past two decades, phenotypic and PCR-based methods have been developed to better characterize the structure, dynamics and diversity of soil microbial communities. The different methods address different questions, and therefore can all be suitable for the monitoring of the effects in soil due to the introduction of bioinoculants, and to give a picture of different aspects of the microbial community. For the detection of microorganisms released in the environment, molecular methods based on PCR techniques that use natural genome polymorphism have largely facilitated and allowed the discrimination at the strain level, of natural and introduced organisms, minimizing the costs and the time efforts (Öpik et al. 2010; Stockinger et al. 2010; Sýkorová et al. 2012). There are several molecular DNA fingerprinting methods that can be adopted to probe the inoculated strains, but they are mainly qualitative and not quantitative. Among the culture-independent methods available, commonly used to investigate soil microbial communities, traditional molecular fingerprinting, sequencing, or combination of different approaches can be used (Trabelsi and Mhamdi 2013; Schwieger and Tebbe 2000; Hirsch et al. 2010; Han et al. 2012).

The fingerprinting method, based on universal bacterial primers, was found not sufficient to discriminate between non-native and native micro-

organism when used singly (Pellegrino et al. 2012). However, combining a community level fingerprinting approach such as T-RFLP, with phylogenetic strain identification after a culture-dependent approach, proved to be a sound approach to highlight differences in community structure and at the same time to track inoculants (Pellegrino et al. 2012). To widen the understanding of the effect of the inoculant on the autochthonous microbial community, the real-time PCR with probes targeting the genes of interest, together with quantifying their copy number, can provide information on the relative abundance of the introduced strains within the microbial community; this approach could be used to follow the dynamics of the microbial community after the application of the biofertilizer (Babić et al. 2008).

The molecular marker-assisted approach, such as T-RFLP, DGGE, TGGE, can also be particularly useful for monitoring purposes. The combination of two culture-independent methods can allow assessing the persistence of microbial inoculants introduced in the soil, also evaluating at the same time, the possible changes occurring at species level for native strains. In this case, the community-level fingerprinting profile can be the preliminary method that allows to define the size of the clone library and the sequencing analysis. Nevertheless, in order to avoid inconsistent results due to the spatial heterogeneity of soil microbial populations, either horizontally or vertically, the soil sampling protocol shall follow a methodology that considers such variability. Successful examples of the application of such methodology can be found in some recent papers. Combining a community-level T-RFLP analysis, with phylogenetic strain identification by culture-dependent approach, made tracking the inoculants possible (Pellegrino et al. 2012). The tracing of an inoculated AMF isolate in the roots of target plants was carried out on the base of a nested PCR protocol (Sýkorová et al. 2012). Habteselassie et al. (2013) used, for the purpose of AMF tracing, the PCR amplification of a target gene followed by clone-assisted or direct sequences analysis. A PCR coupled with a novel combination of NS31 and Glomeromycota-specific LSUClom1 primers targeting the nuclear rDNA cistron, and classified amplicons by T-RFLP were designed to trace two inoculants of arbuscular mycorrhizal fungi discriminating them from native strains in roots up to two year post-inoculation (Pellegrino et al. 2012). Ceccarelli et al. (2010) used sequencing to better trace AMF applied strains showing that the marker-assisted fingerprinting analysis and the associated cloning and sequencing approach represents a multi-approach effective method for traceability of inoculants in soil.

2.3.2 Factors related to the plant

The plants can exert a significant effect on the strain(s) forming the biofertilizer and on their efficacy in promoting growth and yield, which are intimately related to the plant physiological status and phenological phase of growth. Indeed, depending on their nutritional status, plants can modify the release of compounds from the roots resulting in both quantitative and qualitative differences in rhizodeposits (Hartmann et al. 2009; Uren 2007), varying in time and space with respect to the position on the root (Dennis et al. 2010) and growth stage (van Overbeek and van Elsas 2008), which can lead to the selection of specific rhizosphere bacterial communities (Paterson 2003; Marschner et al. 2004; Marschner and Timonen 2005). Furthermore, root exudates contain compounds with stimulatory and inhibitory effect on rhizosphere microorganisms that affect their capacity of establishing beneficial relations with the plant (Hartmann et al. 2009; Bais et al. 2006). Under P-deficiency, plants release more chemical signals stimulating hyphal branching (Akiyama et al. 2005) and colonization (Akiyama et al. 2002) of AM fungi than under P-sufficient conditions. Plants can also influence the functions of soil microorganisms, such as nitrification (Smits et al. 2010). However, root exudates from a long-term monoculture of soybean had little effect on the nitrifier community, but reduced nitrification in the rhizosphere; in contrast, total AMF hyphal length was significantly stimulated by the increased release of

genistein (Wang et al. 2012), a phenylpropanoid compound probably involved in the chemical signaling leading to AMF root colonization (Cesco et al. 2010). Phenolic acids, also exuded by roots, are responsible for the shift in soil microbial communities (Qu and Wang 2008).

However, it has been suggested that rhizosphere microbial communities respond to other rhizosphere carbon pools (e.g. microbial exudates) for the majority of their coexistence with their plant host, thus limiting in reality the role of rhizodeposits in shaping the rhizosphere microbial community (Dennis et al. 2010), therefore also of the strains inoculated with the biofertilizer. Nonetheless, root exudates are likely to be of great importance in initiating the rhizosphere effect in very young seedlings and on emerging lateral roots. In this respect, the application of biofertilizers on seeds and seedlings would increase the efficacy of the treatment.

2.3.3 Factors Related to Soil Conditions

2.3.3.1 Abiotic Interactions

Soil chemical (pH, nutrient content) and physical (texture) characteristics have been found to shape both bacterial and fungal communities (Girvan et al. 2003; Fierer and Jackson 2006; Lauber et al. 2008; Marschner et al. 2004). Soil pH has been found to be the most important predictor of bacterial community structure at the ecosystem level, with higher diversity associated with neutral soils and lower diversity in acidic soils, likely due to the relatively narrow pH growth tolerance of bacterial taxa (Fierer and Jackson 2006; Rousk et al. 2010). The field surveys of AMF communities in a wide range of soil pH suggest that it is also the major driving force for structuring these communities (Dumbrell et al. 2010; Wang et al. 1993), thus affecting the colonization potential, and efficacy, of all kinds of PGPM included in biofertilizers. Adaptations of AMF to abiotic factors such as soil temperature and nutrient availability can strongly influence the effect of the AMF symbiosis on plant growth (Treseder and Allen 2002; Antunes et al. 2011).

2.3.3.2 Interaction with Autochthonous Soil Microorganisms

When introduced into the soil, the biofertilizer strain(s) face the competition from indigenous microorganisms. However, the knowledge of the ecological interactions among soil microorganisms and about the impact of those included into biofertilizers with the soil microbial populations are still limited and do not allow to effectively predict the effect of inoculants introduced with the biofertilizers. Nevertheless, there is a great effort in evaluating these interrelationships and their impact on biofertilizers efficacy, both on the short- and long-term, using methods such as the analysis of soil microbial biomass, soil microbial activity, soil microbial community structure and diversity (Trabelsi and Mhamdi 2013). It has been demonstrated that inoculation with products containing different PGPM (e.g. fluorescent pseudomonad, symbiotic and free-living N-fixing bacteria, AM fungi, etc.) affects in different ways various taxonomical or functional groups of autochthonous soil microorganisms. The application of inocula based on N-fixing bacteria was either increasing (Trabelsi et al. 2011) or strongly reducing the local bacterial community structure and diversity (Trabelsi et al. 2012), also when the inoculation was carried out with a consortia of strains (Naiman et al. 2009; de Salamone et al. 2010). A symbiotic N-fixing strain was shown to particularly affect a specific group of Proteobacteria (Robleto et al. 1998). Many studies have confirmed a high degree of specificity of the bacteria species associated with the AMF that was reflected on the increased presence of these species after inoculation with AMF (Albertsen et al. 2006; Olsson et al. 1996; Marschner and Timonen 2006). However, inoculation with AMF also significantly affected the general development of rhizospheric bacterial and fungal biomass (Linderman 1988). Once established successfully, introduced AMF showed to decrease the species richness of indigenous AM fungal communities in host roots (Koch et al. 2011).

The selection of strains expressing features that support the colonization process, and the "fight" for the roots' environment, is key to

assure the efficacy of any biofertilizer. In this respect, quorum sensing confers an enormous competitive advantage on bacteria, improving their chances to survive (e.g. through biofilm formation) and the ability to explore more complex niches (Gera and Srivastava 2006) even by 'swarming' (i.e. moving in the soil owing to motility – Fray 2002). Such characteristics are strongly related to the need of assuring a minimum population level of the initial PGPR inoculum to promote plant growth (Persello-Cartieaux et al. 2003).

The efficacy of the biofertilizers seems to be also mediated by protozoan grazing, particularly by naked amoeba, which is the most important bacterial grazer in soil (Bonkowski 2004). An increase of the bacterial and fungal feeding nematodes population was observed after application of a biofertilizer composed on both AMF and PGPR (Malusà et al. 2012). Wheat rhizosphere colonization by two *Pseudomonas* species and *Bacillus subtilis* was substantially reduced by three species of nematodes (*Caenorhabditis elegans*, *Acrobeloides thornei* and *Cruznema* sp.) (Knox et al. 2003).

However, it must be underlined that the observed relationships between indigenous and introduced microorganisms would depend largely on the techniques used to address the dynamics of soil microbial communities (Trabelsi and Mhamdi 2013). Indeed, although the number of microbial taxa could be clearly identified through novel metagenomic approaches combined with culture-dependent method, it is still very difficult to identify which functions are attributable to a specific microorganism or group, what are the metabolic potential of soil microbial communities in response to inoculation, and what is the link between the effects on soil microbial communities structure and the functional capabilities of soil microbial population. The study of genes coding for important enzymatic activities or key genes in the interaction process between the inoculant and native microbial population may contribute to gain such knowledge, which could unveil possible functions for the application of biofertilizers specifically designed for particular soil/crops.

2.3.4 Factors Related to Farmers' Practices

2.3.4.1 Fertilization

Fertilization is surely the agronomical practice that affects the efficacy of biofertilizers the most. The application of large quantities of mineral fertilizers has profound effects on soil microorganisms (Gosling et al. 2006; Johansson et al. 2004) and is thus expected to strongly affect the inoculated ones. Long-term application of mineral nitrogen has been shown to reduce soil microbial activity, with both quantitative and qualitative effects on soil bacterial and AMF communities which negatively impacted natural mycorrhizal colonization of roots (Mäder et al. 2002; Johnson et al. 2005; Hartmann and Widmer 2006; Oehl et al. 2004; Toljander et al. 2008). P-accumulation in the soils due to 10-year (Jensen and Jakobsen 1980) or 90-year application of P fertilizers (Cheng et al. 2013) or irrigation with wastewater (Ortega-Larrocea et al. 2001) decreased the spore density, colonization and communities of AM fungi. However, a lower level of differences was observed in sporulating AMF diversity despite 70 years of different soil fertilization regimes (Antunes et al. 2012). Duan et al. (2010) found low AMF colonization levels in maize, soybean, and wheat grown on fertilized soils. The kind of nitrogen fertilizer used can also impact on the AMF community: the occurrence of *Glomus intraradices*, a nitrophilic taxon (Jumpponen et al. 2005), among the most frequent taxa in arable soils (Hijri et al. 2006), was drastically reduced by ammonium nitrate while it was favored by calcium nitrate inputs (Toljander et al. 2008). However, in case of AMF, it has been suggested that the fertilizer rate might have a larger impact than fertilizer nature, mineral or organic, under some conditions (Beauregard et al. 2013).

Nevertheless, in terms of expected efficacy of AMF-based biofertilizers, it is important to consider that the overall fertility of the soil is supposed to regulate the kind of relation between the AMF and the plant. According to the trade balance model (Johnson 2010), parasitic, commensalism or mutualistic outcomes in the AMF symbiosis might be determined according to the

relative abundance or availability of N and P and their interaction with carbon supply and demand among plants and fungi. When N and P are available in sufficient amounts, then AM fungi are more likely to cause growth depression; on the other hand with sufficient N availability, but limited P, the plant benefits from the mutualistic symbiosis (Johnson 2010).

Organic fertilizers generally affect rhizosphere microorganisms positively, though this is not necessarily a favorable condition for inocula introduced with biofertilizers. Root colonization by AMF and development of AM fungal mycelia in soil can be stimulated by amendment of different organic substrates (Gryndler et al. 2005, 2006). Manure application can induce a general increase of bacteria and AMF richness (Esperschutz et al. 2007; Toljander et al. 2008), but can differently impact on specific groups of rhizosphere microorganisms such as denitrifying, aerobic N-fixing or sulfate reducing bacteria (Enwall et al. 2005). Compost treatments increased the frequency of Gram-positive bacteria to more than 80 % of total isolates and to a major frequency of rhizobacteria populations exhibiting PGPR characters (Viti et al. 2010). Application of two liquid organic fertilizers, derived from alternative sources of organic matter (a stillage and a vermicompost extract), with strikingly different composition and nature, differentially affected the size and biodiversity of rhizospheric Archaea and Eubacteria populations even after a short period of the plant growth, in contrast with common mineral fertilizers (Canfora et al. 2015). However, not all organic fertilizers can exert positive effects on AMF bioinoculants. Sewage sludge applications, for example, proved to reduce AMF richness and strongly altered the local bacterial community (Esperschutz et al. 2007; Toljander et al. 2008).

Considering that higher efficacy of colonization and activity of PGPM is expressed under low nutritional conditions, it is thus advised to reduce, but not to eliminate, the quantity of chemical fertilizers applied to favor the establishment of inoculated strain(s). A reduction by 20–50 % of chemical fertilizers has been proved feasible with several crops (Adesemoye et al. 2009; Jeffries et al. 2003). A medium level of N fertilization resulted in a higher N uptake from mycorrhizal plants with respect to high or low N fertilization rates (Azcón et al. 2008). In case of PGPR, when two strains of *Pseudomonas fluorescens* were tested on wheat in combination with varying levels of N, P, and K (at 0, 25, 50, 75, and 100 % of recommended doses), the efficacy was reduced with the increasing rates of NPK added to the soil and the maximum nutrient use efficiency was recorded with the 25 % of recommended NPK fertilizers dose (Shaharoona et al. 2008).

The use of biofertilizers can also allow utilizing different inorganic fertilizers, with lower nutrient availability, thus cheaper for farmers in comparison to synthetic fertilizers. For example, co-inoculation of PSB and KSB together with direct application of phosphate and potassium rocks, characterized by low solubility, increased yield and N, P and K uptake with different vegetable plants grown on soils deficient in P and K (Han and Lee 2005; Supanjani et al. 2006; Vassilev et al. 2006a).

2.3.4.2 Other Soil Management Practices

The structure of the soil microbiome is generally influenced by agricultural management practices (Bernard et al. 2012; Lumini et al. 2011; Reeve et al. 2010; Watt et al. 2006), with contrasting effects when comparing intensive and more environmental-friendly systems: the higher the management intensity (i.e. high inorganic fertilization, no rotation, deep tillage) the lower the microbial diversity (Franke-Snyder et al. 2001; Jansa et al. 2002, 2003; Oehl et al. 2003, 2004). Twenty years of organic management altered soil bacterial and fungal community structure compared to continuous conventional management with the bacterial differences caused primarily by a large increase in diversity (Berthrong et al. 2013). Practices, such as tillage, pest management, combined mineral and organic fertilization, and water regime can modify the efficacy of AMF inoculation (Lumini et al. 2011; Van Der Gast et al. 2011; Malusà et al. 2013; Alguacil et al. 2014). Regular disturbance by plowing in the arable soils strongly reduce AMF survival

(Helgason et al. 1998). Furthermore, some AMF taxa like *Acaulospora*, *Gigaspora*, *Paraglomus*, and *Scutellospora* appear to be more sensitive to some soil management practices (e.g. tillage) (Hijri et al. 2006; Maherali and Klironomos 2007) probably due to fewer intramycelial anastomoses (hyphal fusions) with respect to *Glomus* species (De La Providencia et al. 2005), a feature that could lead to using different species for biofertilizers adapted to specific crops. Several investigations on the diversity of AMF communities in conventional versus low input agricultural sites concluded that the status of nutrients and soil disturbance play a more influential role in homogenizing fungi diversity than any differences due to the employed farming systems (Franke-Snyder et al. 2001; Viti et al. 2010).

Finally, when considering the practice of substrate preparation for potted crops (e.g. in nursery), it was found that the characteristics of the peat used could differently affect root colonization by AMF (Linderman and Davis 2003; Ma et al. 2007).

2.3.4.3 Application Methods

Farmers need to better understand how microorganisms are acting in soil in order to learn the appropriate methods to perform a successful crop inoculation (Date 2001). The method of application can indeed affect the performance of the biofertilizer (Deaker et al. 2004). However, very little work has been done to assess and optimize the application of biofertilizers, in order to meet the farmers' requirement of using technologies already available in the farm or to verify how much the application method utilized can affect the viability of the distributed inocula (Bashan et al. 2014; Malusà et al. 2012). Among the few efforts in this regard can be mentioned by the development of machines to apply biofertilizers having different physical form (Malusà and Sas Paszt 2009).

The already available machines can be normally used to distribute biofertilizers, particularly granulated formulations. However, some machines have been developed for their distribution, by small adaptation of existing machines to be used in horticultural crops (Wawrzyńczak et al. 2011), or for specific purposes, e.g. to inject a slurry containing AMF to inoculate big trees (Symbiom© Inject System), which have shown to support a better performance of the biofertilizer (Hołownicki 2014). Application of inoculants by seed treatment or in furrows by mixing the inocula with soil or vermicompost provided comparable efficacy with regard to grain and straw yields in *Cicer* (Bhattacharjya and Chandra 2013). The application of liquid formulations with a normal sprayer based on hydraulic atomization system only slightly affected bacteria viability, but a prolonged working time reduced the amount of living cells up to 50 % (Świechowski et al. 2012). Effect of water volume and adjuvants were also affecting the amount of spores delivered and their efficacy in case of a fungus (Bailey et al. 2007). Foliar application can also be considered for PGPM application, particularly for endophytic species. Examples of growth and yield promotion using of such application method were demonstrated with several fruit species (Esitken et al. 2003; Pirlak et al. 2007; Sudhakar et al. 2000).

Since the recovery of the inoculated strains in the soil or on root rhizosphere was found to be limited to 30–40 days after inoculation in case of PGPR (Bashan et al. 1995), it would be more efficient to foresee repeated applications (2–4) during the growing season, with an interval of 3–4 weeks. This is not considered an additional drawback for biofertilizers in comparison to the inorganic ones, since normally, even for cereal crops, at least two fertilization treatments are performed, also to fulfill legal requirements or quality standards (e.g. Directive 676/91/CE concerning the protection of waters against pollution by nitrates).

2.4 Conclusion

The global market for biofertilizers was estimated to be worth about five billion USD in 2011 and is forecasted to double by 2017 (Marketsandmarkets 2013), actively in Latin America, India and China (Fuentes-Ramirez and Caballero-Mellado 2005; Bashan and de-Bashan

2010; Bashan et al. 2014). Improvement of quality standards of production and a clear legal framework that guarantees both manufacturers and farmers are needed to sustain such potential economic development.

Considering that, in general, 60–90 % of the total applied fertilizer is lost and only 30–50 % of applied N fertilizers and 10–45 % of P fertilizers are taken up by crops (Adesemoye et al. 2008, 2009), the application of biofertilizers can play a key role to develop an integrated nutrient management system, sustaining agricultural productivity with low environmental impact (Adesemoye et al. 2009; Adesemoye and Kloepper 2009; Malusà and Sas Paszt 2009). However, even though in some cases the application of biofertilizers resulted in an increased yield over respective un-inoculated controls in the presence of 100 % of recommended fertilizer doses, we believe that a valid target for this practice would be reaching the same crop productivity obtained without biofertilizers, but with a significant reduction of mineral fertilizers use. Biofertilizers have the potential to help reducing the buildup, leaching, or runoff of nutrients from fields when used in the framework of an integrated nutrient management system, participating in nutrient cycling and benefiting crop productivity (Singh et al. 2011). It has been demonstrated that such approach, combining in different ways, depending on the crops, the use of organic fertilizers and no or limited tillage, is promising and can support an economically and environmentally sustainable management of the crops (Adesemoye et al. 2009; Grzyb et al. 2012; 2013).

More impetus for a wider and effective use of biofertilizers can derive from recent knowledge on microorganisms and technological development. Use of strains cooperating with autochthonous microorganisms, such as endophytic bacteria (Reinhold-Hurek and Hurek 2011; Ryan et al. 2008), or exploiting the synergies with microbial communities (Bernard et al. 2012), as well as the inclusion of protozoa in the formulation of biofertilizers (Bonkowski 2004; Ronn et al. 2002) could also be key for the development of new kinds of biofertilizers. The observation that dimethyl sulfide, a volatile organic compound, is released by legumes to attract nematodes that transport rhizobia toward the roots is also supporting a wider use of the different microorganisms forming the soil web (Horiuchi et al. 2005).

New kinds of biofertilizers can benefit from the inclusion in the inoculum of yeasts, since isolates from genera such as *Williopsis*, *Saccharomyces*, *Candida*, *Meyerozyma* and *Pichia* have been shown to promote plant growth and nutrition with different crops (Amprayn et al. 2012; Agamy et al. 2013; El-Tarabily and Sivasithamparam 2006; Xiao et al. 2013) also with an integrated nutrient management (Nakayan et al. 2013).

The use of non-obligate endosymbiont mycorrhizal fungi, of the order Sebacinales, could ease the production process of this kind of biofertilizers, which have shown beneficial effect also in association with PGPR and with non-mycorrhizal species (Kumar et al. 2012).

New kind of additives could derive from biological substances that are involved in the colonization of roots such as the strigolactones synthetic analogs for the AMF–plant symbiosis (Ruyter-Spira et al. 2011), or that can support the root colonization by inoculated microorganisms such as vitamins (Palacios et al. 2014).

Biofertilizers could also be developed for in-vitro grown plant material leading to enhanced growth of seedlings, being more resistant to biotic and abiotic stresses (Sekar and Kandavel 2010), as well as for their quantitative and/or qualitative enhancement of plant secondary metabolites content in medicinal plants (Zubek et al. 2012).

From the data presented, it emerges that the several biotic, abiotic and anthropogenic factors pose challenges in successful application of commercial biofertilizer and are responsible for the efficacy of the biofertilizers as a field practice. Mathematical simulations showed that the most significant factors affecting PGPR survival, and thus the ability of providing beneficial effect to plants, were the competition with autochthonous bacteria, the compatibility with the exuded compounds by the plant host (rhizodeposition) and their ability to utilize them (Strigul and

Kravchenko 2006). On the other hand, there are several tools and actions which can be already utilized and implemented to improve the field efficacy of biofertilizers. The assurance of efficacy for a biofertilizer in a particular soil with a specific variety of crop is thus a complex task, which shall be considered by researchers, manufacturers, agricultural advisors and farmers when designing and applying a specific biofertilizer: a challenge to transform the fertilization with these products into a common practice for twenty-first century agriculture.

Acknowledgments The work has been supported by a grant to E.M. from the EU Regional Development Fund through the Polish Innovation Economy Operational Program, contract N. UDA-POIG.01.03.01-10-109/08-00.

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Revisiting Action of Bioinoculants: Their Impact on Rhizospheric Microbial Community Function

3

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Abstract

Bioinoculants have been known to promote plant growth and grain yield by more than one mechanism, though it has been difficult to pinpoint them. The contribution of the impact exerted by these live microorganisms on the resident rhizospheric microbial community functioning in enhancing plant growth cannot be ruled out. The chapter aims to critically evaluate the studies that throw light on such non-target effects of bioinoculants and to bring out the existing research gaps in the area. Also, markers and methodologies, which could be good indicators of soil functioning, have been highlighted for the benefit of workers probing into similar problems.

Keywords

Bioinoculants • Rhizosphere • Microbial communities

3.1 Introduction

The need of the hour in agriculture is to turn to environmentally sustainable means. With limited resources, increasing population, and growing concern over application of toxic, expensive chemicals, and their long-term effects, alternatives for safe means of enhancing crop productivity is a major challenge. Bioinoculants, which are live microorganisms with the potential to improve plant's growth and enhance crop's productivity, have proven to be an efficient and cost-effective method. Moreover, being live organisms that can divide, most of the time there is no need for repeated application.

The mechanisms of action of bioinoculants can be broadly classified under three categories: production of plant growth promoting substances, nutrient acquisition, and biocontrol. In fact, the traits do not function independently but in a synergistic fashion with multiple mechanisms working to result in the observed enhancement in plant growth (Bashan and Holguin 1997). In the last decade it has been established that the bioinoculants have more than just direct effects on the plants. The mode

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_3

of their interaction with resident microbiota in the rhizosphere of crop also plays a crucial role in the overall effect of plant growth promotion and yield enhancement. It is only recently that scientists have started understanding the significance of these interactions as possible mechanisms of plant growth promotion (Kang et al. 2013).

When applying microorganisms as bioinoculants, in numbers larger than what are normally present in the rhizosphere, the aspect of their survival and persistence is crucial, as they are to adjust to an already established microbial community. Together with facing competition from the resident members, there is bound to be disturbance, even if it is transitory, in the structure and function of rhizospheric microflora. Though they have been categorized as "safe" by virtue of having biological origin, their "non-target" effects on the dynamics of rhizospheric microbial communities have been a largely ignored aspect till recently. Such effects could be both positive (leading to enhanced nutritional status of the soil and improved grain yield) or negative (showing deleterious effect on beneficial microbes leading to declined condition of the soil for next season crop). Hence, their safety, in terms of their impact on microbially-mediated soil processes, is an issue that requires critical evaluation. A thorough understanding of the interactions of the introduced strain with the native rhizospheric community is also essential to ensure the survival and efficacy of the bioinoculants.

While the impact of bioinoculants on microbial community structure has been fairly well worked upon and reviewed (Castro-Sowinski et al. 2007; Sharma et al. 2012; Bajsa et al. 2013; Trabelsi and Mhamdi 2013), relatively less understood is the resulting impact on soil processes mediated by microorganisms in rhizosphere. In microbial ecology "structure" refers to the community composition, i.e. what is present, where and in what numbers. Microbial community "function", however, refers to the processes carried out by microbial community members.

3.2 Scope of the Chapter

In the chapter, we have discussed the impact exerted by bioinoculants on microbial community function at the level of system's potential (gene level), and on active microbiota (enzyme level) performing different soil functions. This is followed by an introduction to various other tools with potential to be employed in studying microbial community function in the context of non-target effects of bioinoculants. We conclude with a critical summary derived from reports till now, and the future perspective. The chapter excludes studies reporting impact of microbial bioinoculants on microbial community structure, and soil nutrient status. Also, it is outside the purview of the chapter to review the methodologies which have been applied for monitoring microbial community function in detail. However, an attempt has been made to present conventional as well as state-of-art tools that are being used / have the potential of application to address such questions.

3.3 Methodologies to Study Microbial Community Function

Study of microbial community dynamics has been conventionally performed using enumeration technique of culturing microorganisms on general and selective media. Its simplicity still makes it a popular tool. Community-level physiological profiling (CLPP) is an important tool based on the catabolic potential of active microorganisms thereby giving a clear picture of the microbial community function. It is an efficient technique to target the functional diversity of microorganisms with regard to the substrate utilized for metabolism. However, these techniques restrict to <1 % of the microbial members, which are culturable (Amann et al. 1995). Also, with the fast-growing organisms out-competing the slow growers, the technique leads to introduction of biases. Overall, using only cultivation-dependent tool for assessment of microbial community in the rhizosphere leads to an under-estimation of

the same. Molecular microbiology tools have made it possible to analyze the total microbial community structure and function, thereby overcoming the limitations involved in cultivation of microorganisms. By extracting markers like lipids, proteins and nucleic acids directly from rhizospheric samples, it has been possible to not only generate profiles, which provide an instant picture of the extent of changes in microbial communities, but also to identify key members. While studies at the level of DNA give information on the total "potential" of the system, the rather tedious studies at the level of RNA, especially mRNA, provides an insight into particular functions. The next in the flow of information is analysis performed at the level of enzymes. This has been the most common means of targeting soil functions. The measurement of enzymatic activities involved in cycling of important soil nutrients can be early and efficient indicators of changes in soil fertility (Ceccanti and García 1994).

3.4 Effects of Bioinoculants on Microbially Mediated Soil Processes at Gene Level

Nitrogen (N) is one of the most important nutrient elements in all living beings and serves as an important building block of proteins, nucleic acids (DNA and RNA) and other cellular components. Despite the fact that majority of the air we breathe is mainly composed of N_2 (78 % of the atmosphere), it is not available to plants due to their inability to metabolize it. Because of this, nitrogen is often a major limiting nutrient, and nitrogen cycle is a critical component of the biogeochemical cycles occurring in the environment. Thus, the free nitrogen in the atmosphere has to be fixed by microorganisms present in the soil into forms that can be metabolized by the plants.

Because of its great significance in ecology, nitrogen cycle is well studied and documented at the genetic level. Ample literature is available describing the gene sequences, primer details, PCR conditions and organisms involved at each step of the N cycle. However, limited studies have targeted the cycle to assess non-target effects of bioinoculants at gene level (Table 3.1).

The first extensive study employing markers for different steps in nitrogen turnover to analyze non-target effects of bioinoculants was conducted by Babić et al. (2008). After seed inoculation of alfalfa (*Medicago sativa* L.) with rhizobial bacterium, *Sinorhizobium meliloti*, and growth of the plants in pot under controlled conditions, the abundance of all genes involved in nitrogen cycle were found to be affected; *nif*H gene copy numbers were found to be positively affected in rhizosphere at late flowering stage when treated with the bioinoculant.

Gupta et al. (2012) reported more than four-fold increase in *nif*H gene copy number in *Cajanus cajan* rhizosphere with the application of a microbial consortium comprising *Bacillus megaterium*, *Pseudomonas fluorescens* and *Trichoderma harzianum* over un-inoculated rhizospheric soil in pot experiments. Since none of the inoculated strains carried *nif*H gene, the study clearly highlighted the non-target effect of the microbial consortium. It needs to be mentioned here that the three inoculants together (compared to single and dual inoculations) performed the best in terms of grain yield enhancement in *Cajanus cajan*. The markers employed to study the agriculturally undesired process of denitrification also displayed an increase in their gene copy numbers; however, the fold enhancement observed was much smaller compared to that for the beneficial process of nitrogen fixation.

The two studies described above were quantitative with no attempt made to evaluate the impact of bioinoculants on the diversity of microbial members involved in nitrogen cycle. When a qualitative study was performed to target the diversity of diazotrophs employing terminal restriction length polymorphism (tRFLP) in rice upon application of a commercial formulation with *Pseudomonas fluorescens* and *Azospirillum brasilense* under field conditions, no changes could be observed in the patterns as compared to control (García de Salamone et al. 2012). Contrasting results were obtained in the rhizosphere of soya bean upon binary application of

Table 3.1 Genes employed as marker to study the impact of bioinoculants on microbial community function

Plant system	Bioinoculants applied	Genes analyzed and methodology employed	Reference
Alfalfa (*Medicago sativa*)	*Sinorhizobium meliloti*	qPCR of archaeal *amoA*, bacterial *amoA*, *nirK*, *nirS*, *nosZ*, *nifH*	Babić et al. (2008)
Pigeon pea (*Cajanus cajan*)	*Pseudomonas fluorescens, Bacillus megaterium, Trichoderma harzianum*	qPCR of archaeal *amoA*, bacterial *amoA*, *nirK*, *nirS*, *narG*, *napA*, *nifH*	Gupta et al. (2012)
Rice (*Oryza sativa*)	*Azospirillum brasilense*, *Pseudomonas fluorescens*	*nifH* gene amplification and T-RFLP of its amplicons	García de Salamone et al. (2012)
Soya bean (*Glycine max*)	*Bradyrhizobium japonicum, Bacillus megaterium*	Clone library of amplicons of *nifH*	Kravchenko et al. (2013)

Bradyrhizobium and *Bacillus* in field conditions (Kravchenko et al. 2013). When clone libraries were generated with *nifH* amplicons, a higher diversity was found in the treatments, compared to control. While it can be said with some confidence that bioinoculants lead to an increment in *nifH* gene, it is still not clear if the enhancement in these numbers is because of flourishing existing diazotrophic population or selection of newer members.

Other important nutrient cycles occurring in the soil include carbon (C), phosphorus (P) and sulfur (S) cycles. Studies have also been performed describing the reaction steps and genes involved in these cycles but to a lesser extent than that of nitrogen cycle, and so is not completely understood in terms of its genetic basis. There are, to the best of our knowledge, no studies on effect of bioinoculants on cycles other than nitrogen. However, for the benefit of the readers we have compiled all characterized genetic markers for N, P, S and C cycle that have been employed in microbial ecology (Table 3.2). It must be noted that mere presence of a gene does not assure its expression and activity. Hence it is important to perform analysis at the next levels, viz transcriptomic and proteomic.

The only report trying to answer the question at the transcript level has been performed on *Cajanus cajan* with application of *Bacillus megaterium*, *Pseudomonas fluorescens* and *Trichoderma harzianum*, individually and in different combinations (Gupta 2014). Comparison of these effects was made with that of chemical fertilizers applied at recommended dose, in a pot experiment conducted under field conditions. A comprehensive analysis of the nitrogen cycle genes and transcripts was done targeting the steps of nitrification, nitrogen fixation and denitrification. Triple inoculation showed enhancement in *nif*H (nitrogen fixation) and *amo*A (nitrification) transcripts by 2.7 and 2.0 folds, respectively. This work goes a step further in confirming that the increase in *nifH* gene as reported by Gupta et al. (2012) was not only because of increase in diazotrophic population but also because the gene was being transcribed.

3.5 Effects of Bioinoculants on Microbial-Mediated Soil Processes at Enzyme Level

A number of studies have assessed the changes in metabolic properties of soil microbes as a result of application of bioinoculants by targeting soil enzymes. The effect bioinoculants exert on rhizospheric microbial activity is crucial for plant's growth as it decides the availability of nutrients for plant (Kohler et al. 2007). Spectrophotometric analysis of enzymes like dehydrogenase, catalase, superoxide dismutase, urease, chitinase, phytase and protease, colorimetric estimation of enzymes like phosphatases and cellulases, and quantification of nitrogenase activity by gas chromatography has been widely employed. The significance of different enzymes in relating specific microbial community function and soil processes

Table 3.2 Potential genetic markers to study major soil biogeochemical cycles

Process	Marker(s)	Enzyme(s) coded for	Studies employing the marker(s)
N CYCLE			
Nitrogen fixation	*nif* genes	Nitrogenase	Zehr et al. (2003), Babić et al. (2008), Gupta et al. (2012)
Nitrification			
Ammonium oxidation	*amo*A, *amo*B, *amo*C, *hao*	Ammonia monooxygenase, hydroxylamine oxidoreductase	Rotthauwe et al. (1997), Bergmann et al. (2005), Starkenburg et al. (2006), Pester et al. (2014)
Nitrite oxidation	*nxr*B, *nxr*A	Nitrite oxidoreductase	
Denitrification			
Nitrate reduction	*nar*G, *nap*A	Nitrate reductase	Zumft (1997), Philippot (2002)
Nitrite reduction	*nir*K, *nir*S	Nitrite reductase	Philippot (2002)
Nitric oxide reduction	*nor*B, *nor*C	Nitric oxide reductase	Philippot (2002)
Nitrous oxide reduction	*nos*Z	Nitrous oxide reductase	Philippot (2002)
Dissimilatory nitrate reduction to ammonium (DNRA)	*nap*A	Periplasmic nitrate reductase	Papaspyrou et al. (2014)
	*nrf*A	Cytochrome C nitrite reductase	Rütting et al. (2008)
Nitratification/Anaerobic ammonia oxidation (Anammox)	*hzo, hzf*	Hydrazine hydrolase and dehydrogenase	Strous et al. (2006), Li and Gu (2011)
C CYCLE			
CO_2 fixation	*cbb*L/*cbb*M, *rbc*L	Ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO)	Wawrick et al. (2002), Frias-Lopez et al. (2008), Yousuf et al. (2014)
S CYCLE			
Sulfur metabolism	*aps*A	Adenosine phosphosulfate reductase subunit α	Keshri et al. (2013)
	*apr*A	Adenylylsulfate reductase subunit α	Meyer and Kuever (2007), Pradel et al. (2013)
Sulfur metabolism: arylsulfonate desulfonation	*asf*A	Arylsulfonatases	Mirleau et al. (2005), Schmalenberger et al. (2010)
Sulfur oxidation	*sox*B	Sulfate thiohydrolase	Anandham et al. (2008)
Sulfite reduction	*dsr*AB	Dissimilatory sulfite reductase	Geets et al. (2006), Liu et al. (2009)
P CYCLE			
Phosphate solubilization	*ppx*	Exopolyphosphatase	Lindner et al. (2009)
	ppk	Polyphosphate kinase	Chouayekh and Virolle (2002)
	acpA	Acid phosphatases	Jaharamma et al. (2009)
	pho	Alkaline phosphatase	Jaharamma et al. (2009)
	phyA	Phytases (*myo*-inositol hexakisphosphate phosphohydrolases)	Pasamontes et al. (1997), Berka et al. (1998)
	*gab*Y/ *mps*	Pyrroloquinoline quinone (PQQ) synthase	Jaharamma et al. (2009), Behera et al. (2014)

has been reviewed earlier (Caldwell 2005; Das and Verma 2011). Broadly, dehydrogenases are indicative of microbial redox systems so serve as a parameter for microbial oxidative activities. Phosphatases (acid and alkaline) release inorganic P from organic compounds (P mineralization), thereby being of importance in soil phosphorus cycle, while proteases are a good measure of N mineralization.

So far, one common observation by all research groups is a significant enhancement in the level of soil enzymes upon application of bioinoculants (Table 3.3). Irrespective of the kind of inoculant applied (cyanobacterial, bacterial or

Table 3.3 Enzymatic markers employed to study the effects of bioinoculants on function of rhizospheric microbial community

S. No.	Bioinoculants applied	Plant system	Enzyme(s)	Reference(s)
1.	*Azospirillum brasilense, Azotobacter chroococcum*, AM fungi	Pomegranate (*Punica granatum*)	Dehydrogenase, nitrogenase, alkaline phosphatase, hydrolysis of fluorescein diacetate (FDA)	Aseri et al. (2008)
2.	*Pseudomonas jessenii, Pseudomonas synxantha*, AM fungi	Wheat (*Triticum aestivum*), Rice (*Oryza sativa*), Black gram (*Vigna mungo*)	Dehydrogenase, alkaline and acid phosphatase, urease	Mader et al. (2011)
3.	*Methylobacterium oryzae, Azospirillum brasilense, Burkholderia pyrrocinia*	Tomato (*Lycopersicon esculentum*), Red pepper (*Capsicum annuum*), Rice (*Oryza sativa*)	Nitrogenase, phosphatase, urease	Madhaiyan et al. (2010)
4.	*Azotobacter chroococcum, Azospirillum brasilense, Pseudomonas fluorescens, Bacillus megaterium*, VAM	Wheat (*Triticum aestivum*)	Dehydrogenase, phosphatase	Parewa et al. (2014)
5.	*Azospirillum brasilense*, AM fungi: *Glomus clarum*	Faba bean (*Vicia faba*)	Acid phosphatase, alkaline phosphatase, nitrogenase	Rabie and Almadini (2005)
6.	*Rhizobium* sp., *Pseudomonas fluorescens*	Common bean (*Phaseolus vulgaris*)	Nitrogenase	Samavat et al. (2012)
7.	*Thiobacillus* sp., AM fungi	Onion (*Allium cepa*), Maize (*Zea mays*)	Dehydrogenase	Mohamed et al. (2014)
8.	3 bacterial and 3 cyanobacterial strains	Wheat (*Triticum aestivum*)	Dehydrogenase, FDA hydrolase, alkaline phosphatase, nitrogenase	Nain et al. (2010)
9.	*Azospirillum brasilense, Bacillus sphaericus*	Banana (*Musa* spp.)	Nitrogenase	Baset Mia et al. (2010)
10.	*Azotobacter chroococcum, Bacillus circulans* and AM fungi	River red gum (*Eucalyptus camaldulensis*)	Dehydrogenase, nitrogenase	Al-Hadad et al. (2014)
11.	Organic nutrient management including *Bacillus megaterium, Azospirillum*	Turmeric (*Curcuma longa*)	Dehydrogenase, ß-glucosidase, acid phosphatase, protease, arylsulfatase	Dinesh et al. (2010)
12.	*Bacillus pumilus*	Faba bean (*Vicia faba*)	Catalase	Kang et al. (2013)
13.	Effective microorganisms™	Red clover (*Trifolium pratense*), Oat (*Avena sativa*)	Dehydrogenase, FDA hydrolase	Park and Kremer (2007)

fungal), type of plant (legumes, cereals, vegetables or fruits), condition of plant growth experiment (pot or field), the same trend has been invariably observed. Also, dual or multiple inoculations have been observed to enhance the levels more than single inoculations (Aseri et al. 2008; Madhaiyan et al. 2010; Samavat et al. 2012; Al-Hadad et al. 2014). However, co-inoculation involving *Rhizobium* as one of the partners has been shown to have either positive or negative impact on its symbiotic abilities depending on the strain of *Rhizobium* (Lucas Guarcia et al. 2004; Samavat et al. 2012).

Biocontrol agent *P. fluorescens* F113 did not exert any impact on eight enzymes of the P and S nutrient cycles when applied to sugar beet under field conditions (Naseby et al. 1998). This was observed when sampling was performed 181 days after application of the bioinoculants. However, the same group observed changes in enzyme activity levels when the study was performed in microcosms and sampling done after 21 days of growth of plant (Naseby and Lynch 1998). They credited this discrepancy to majority of *Pseudomonas* strains being r-strategic and hence exhibiting transient effects in short-term experiment.

In a pot experiment conducted by Nain et al. (2010) with wheat, different combinations of three bacterial and cyanobacterial isolates were used with N, P and K fertilizers. The crop yield enhancement was approximately 48 % by the application of bacterial and cyanobacterial isolates. More than 50 % enhancement in alkaline phosphatase and dehydrogenase activities was observed in bioinoculant-treated samples as compared to controls. Also, the activity of dehydrogenase and FDA hydrolysis positively correlated with soil microbial carbon.

With the development of BIOLOG plates in 1980s, community level physiological profiling (CLPP) has been widely employed in microbial ecology. However their application to evaluate the effect of bioinoculants has been limited. When Park and Kremer (2007) applied Effective Microorganisms™ in red clover and oats, despite observing its effect on dehydrogenase activity and FDA hydrolysis, no difference could be observed in BIOLOG assay. Similarly Javoreková et al. (2015) assessed the effects of AZOTER on arable soil. They used PCR-DGGE technique to characterize the difference in microbial community structure and could observe changes. However, the community metabolic diversity assessed using BIOLOG was influenced by the incubation time, but not by application of biofertilizers. The reason for this insensitivity of BIOLOG to changes in microbial community functioning, despite other techniques having better resolutions, has been attributed to metabolic redundancy (Konopka et al. 1998). By estimating the community level response to a particular substrate one cannot estimate the structural changes in the microbial community in the sample being investigated. The method does not provide resolution to identify sensitive organisms causing the change in physiological profiles.

3.6 Other Potential Techniques

In the last decade a number of advanced techniques have come up which correlate microbial community structure with its function. Though these techniques have rarely been used to address the question of non-target effects of bioinoculants on microbial community function, they hold promise in unraveling this aspect.

Microautoradiography, combined with fluorescence in situ hybridization (FISH-MAR), is an excellent means of assessing the uptake of radioactively labeled chemicals by microbes. Stable isotope probing (SIP) is a tool bringing together isotope labeling followed by molecular analysis of phospholipids or nucleic acids extracted from the community. Recent development to SIP has been employing RNA and proteins as markers, which goes a step further in linking the microbial community structure with its function (Manefield et al. 2002; Jehmlich et al. 2010). In fact we are now witnessing various conjunctions of tools, one of the most recent one being SIP and metagenomics (Chen and Murell 2010). This interesting combination is not only efficient in detecting less abundant members, but also analyzing metabolic diversity.

The advent of whole community analysis approaches involve study of microbial community function using meta-omics tools like metagenomics, metatranscriptomics, metaproteomics and metabolomics, which have provided a new window into this hidden microbial world. Functional gene arrays (FGA) developed with probes for key functional genes are useful in simultaneous analysis of different processes. The most comprehensive FGA till date is GeoChip 3.0 (He et al. 2010) targeting >45,000 functional genes encompassing different biogeochemical cycles.

A number of recent papers have critically reviewed the plethora of techniques available for studying microbial community structure and function together with their advantages and pitfalls (Spiegelman et al. 2005; Kreuzer-Martin 2007; Sørensen et al. 2009; Rastogi and Sani 2011; Guttman et al. 2014).

3.7 Conclusion

Challenges in linking microbial communities' structure and ecosystem functioning have been a major hindrance in getting a complete picture of the indirect mechanisms of plant growth promotion by bioinoculants. With most of the studies focusing on the impact of these biological agricultural amendments using conventional culture-dependent tools or enzyme assays, and only a handful of papers employing genomic tools to understand these phenomena, it becomes all the more difficult to draw conclusions. Transcriptomic approach has not yet been applied to address the issue (except by Gupta 2014), probably because of the cumbersome nature of protocol for extraction of mRNA from soil samples. Source of most of the enzymes that have been employed in such studies could be plant and microbial; hence, the effects observed cannot be solely attributed to microbiota. Moreover, enzymes being stable in soil matrix do not represent the exact status of sampling. While it is clear that introduction of bioinoculants leads to a significant increment in all soil enzymes tested, a snapshot of the cumulative effects can only be assessed by employing a polyphasic approach. It is evident from the reports that the mechanisms involving plant growth promotion by bioinoculants include, besides their direct effect, their interaction with resident microbial community and the resulting impact on soil functioning. There is, therefore, a need to critically evaluate such non-target effects of bioinoculants at genomic, transcriptomic and proteomic level, and to validate such results at the field level before their release in agriculture.

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Phenazine-Producing *Pseudomonas* spp. as Biocontrol Agents of Plant Pathogens

4

Tanya Arseneault and Martin Filion

Abstract

Soils that are suppressive to diseases have often been shown to contain high levels of fluorescent *Pseudomonas* spp. that produce a variety of secondary metabolites, including antibiotics such as hydrogen cyanide, diacetylphloroglucinol (DAPG) and phenazines, among others. Phenazine-producing *Pseudomonas* spp. show promise for use as successful biocontrol agents against many diseases affecting several agricultural crops. The production of different types of phenazines (phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), hydroxyphenazines (OH-PHZ) and pyocyanin (PYO)) has been shown to be directly involved in the reduction of several diseases caused by fungi, oomycetes and bacteria, in a variety of geographical locations. Phenazines can also be highly important in fluorescent *Pseudomonas* spp. physiology and have the potential to increase fitness of the producing strains by affecting traits such as biofilm formation and iron acquisition. The high capacity for soil colonization as well as the robustness and competitiveness of fluorescent *Pseudomonas* spp. show potential for their increased use in commercial applications. However, further studies are needed to determine the optimal conditions under which these bacteria can persist and produce phenazines under natural soil conditions, and their implication at the molecular, physiological, and ecological levels.

Keywords

Pseudomonas • Biocontrol agents • Phenazine • Biofilm • Phytopathogens

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_4

4.1 *Pseudomonas* spp.: Promising Biocontrol Agents (BCA)

Pseudomonas spp. are aerobic, Gram-negative bacteria that are ubiquitously found in soils, especially in the rhizosphere, which consists of the first few millimeters of soil adhering to plant roots. They are particularly well suited for plant root colonization. Several excellent reviews on the plant growth-promoting capacity as well as the biocontrol potential of these rhizobacteria have shown that *Pseudomonas* spp. are of high interest in an agricultural setting (Couillerot et al. 2009; Haas and Défago 2005; Weller 2007; Weller 1988, among others). Their ability to metabolize a wide array of nutrients, their rapidity and ease of growth and their natural abundance in a variety of plant–soil environments (Mark et al. 2006; Mercado-Blanco and Bakker 2007; Weller 2007) make them promising organisms for the development of biocontrol and biofertilizer products. Furthermore, studies on several natural disease-suppressive soils have found that these are often rich in antibiotic-producing fluorescent *Pseudomonas* spp. (reviewed by Weller et al. 2002; Raaijmakers et al. 1997; Mazurier et al. 2009).

Fluorescent *Pseudomonas* spp. are a group of well-studied bacteria belonging to different closely related species of *Pseudomonas* spp., named for their production of pyoverdines, fluorescent compounds that act as siderophores and are essential to iron scavenging and uptake, among other physiological roles (Visca et al. 2007). Species belonging to this group include *Pseudomonas fluorescens*, *P. chlororaphis* (or *P. aureofaciens*), *P. aeruginosa, P. syringae* and *P. putida.* Fluorescent *Pseudomonas* spp. are aggressive root colonizers. Some strains are even able to form robust endophytic micro-colonies in the intercellular spaces of plant root cells (Couillerot et al. 2009; Mark et al. 2006). Furthermore, they possess adaptability with regard to motile and sessile lifestyles, being able to use flagella to actively colonize plant roots and then form an immotile and resistant biofilm. During biofilm formation, the bacteria attach to plant roots using several molecules, including adhesins and exopolysaccharides. This switch in finely tuned by many genetic regulators that respond to environmental conditions (Ramey et al. 2004; Baraquet et al. 2012) and involves cell-to-cell communication using chemical signals (Davies et al. 1998). Adequate colonization is essential for fluorescent *Pseudomonas* spp. to interact with the host plant and to provide growth promotion and/or biocontrol (Chin-A-Woeng et al. 2000).

The growth-promoting capabilities of several fluorescent *Pseudomonas* spp. have been frequently observed and can be substantial in improving plant health and/or crop yield. For example, the beneficial effect of inoculating potato plants with *Pseudomonas* spp. has been documented many decades ago (Kloepper et al. 1980), increasing by twofold shoot and root formation in 1-month-old plants (Burr et al. 1978). Fluorescent *Pseudomonas* spp. can achieve this effect using several mechanisms, which include, among others, increasing the availability of nutrients (via nitrogen-fixation and phosphate solubilization, among others) and producing growth hormones (mainly auxins and gibberellins) (reviewed by Vessey 2003; Lugtenberg and Kamilova 2009).

When populations of fluorescent *Pseudomonas* spp. reach a certain threshold, the organisms often have the ability to produce several antibiotics, as well as other secondary metabolites, which can target a wide array of plant pathogens and enable biocontrol (Couillerot et al. 2009). The quorum-dependent regulation of their production relies mainly on the two-component GacA/GacS system found in most Gram-negative bacteria (Heeb and Haas 2001). The most studied antibiotics or antimicrobial compounds produced by fluorescent *Pseudomonas* spp. are: 2,4-diacetylphloroglucinol (DAPG) (Couillerot et al. 2009; Keel et al. 1996), hydrogen cyanide (HCN) (Voisard et al. 1989), pyrrolnitrin (Burkhead et al. 1994), pyoluteorin (Zhang et al. 2010) and phenazines (Chin-A-Woeng et al. 2003; Mavrodi et al. 2006). More specifically, phenazines have been shown to be important secondary metabolites implicated in biocontrol in

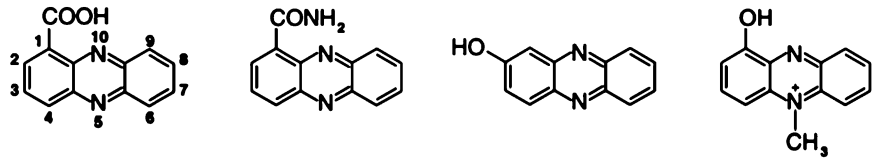

Fig. 4.1 Phenazines commonly produced by fluorescent *Pseudomonas* spp. of biocontrol interest: phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), 2-hydroxyphenazine (2-OH-PHZ) and pyocyanin (PYO)

fluorescent *Pseudomonas* spp. (Chin-A-Woeng et al. 2003), in addition to other physiological roles. The most commonly encountered phenazines produced by fluorescent *Pseudomonas* spp. include phenazine-1-carboxylic acid (PCA), phenazine-1-carboxamide (PCN), hydroxyphenazines (OH-PHZ) and pyocyanin (PYO) (Fig. 4.1).

Fluorescent *Pseudomonas* spp. display inherent mechanisms of competition such as iron scavenging and niche competition through aggressive colonization (Lugtenberg and Kamilova 2009). The production of phenazines can also contribute to competition leading to biocontrol by killing the pathogen directly. However, phenazines can also contribute to biocontrol though other mechanisms such as inducing signaling mechanisms in the plant to increase defense responses (ISR), as it has been shown for pyocyanin (Audenaert et al. 2002), and even reducing pathogen virulence through transcriptional alteration of key pathogenesis gene expression in the pathogen, as it has been shown for PCA (Arseneault et al. 2013).

4.2 Evidence for Phenazines as Important Molecules in Controlling Plant Pathogens

A very convincing example of the importance of phenazines for disease control comes from the study of a wilt-suppressive soil in Châteaurenard, France (Mazurier et al. 2009). While studying the abundance and diversity of phenazine-producing fluorescent *Pseudomonas* spp. in disease-suppressive and conducive soils, the authors found that phenazine-producing (Phz+) strains were only found in the suppressive soil.

Of the many *Pseudomonas* strains that are isolated from soils to be tested as BCAs, several studies have noted that phenazine production can be crucial to biocontrol. Some studies have explicitly linked biocontrol ability of several fluorescent *Pseudomonas* strains to the production of one or many phenazine compounds (Table 4.1), either by producing isogenic mutants deficient in phenazine production (Anjaiah et al. 1998; Arseneault et al. 2013; Audenaert et al. 2002; Chin-A-Woeng et al. 1998; D'aes et al. 2011; Tambong and Höfte 2001; Thomashow et al. 1990; Upadhyay and Srivastava 2011; Yang et al. 2011), or by directly assessing the effect of phenazine on plant pathogens (Bardas et al. 2009; Hu et al. 2014; Jasim et al. 2014; Raio et al. 2011). The strains utilized in the studies listed have been shown to colonize a variety of agricultural crops, showing the wide applications of phenazine-producing fluorescent *Pseudomonas* spp. In terms of the pathogens targeted by these *Pseudomonas* strains, most of the studies have been accomplished using fungal pathogens; however, recent experiments have shown that a bacterial pathogen, *Streptomyces scabies*, can also be controlled with the PCA-producing *Pseudomonas* sp. LBUM223 (Arseneault et al. 2013). Oomycetes such as *Pythium* spp. can also be controlled by phenazine-producing *Pseudomonas* spp. (Tambong and Höfte 2001; Jasim et al. 2014). Additionally, although the involvement of phenazines was not directly assessed, it has been shown that several strains of phenazine-producing *P. aeruginosa* were able to control the root-knot nematode *Meloidogyne javanica* and reduce disease both under controlled and field conditions (Ali Siddiqui et al. 2001).

Table 4.1 *Pseudomonas* strains with biocontrol capacity associated with phenazine production, under controlled (C) and field (F) conditions

Biocontrol strain	Phenazine produced	Pathogen	Controlled disease	Crop	Conditions	Reference
P. chlororaphis PCL1391	PCN	*Fusarium oxysporum* f. sp. *radicis-lycopersici*	Root rot	Tomato	C	Chin-A-Woeng et al. (1998)
		Colletotrichum lindemuthianum	Anthracnose	Bean	C	Bardas et al. (2009)
P. aeruginosa PNA1	PCA, PCN	*Fusarium oxysporum* f. sp. *ciceris*	Fusarium wilt	Chickpea	C	Anjaiah et al. (1998)
		Pythium myriotylum	Root rot	Cocoyam	C	Tambong and Höfte (2001)
P. aeruginosa 7NSK2	PYO	*Botrytis cinerea*	Gray mold	Tomato	C	Audenaert et al. (2002)
Pseudomonas sp. CMR12a	PCA, PCN	*Rhizoctonia solani*	Root rot	Bean	C	D'aes et al. (2011)
P. fluorescens Psd	PCA	*Fusarium oxysporum*	Fusarium wilt	Tomato	C	Upadhyay and Srivastava (2011)
Pseudomonas sp. HC9-07	PCA	*Gaeumannomyces graminis* var. *tritici*	Take-all	Wheat	C	Yang et al. (2011)
P. aeruginosa strain	PCA	*Pythium myriotylum*	Rot	Ginger	C	Jasim et al. (2014)
P. fluorescens 2-79	PCA	*Gaeumannomyces graminis* var. *tritici*	Take-all	Wheat	C & F	Thomashow et al. (1990)
P. chlororaphis 30-84	PCA	*Gaeumannomyces graminis* var. *tritici*	Take-all	Wheat	C & F	Thomashow et al. (1990)
Pseudomonas sp. LBUM223	PCA	*Streptomyces scabies*	Common scab	Potato	C[1] & F[2]	[1]Arseneault et al. (2013);[2] Arseneault et al. (2015)
P. chlororaphis Pcho10	PCN	*Fusarium graminearum*	Fusarium head blight	Wheat	C & F	Hu et al. (2014)
P. chlororaphis M71	PCA, 2-OH-PHZ	*Seiridium cardinale*	Bark canker	Cypress	F	Raio et al. (2011)

Field studies have demonstrated the biocontrol ability of different phenazine-producing fluorescent *Pseudomonas* spp. in many geographic locations, spanning from North America (Thomashow et al. 1990; Powell et al. 2000; Arseneault et al. 2015) to Europe (Raio et al. 2011) to Asia (Hu et al. 2014). While some registered biocontrol treatments, or others to come, may not be able to be distributed internationally due to regulation issues, commercial products based on phenazine-producing *Pseudomonas* show promise to be used in various geographic locations and under different climatic conditions.

Although phenazine-producers are often potent biocontrol agents, in some cases the contribution of phenazines to disease control has not been specifically verified, or molecules other than phenazines are responsible for biocontrol. For example, it has been shown that the production of PCA and hydroxyphenazine by *P. chlororaphis* PA23 is not essential to control *Sclerotia sclerotiorum*, which seems mainly mediated by the production of pyrrolnitrin. However, the phenazines produced by PA23 contribute to biofilm formation and thus could improve its survival in the environment (Selin et al. 2010). The roles of phenazines can therefore be multiple – acting as an antibiotic against pathogens, being involved in physiology and even acting as a molecular signal.

4.3 Phenazines

4.3.1 Chemical Forms

Phenazines are electron shuttles that exhibit unique redox properties (Mavrodi et al. 2006). They exist in several chemical forms, the base of which is PCA (Fig. 4.1), which can then be converted into other forms (PCN, PYO, OH-PHZ) through the genetic expression and production of different enzymes, which vary among fluorescent *Pseudomonas* species. While *P. fluorescens* only produces the yellow-pigmented PCA, other species or strains can produce one or more additional forms, including: the orange-dark red hydroxyphenazines (OH-PHZ), the green-pigmented phenazine-1-carboxamide (PCN) and the blue-green pyocyanin (PYO) (5-methyl-1-hydroxyphenazine) (reviewed by Mavrodi et al. 2006). All have been shown to possess broad antifungal activity *in vitro*, although PCN and OH-PHZ seem to have the greatest overall efficacy (Chin-A-Woeng et al. 1998; Mavrodi et al. 2006). Their activity is affected by physicochemical factors such as pH (Ownley et al. 1992) and solubility, which can play a role in aqueous environments, as hydroxyphenazines are more soluble in water than other chemical forms (Chin-A-Woeng et al. 2003).

4.3.2 Production and Regulation

The enzymes necessary for the biosynthesis of phenazines are encoded by the Phz operon, the core of which includes *phzA, B, C, D, E, F*, and *G* genes that are highly conserved among all producing *Pseudomonas* species (Mavrodi et al. 2010). The first step in the biosynthesis of phenazines requires the accumulation of chorismic acid, which is then sequentially modified by PhzE, PhzD, PhzF, PhzB and PhzG to produce PCA. The redirection of the shikimate pathway towards the biosynthesis of the chorismate necessary for PCA biosynthesis is catalyzed by PhzC (reviewed by Mavrodi et al. 2013 and Blankenfeldt 2013). The exact role of PhzA, a copy of PhzB with 80 % sequence identity, is unknown, although it does not have the same activity as PhzB due to mutations in the enzyme's active site (Ahuja et al. 2008; Mavrodi et al. 2013). Additional *phz* genes present among different fluorescent *Pseudomonas* spp., either adjacent to the operon or elsewhere in the genome, encode enzymes that can chemically modify PCA into other forms of phenazines (Mavrodi et al. 2006). Hydroxylases encoded by PhzO (Delaney et al. 2001) and PhzS (Mavrodi et al. 2001) convert PCA into hydroxyphenazines, while a methyltransferase encoded by PhzM acts with PhzS to produce pyocyanin (Mavrodi et al. 2001). PhzH, similar to an asparagine synthase, is responsible for the conversion to PCN (Mavrodi et al. 2001).

Phenazine production is dependent on quorum-sensing, and is only activated once a critical mass of bacterial cells and their signals have accumulated. Quorum-sensing is an efficient means of communication for bacteria to synchronize their metabolism in order to act collectively. Most studies on the regulation of phenazine production among biocontrol strains have been accomplished using *P. chlororaphis* 30–84, and some using *P. fluorescens* 2–79, therefore more research should be accomplished on additional strains to confirm the current model, of which a simplified version is presented. The Phz operon possesses two regulatory genes: *phzI* and *phzR* (Mavrodi et al. 2006), members of the Lux type transcriptional regulators. PhzR activates the expression of the Phz operon (Pierson et al. 1994) in response to the accumulation of acyl-homoserine lactones (acyl-HSLs) produced by PhzI (Khan et al. 2007). PhzR can also respond to acyl-HSLs produced by other bacteria in a microbial community (Pierson et al. 1998), which can be highly relevant in an agricultural soil environment where microbial communities are diverse. The PhzR/I system is itself regulated by another two-component system: GacS/GacA, an important system that controls the production of many secondary metabolites in Gram-negative bacteria and is crucial to biocontrol activity (reviewed by Heeb and Haas 2001). In many fluorescent *Pseudomonas* spp., the Gac system operates through small RNA-binding proteins (RsmA and RsmE) (Reimmann et al. 2005). Although the signaling mechanisms involved are not completely elucidated, it is thought that in *P. chlororaphis* 30–84, the Gac-Rsm pathway acts with another signal transduction system (RpeA/RpeB) to activate phenazine production in response to environmental signals (Wang et al. 2012, 2013). The RpeA/RpeB system seems to respond to the metabolic state or cellular stress in order to regulate phenazine production (Wang et al. 2012). In addition to being regulated by inter-bacterial communication, there is evidence that in *P. aeruginosa*, pyocyanin itself can act as an intercellular signal, directly inducing the expression of genes associated with quorum-sensing (Price-Whelan et al. 2006). The biosynthesis of phenazine relies on the regulation of many systems and factors, including many different molecular signals, some of which still remain uncharacterized.

4.3.3 Physiological Implications for Phenazine Producers

Studies have shown that phenazine production by fluorescent *Pseudomonas* spp. contributes to their ecological competence in soil (Mazzola et al. 1992). Furthermore, the high conservation of the *phz* operon among these bacteria (Mavrodi et al. 2010) suggests that there is an evolutionary pressure to maintain an intact production of phenazines, and that it may be crucial to thrive in the environment (Mavrodi et al. 2013).

The production of phenazines has been linked to the establishment and maturation of biofilms on wheat seeds and plant roots by *P. chlororaphis* 30–84 (Maddula et al. 2006; 2008). Biofilms are resistant structures in which bacteria are agglomerated in a complex matrix consisting of exopolysaccharides, proteins and nucleic acids, and are highly influenced by water and nutrient availability when associated with underground plant tissues (reviewed by Ramey et al. 2004). Although mutated strains (Phz-) unable to produce phenazines can still form biofilms, their structure and appearance is generally altered and influenced by the phenazine compounds that are, or are not, being produced (Maddula et al. 2008).

The electron shuttling capacity of phenazines also has an effect on the metabolism of fluorescent *Pseudomonas* spp. biofilms. It is thought that the dense structure of biofilms creates an oxygen gradient that could be problematic to the aerobic bacteria that are in the center of the aggregate, an area of low oxygen content. However, it has been suggested that phenazines, and in particular pyocyanin, could act as electron acceptors in place of oxygen for the accumulating NADH, increasing concentrations of NAD+ (Price-Whelan et al. 2007). This would allow for the survival of cells under anaerobic conditions (Wang et al. 2010). Another effect of phenazines on metabolism involves iron uptake.

P. chlororaphis PCL1391, which produces PCN, can effectively reduce and utilize poorly dissolved iron and manganese oxides, while its PCN-mutant is not able to do so (Hernandez et al. 2004). This is clearly an advantage in soil, where iron is limited and scavenged using siderophores. This ability is even more meaningful in the event that siderophores are not produced. This was shown in a siderophore-deficient *P. aeruginosa* strain, unable to form biofilms, that could use added PCA for the reduction of Fe^{3+} and uptake of the resulting Fe^{2+}, restituting the ability to form biofilms (Wang et al. 2011).

4.4 Mode of Action

The ability of phenazines to shuttle electrons is at the heart of their biological and chemical activities. Although some explanations as to how these molecules affect plant pathogens have been proposed, the exact mechanisms involved in biocontrol remain unclear. Phenazines are thought to cross the cell membrane of the pathogen and act as a reducing agent, interfering with the electron transport chain and generating several toxic reactive oxygen species (superoxide radicals and hydrogen peroxide) (reviewed by Chin-A-Woeng et al. 2003; Hassan and Fridovich 1980).

One of the best studied phenazines is pyocyanin (Jayaseelan et al. 2014), as its production by *P. aeruginosa* is associated with pathogenesis in opportunistic lung infections in patients with cystic fibrosis (Lau et al. 2004). PYO has been shown to have many detrimental effects on human cells, including inactivation of vacuolar ATPases (Ran et al. 2003) and inhibition of catalase activity (O'Malley et al. 2003). In microorganisms, pyocyanin seems to inhibit bacterial growth in several species by interacting with the respiratory chain, disabling energy-dependent metabolic processes, such as active membrane transport (Baron et al. 1989). Pyocyanin has recently been shown capable of binding to extracellular DNA (Das et al. 2013), and is also the only phenazine currently known to induce systemic resistance in plants, leading to better disease control (Audenaert et al. 2002). Among other phenazines, chemically synthesized PCN analogs, which are being studied as potential cancer treatments, are shown to be DNA intercalating, inhibiting topoisomerase I and II, and subsequently cell division (Gamage et al. 2002). The potential of phenazines to bind DNA could also lead to interactions with coding DNA, and affect genetic transcription to varying extents (Mavrodi et al. 2006).

4.5 Phenazine-Producing *Pseudomonas* under Natural Field Conditions

An excellent review by Mavrodi and colleagues, in which the authors develop what is currently known about the ecology, diversity and prevalence of phenazine-producing (Phz+) *Pseudomonas* spp., especially under natural conditions, has been recently published (Mavrodi et al. 2013). In the past years, these researchers have characterized phenazine production and population biology among saprophytic *Pseudomonas* spp. indigenous to over 80 dryland fields used for cereal crops in the northwestern USA. They found that there was a direct relationship between the amount of PCA extracted from the rhizosphere and the populations of Phz+*Pseudomonas* spp. naturally present, which was the first demonstration of a significant accumulation of phenazine in agricultural fields (Mavrodi et al. 2012a). Furthermore, PCA seemed to be produced in amounts seemingly sufficient (estimation of 100 μM localized) for signaling as well as for the inhibition of sensitive pathogens. They also showed that plant colonization by phenazine-producing *Pseudomonas* spp. depends on soil water content, being negatively correlated with annual precipitation (Mavrodi et al. 2012a) and irrigation (Mavrodi et al. 2012b). Most sampled fields had mean indigenous populations of phenazine-producing *Pseudomonas* spp. between 10^3 and 10^7 CFU/g of root (Mavrodi et al. 2012a), while it has been estimated that the level of *Pseudomonas* spp. required for biocontrol is generally between 10^4 and 10^6 CFU/g soil (Haas and Défago 2005) or 10^5 to 10^6 CFU/g root

(Raaijmakers and Weller 1998), suggesting that there is a good level of disease protection in the fields sampled, in addition to the detection of high levels of PCA. A follow-up study that sought to characterize the diversity of the Phz+*Pseudomonas* spp. present using BOX-PCR fingerprinting showed that 31 distinct phylogenetic groups related to *P. fluorescens* were found (Parejko et al. 2012). Geography and other factors such as soil characteristics, have clearly a role to play in bacterial populations and diversity, as the results obtained by Parejko et al. (2012) significantly differed from a study on the wilt-suppressive Châteaurenard soil (Mazurier et al. 2009), in which Phz+ strains were rather found to be genetically related to *P. chlororaphis.* Soil type has been shown to be a major factor, as identical fluorescent *Pseudomonas* spp. communities used to inoculate the same plant species in two different sterilized field soils resulted in significantly different bacterial communities (Latour et al. 1999). Another aspect of soil properties that is capable of influencing indigenous *Pseudomonas* spp. communities, both in abundance and diversity, is the application of fertilizers, which favors some strains and hinders others when verified in natural fields (Tambong and Xu 2013).

With regards to field-inoculated Phz+ fluorescent *Pseudomonas* spp., at least one registered BCA has been studied. BioJect Spot-Less (*P. aureofaciens* TX-1) (Eco Soil Systems Inc., San Diego, CA) is used to treat dollar spot in turf, a fungal disease caused by *Sclerotinia homoeocarpa*. PCA production by *P. aureofaciens* TX-1 was shown to be essential to disease control (Powell et al. 2000). The BCA is applied almost daily during summer months using a modified irrigation system. A study on the fate of TX-1, once applied, has revealed that the strain was well established in the rhizosphere and could even be detected after the following winter (Sigler et al. 2001). Other commercial fluorescent *Pseudomonas* spp.-based BCAs exist, although phenazines are not the main antagonistic molecules marketed. One of the most widely used registered *Pseudomonas*-based biopesticide is Cedomon®, formulated using *P. chlororaphis* MA 342. This BCA has been proven efficient at controlling a wide range of fungal diseases in cereal crops (Johnsson et al. 1998) by producing 2,3-deepoxy-2,3-didehydrorhizoxin, a fungicide. Another registered fluorescent *Pseudomonas*-based BCA is BlightBan® A506 (*P. fluorescens* A506) (Nufarm, Burr Ridge, IL), which is used as a foliar spray to treat fire blight and frost damage in several fruit trees as well as tomato, potato and strawberry. The use of fluorescent *Pseudomonas*-based BCAs can therefore be attractive as they can target a wide array of pathogens in several different crops. This also shows that in addition to their use as soil-based BCAs for agricultural crops, fluorescent *Pseudomonas* BCAs can also be inoculated on leaves (blossoms) and turf, extending the range of environments in which biocontrol can be achieved.

4.6 Considerations in Developing a Phz+Pseudomonas-Based BCA for Field Application

The half-life of PCA is very low in soil (3.4 days) (Mavrodi et al. 2013), showing the importance of constant *in situ* production of phenazines by directly inoculating and assuring adequate colonization of Phz+*Pseudomonas* strains in the field. Figure 4.2 suggests a pipeline of factors to study for each potential *Pseudomonas* strain to be developed into an efficient commercial biocontrol treatment. Field trials should be conducted in a variety of geographical regions and soil types, and for each *Pseudomonas* strain of biocontrol interest, all targeted pathogens should be tested *in vitro* and *in planta* prior to field assays in order to more easily determine the potential mechanism(s) of biocontrol involved. This can be done by identifying antibiotics and others active molecules being produced by the strain of interest, and confirming their role in biocontrol by producing mutants deficient in their production. A detailed review providing insight on how to efficiently screen for bacterial biocontrol strains has shown the importance of verifying several of these factors to increase the odds of obtaining a field-competent BCA (Pliego et al.

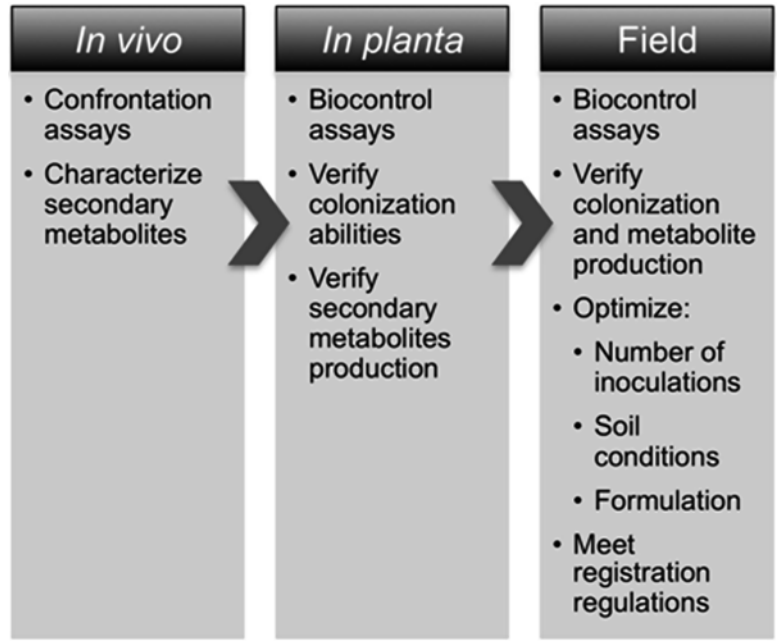

Fig. 4.2 Pipeline for developing an efficient biocontrol treatment for agricultural use

2011). Determining the mechanism of biocontrol is extremely useful in optimizing performance and understanding how a pathogen will respond to a BCA. For example, the success of a BCA that acts using antibiosis can be tracked during the growing season by assessing the reduction in soil pathogen populations; however, if a reduction of virulence is the mechanism utilized, one can expect an absence of change in pathogen populations. In addition, such an approach can help in elaborating a synergistic multi-strain biocontrol treatment that targets several different mechanisms of biocontrol, possibly improving disease reduction. Such combinations have been shown to be successful; for example, the use of two antibiotic-producing strains of *P. fluorescens*, one producing PCA and the other DAPG, increased the level of biocontrol of root rot of strawberry (*Phytophthora cactorum*) compared to each strain used alone (Agusti et al. 2011). Combinations of strains of *P. fluorescens* have also been effective against potato storage diseases, their biocontrol ability even increasing when grown in culture together compared to being blended prior to inoculation (Slininger et al. 2010). However, some argue that these combinations do not necessarily synergistically contribute to biocontrol, as each inoculated bacterium is diluted among, and directly competes with the other strains present (Lugtenberg and Kamilova 2009). These results illustrate the need for additional testing and optimization when combination treatments are used.

Following *in vivo* and *in planta* validation of biocontrol activity, there are important considerations for developing successful and consistent *Pseudomonas* spp. biocontrol agents for agricultural use (Fig. 4.2), to ensure that the bacterium is sufficiently delivered in good condition and at the right time. The complexity of different biotic and abiotic factors in a field setting compared to controlled experiments can likely affect the behavior of an inoculated *Pseudomonas* strain, as some genes involved in fitness and metabolism in *P. fluorescens* have been shown to be specifically expressed in soil environments (Varivarn et al. 2013).

4.7 Formulation, Number of Applications, Time of Application

Consideration must be given to the carrier or encapsulation method used to deliver *Pseudomonas* spp. to the field; they must be sta-

ble, ideally at room temperature, and easily applicable to the field. *Pseudomonas putida* cells have been shown to be quite resistant in carbon- and nitrogen-depleted media at 30 °C, almost 100 % of bacteria surviving and being able to be revived in nutrient-rich media after 1 month in these minimal media conditions (Givskov et al. 1994). This could prove useful in BCA formulation, as the bacterium have a good shelf life in these conditions. BCAs could be applied directly using liquid media, as seed coatings, or being encapsulated in a biodegradable matrix such as natural polysaccharides (alginate, agar, cellulose, gums, lignin, etc), polypeptides (gelatin), lipids (waxes), biopolymers (lignin) or synthetic polymers (reviewed by Vemmer and Patel 2013).

The timing of inoculation is crucial to avoid onset of disease, and can vary depending on the crop and on the pathogen, and must therefore be determined for each targeted plant disease. The frequency of application can also vary depending on these factors, along with the ability of the applied *Pseudomonas* strain to colonize and persist on plant roots and in the rhizosphere. Multiple applications can be necessary, as a difficulty in maintaining plant root populations has been observed with several *Pseudomonas* strains inoculated in the field, being reduced by as much as 10^5 after 4 months of growth (Viebahn et al. 2003). This is particularly relevant when the BCA is applied as a seed coating while disease onset occurs many weeks or months after sowing. For example, disease onset of common scab of potato occurs as new tubers are formed, several weeks after planting depending on cultivars and conditions (Khatri et al. 2011), and could require additional treatments at that time if the initial inoculated bacteria do not sufficiently maintain their populations.

4.8 Determining Favorable Soil Properties and Environmental Conditions for Biocontrol

Biocontrol ability is closely linked to the capacity of Phz+*Pseudomonas* spp. to adequately colonize and produce phenazines where they are needed. Populations of inoculated *Pseudomonas* spp. must be determined to ensure that they are properly established. One important aspect of colonization ability is the capacity to produce endophytic colonies; many *Pseudomonas* strains have been shown able to colonize plant cells. Selecting for this trait when screening for potential biocontrol bacteria can provide assurance that the strain is extremely competent and could potentially better persist in an agricultural soil (reviewed by Sturz et al. 2000). Biocontrol ability can vary greatly due to abiotic factors such as precipitation, temperature, pH, mineral content and soil composition. A study, using a PCA-producing *P. fluorescens* strain and steamed soil from ten different fields, positively correlated biocontrol of take-all in wheat to soil content in ammonium, percentage of sand, soil pH, sodium, sulfate-sulfur and zinc, while negatively correlating to cation-exchange capacity (CEC), exchangeable acidity and soil content in iron, manganese, percentage of clay, percentage of organic matter, percentage of silt, total carbon, and total nitrogen (Ownley et al. 2003). Their statistical model showed that among these factors, the six most important soil properties were ammonium, CEC, iron, percentage of silt, soil pH and zinc. This illustrates why biocontrol treatments must be validated in a variety of soils, geographical locations and crops to account for these factors.

Determining *Pseudomonas* spp. soil populations is also required to indicate if biosynthetic gene expression and production of quorum-dependant molecules, such as phenazines, can occur. Secondary metabolism in *Pseudomonas* spp. relies on quorum-sensing, and is highly active when these bacteria are assembled in biofilms (Fuqua et al. 2001; Heeb and Haas 2001). To improve resistant biofilm formation around plant roots, conditions favoring a switch from a mobile to a sessile lifestyle must be determined (Ramey et al. 2004). One recently identified regulator is FleQ, whose role remains to be further characterized (Baraquet et al. 2012).

In order to evaluate phenazine production in soil, chemical extractions can be used to measure phenazine quantities (as done by Mavrodi et al.

2012a). The expression of the phenazine biosynthetic operon can also be quantified by RT-qPCR to determine if transcription has been initiated. Phenazine production can be affected by many factors. In *P. fluorescens* 2–79, PCA production has been shown to be very sensitive to pH and temperature under *in vitro* conditions, the highest production occurring at pH 7 and between 25 and 27 °C (Slininger and Shea-Wilbur 1995). The study also found that carbon sources had an effect on PCA accumulation, being higher in glucose-containing media than glycerol, xylose or fructose. Similarly, PCN production by *P. chlororaphis* PCL1391 is dramatically decreased when temperature is reduced (from 21 to 16 °C) and pH is lowered from 7 to 6 (van Rij et al. 2004). In addition, low magnesium concentrations increased PCN production, while salt stress and low concentrations of ferric iron, phosphate, sulfate and ammonium hinder its production. The effect of growth conditions on the production of different antibiotics should also be taken into account. For example, while the presence of glucose in media favored the production of phenazines by *P. chlororaphis* O6, it reduced the production of pyrrolnitrin, which is essential to the biocontrol against *Rhizoctonia solani* (Park et al. 2011). Although some optimization of conditions favoring phenazine production has been accomplished *in vitro*, almost nothing is known of the synthesis and degradation dynamics occurring under natural conditions (Mavrodi et al. 2013). Although phenazine-producing *Pseudomonas* spp. are ubiquitously found in all types of soils worldwide, there are strains that are adapted to particular soil types and environmental conditions. One could then assume that a potential *Pseudomonas* spp. BCA would perform better in the environment from which it was isolated. While some changes or amendments can be applied to fields to provide more favorable conditions (increase in mineral, nutrient, and water content), some factors (soil type, temperature) cannot easily be modulated. This suggests that, although the ideal BCA would work in many soil types against many pathogens in different geographical areas, it is more likely that several adapted BCAs will be required to achieve widespread successful biocontrol under different conditions.

It is also of interest to determine if other secondary metabolites produced by *Pseudomonas* spp. can also act synergistically with phenazines to ensure biocontrol ability. For example, it has been shown that biosurfactants produced by *P. aeruginosa* PNA1, in addition to phenazines, were essential for the biocontrol activity of the strain against pathogenic *Pythium* spp. (Perneel et al. 2008). In the biocontrol strain *Pseudomonas* sp. CMR12a, the production of cyclic lipopeptides, in addition to phenazine production, also played a significant part in the biocontrol ability and physiology of the strain (D'aes et al. 2011, 2014).

4.9 Assessing Impact on Microbial Ecology

It is impossible to predict the exact impact of the introduction of a particular *Pseudomonas* spp. on existing microbial communities, and therefore it is crucial this should be assessed prior to commercializing a *Pseudomonas* spp.-based product. So far, most studies using more traditional microbial genotyping methods (DGGE, RFLP, AFLP, etc.), seem to indicate that inoculations with *Pseudomonas* spp. of biocontrol interest have a limited impact on the saprophytic microbial ecology of plant root systems (Viebahn et al. 2003; Lottmann et al. 2000; Bankhead et al. 2004); and although multiple inoculations of turf with the phenazine-producing registered bicontrol strain *P. aureofaciens* TX-1 resulted in a transient change of the leaf bacterial community, there were no apparent changes in the rhizosphere communities (Sigler et al. 2001). Ecological impact is an important factor to study in the development of a biocontrol treatment, and with the further development and cost reduction of next-generation sequencing, it will be easier to obtain a more accurate and detailed report on the total microbial diversity present before and after the application of a given *Pseudomonas* spp. strain.

4.10 Future Research

Improvements facilitating the commercialization of *Pseudomonas* spp.-based treatments should be made with regard to regulations. Currently, many countries regulate BCAs using the same criteria as chemical pesticides (Sundh and Goettel 2013). This can lead to setbacks in getting products onto market in a timely fashion and is quite costly, in part due to determining the exact composition of all components of the BCA (including all the molecules produced by the bacterium of interest), which is much more complex than a formulated chemical. For example, the BCA Cedomon took 10 years to reach the market after being submitted for EU registration (reviewed by Velivelli et al. 2014). Registering a bacterium as a biofertilizer has less rigorous criteria, requiring to mainly study the biological and ecological impacts to determine safety, which is advantageous for certain companies or laboratories because it is both less expensive and more rapid to perform. However, despite this advantage, a biofertilizer cannot be marketed as a biopesticide without going through the proper registration channel (Velivelli et al. 2014; Harman et al. 2010). A good review on how the current regulatory framework may have hindered the development of marketed BCAs has been recently published by Sundh and Goettel (2013). A general review of the constraints associated with bioformulation and commercialization of PGPR is also of interest (Arora et al. 2011), and suggests future strategies for increasing the use of BCAs in agriculture. In addition to registration improvements, advances will be needed in optimizing formulation and encapsulation methods to facilitate longer shelf life and easy field application.

4.11 Conclusion

The future of the successful development and commercialization of *Pseudomonas* spp.-based BCAs rests in improving our understanding of the *in situ* conditions required for growth and colonization in field soil, which includes biotic (soil microflora) as well as abiotic factors (humidity, soil composition, etc.). Despite the numerous studies published in this research field, little is known about how *Pseudomonas* spp. behaves in natural soils. This knowledge is essential to develop consistently effective *Pseudomonas spp*.-based commercial products. The use of environmentally sustainable agricultural practices are essential in ensuring food security, and phenazine-producing *Pseudomonas* spp. show high potential for playing an important role in biocontrol by reducing pesticide dependence and allowing the control of various crop diseases.

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Role of Nonpathogenic Fungi in Inducing Systemic Resistance in Crop Plants Against Phytopathogens

5

Shachi Singh

Abstract

Plants are surrounded by a plethora of microorganisms, including fungal strains. Fungi associated with plants are known to exert their beneficial effects by helping them in absorption of water and nutrients and protecting them against harmful microorganisms. Protective effect is generally mediated by performing antagonistic action on pathogens and pests. However, along with their direct effects, they have been shown to trigger defense responses in plants against various pathogenic species, including members of bacterial, fungal and viral groups. This type of resistance mechanism triggered by nonpathogenic microorganisms is termed as induced systemic resistance (ISR) and has been observed in several strains of fungi. Some of the important nonpathogenic fungal strains found to induce ISR in crop plants include mycorrhiza, *Trichoderma* sp., *Penicillium* sp., *Fusarium* sp., *Phoma* sp., etc. They have been shown to trigger defense responses via multiple signaling pathways involving salicylic acid, jasmonic acid or ethylene. Candidate signaling molecules, also known as elicitors, have been recently identified, particularly from *Trichoderma* sp. and shown to protect the plants from pathogens. Thus, with respect to their role in ISR, this chapter highlights the potential of nonpathogenic fungal strains in controlling plant diseases.

Keywords

Fungi • Bioinoculants • Induced resistance • Fungi • Phytopathogens

5.1 Introduction

Diverse arrays of microorganisms are found to be associated with almost every part of a plant species. These microbes interact with them through highly coordinated cellular processes, thereby

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_5

influencing plant growth and development (Stacey and Keen 1996). Their associations could be either beneficial or harmful (Martin and Kamoun 2011). Harmful interaction with pathogenic microorganisms, such as fungi, bacteria, and viruses, is a matter of concern, since they immensely affect crop productivity (Katsy 2014). To protect the crops from these phytopathogens, a variety of chemicals have been designed. Application of such chemicals had significantly improved crop productivity and quality for many years, but the environmental pollution caused by excessive use of agrochemicals has also tremendously increased (Damalas and Eleftherohorinos 2011). Because of their negative effects many of the chemical pesticides used in agriculture have been replaced by natural methods of crop protection. These methods are safer to the environment and people's health. One of such important alternative of the synthetic chemicals, to control phytopathogens, is through biological methods.

Biological control of plant diseases involves the use of living organisms (other than humans) or products derived from them, to reduce or prevent a pathogen. These organisms may occur naturally within the host environment, or may be applied exogenously on the host plant where they can provide protection against the pathogen. Biocontrol organisms work through several mechanisms, such as some produce antibiotics that kill or stop the growth of the pathogens, some are parasites, while others compete with pathogens for available food and other resources (Cook 1993). However, along with these direct effects, they also protect the plants by inducing systemic resistance against phytopathogens.

It has long been observed that when plants survive pathogen infection they develop an increased resistance to subsequent infections. Experiment done by Ross (1961), proved that limited primary infection with Tobacco mosaic virus (TMV), restricted further infection by the pathogen on the infected as well as non-infected plant tissues. This resistance was not only effective against TMV but also on other virus and bacterial pathogens. This type of resistance mechanism was called "systemic acquired resistance" (SAR). Till now, SAR has been demonstrated in many plant species against several plant pathogenic bacterial, fungal and viral strains. The localized resistance was observed to be transferred to distal organs of a plant through emission of molecular signals. Signaling network regulating the local and systemic defense responses were observed to rely on the plant hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Van der Ent et al. 2009).

Besides pathogens, the nonpathogenic microbes have also shown to increase the level of disease resistance in plants (Bittel and Robatzek 2007). The induction of defense by nonpathogenic microorganisms was termed induced systemic resistance (ISR) and has been demonstrated in many plant species. ISR is phenotypically similar to SAR and both are effective against a broad range of diseases caused by viruses, bacteria and fungi. However, from a molecular point of view, ISR differs from SAR. SAR is characterized with an increase in salicylic acid (SA) level, as well as activation of a specific set of pathogenesis-related (PR) genes, encoding proteins with antimicrobial activity, while ISR triggered by nonpathogenic microorganisms is independent of SA accumulation and pathogenesis-related gene activation and typically relies on the JA and ET signaling pathway (Newman et al. 2013).

Plant growth-promoting rhizobacteria (PGPR), particularly strains of *Pseudomonas* and *Bacillus* species, have been extensively studied concerning their ability to trigger systemic resistance in plants (Van der Ent et al. 2009). However, nonpathogenic fungal strains are less investigated for their potential to induce ISR despite their ubiquitous presence around plants. In this chapter, efforts have been made to summarize the role of nonpathogenic fungal species in inducing defense mechanism in plants and providing resistance towards phytopathogens. Signaling molecules triggering defense reactions will also be discussed.

5.2 Types of Fungal Associations in Plants

Both the above- and below-ground parts of the plants are habitat for diverse microorganisms, including fungal species. Associated with plant species, they can be found as epiphytes on the plant surface (Buxdorf et al. 2014) as well as endophytes (Nicoletti et al. 2014) within plant tissues. Epiphytes either reside permanently or casually onto the surface of plants, while endophytes colonize plant tissue internally and asymptomatically within stems, leaves, bark, and roots, for at least some of their lifecycle. Plant roots harbor characteristic assemblages of fungal endophytes that are distinct from those of aboveground plant tissue (Maciá-Vicente et al. 2008; Kernaghan and Patriquin 2011). Both the epiphytic and endophytic microorganisms play an important role for plant health and protection (Kharwar et al. 2010).

Most of the growth-promoting fungal species are observed to reside around roots. Root area could be divided into two zones, the rhizoplane and rhizosphere. Rhizoplane is the root surface zone where microorganisms attach themselves using surface structures, whereas the rhizosphere is a thin layer of soil immediately surrounding plant roots. This is an extremely important and active area for root activity and metabolism.

Plant roots are exposed to a broad spectrum of soil fungi, some of which form mutualistic associations, called mycorrhizas. Mycorrhizas are symbiotic relationships between fungi and plant roots. More than 80 % of the species of higher plants have these relationships, and so do many pteridophytes and some mosses. They are as common on crop plants as in wild plant communities, and in several cases they have been shown to be essential for plant performance (Wehner et al. 2009). In a mycorrhizal association, the fungus obtains at least some of its sugars from the plant, while the plant benefits from the efficient uptake of mineral nutrients and water by the fungal hyphae and sometimes in protecting them against drought or pathogenic attack. Basically two important types of mycorrhizal associations are found in plants, arbuscular mycorrhiza and ectomycorrhiza. In arbuscular mycorrhiza, the fungus penetrates the cortical cells of the roots of a vascular plant (Wehner et al. 2009). The fungi involved are members of the zygomycota and classified in six genera: *Acaulospora*, *Entrophospora*, *Gigaspora*, *Glomus*, *Sclerocystis*, and *Scutellospora*. None of them can be grown in axenic culture, i.e. in the absence of their hosts. Ectomycorrhizas are one in which hyphae of the fungi do not penetrate within the individual cells of the root, but forms a sheath around the root tip. The fungi involved are mainly Ascomycota and Basidiomycota (Bacon and White 2000).

5.3 ISR Activity of Nonpathogenic Fungal Species

Antagonistic action of fungal strains against phytopathogens for the protection of the plants had been extensively studied for a long time; however, their potential to stimulate defense reaction was recently observed, when live cells as well as the extracts or extracelluar products of beneficial fungal strains were able to elicit defense responses in plants. The responses observed include rise of cytosolic H^+ and Ca^{2+}, production of reactive oxygen species (ROS), reactive nitrogen species such as nitric oxide (NO), hypersensitive response, phytoalexin accumulation, the deposition of structural polymers, such as callose and lignin and activation of defense-related genes, followed by protection against pathogens (Whipps 2001; Yedidia et al. 2003; Newman et al. 2013). Some of the well documented ISR-inducing fungi are mycorrhiza, *Trichoderma* sp., *Fusarium* sp., *Penicillium* sp., *Pythium* sp., and *Phoma* sp. Most of them fall in the category of plant growth-promoting fungi (PGPF), widely distributed in the rhizosphere soils (Whipps 2001; Jogaiah et al. 2013).

Initial experiments done on nonpathogenic fungal species, demonstrating ISR, were based on spatial or temporal separation of fungal and pathogenic strains and then observing the protection induced by them. Generally the roots of the plants were treated with fungal strains and

pathogens applied on the shoots or the seed treatment. Protections conferred to the aboveground plant parts without movement of the fungal strains were observed to study ISR. For example, plant defense responses triggered by the biocontrol agent *Trichoderma asperellum* 203 was investigated by inoculating roots of cucumber seedlings with *T. asperellum* in an aseptic hydroponic system (Yedidia et al. 1999, 2003). Aseptic growth condition was maintained to make sure that *T. asperellum* was the only microorganism surrounding the roots. The fungus was found to colonize epidermis and outer cortex of the roots. Defense mechanisms were induced by the fungus and were evident by observing the deposition of callose and cellulose in the epidermal and cortical cell-walls, responsible to strengthen the walls against pathogen infiltration. This strengthening of the cell wall was found beyond the sites of fungal penetration (Yedidia et al. 1999), indicating systemic induction of defense reactions. Final outcome of this fungal attachment was a reduction in the disease symptoms. A similar relationship was observed in the colonization of the roots of *Arabidopsis thaliana* by *Trichoderma*-34, which rendered the leaves more resistant to the bacterial pathogen *Pseudomonas syringae pv. tomato*, the biotrophic oomycete *Hyaloperonospora parasitica* and the necrotrophic fungus *Plectosphaerella cucumerina* (Segarra et al. 2009). Another *Trichoderma* sp., *T. harzianum* T39 caused a reduction in the disease symptom caused by *Botrytis cinerea* in several crop plants (De Meyer et al. 1998). Sterilized sections of the plant stems when harvested and placed on *Trichoderma*-specific agar medium, showed no growth of *Trichoderma* mycelium (Segarra et al. 2009). However, when root tissue with adhering rhizosphere of *Trichoderma*-treated plants was planted on the *Trichoderma*-specific medium, massive outgrowth of mycelium was detected. These results demonstrated that the fungus colonized the rhizosphere or root tissue, but did not spread into the aboveground parts (Segarra et al. 2009). Moreover, fungal strains were not shown to antagonize phytopathogens, when grown in dual cultures (Shoresh et al. 2005). These observations indicated that the protective effect conferred by *Trichoderma* to the plant against the pathogen infection was not due to direct antagonism but rather a plant-mediated phenomenon. Till date, several strains of *Trichoderma* sp. have been shown to trigger ISR in various dicot and monocot plants including members of Graminaceae, Solanaceae and Cucurbitaceae against major plant pathogens (Table 5.1).

The protection afforded by the *Trichoderma* sp. was associated with the accumulation of mRNA of defense genes, such as the phenylpropanoid pathway genes encoding phenylalanine ammonia lyase (*PAL*) or the lipoxygenase pathway gene encoding hydroxyperoxide lyase (*HPL*), followed by phytoalexin accumulation (Yedidia et al. 2000, 2003; Segarra et al. 2007). Higher activities of pathogenesis-related proteins, such as chitinase, β-1,3-glucanase, cellulase and peroxidase in roots as well as leaves (Yedidia et al. 1999, 2000; Segarra et al. 2007), were observed. Seed treatment with *T. virens* was found to stimulate synthesis of terpenoids phytoalexin in cotton roots (Howell et al. 2000). Similarly pepper seed and root treatments with *T. harzianum* spores significantly reduced stem necrosis caused by *Phytophthora capsici* and showed capsidiol accumulation in the inoculated sites (Ahmed et al. 2000; Sriram et al. 2009). Capsidiol is the principal phytoalexin synthesized by pepper plants exposed to infection or tissue damage (Ahmed et al. 2000). Induction of PR-proteins peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) in turmeric plants treated with *T. viride* was reported (Ushamalini et al. 2008).

Apart from *Trichoderma* strains, many other nonpathogenic fungal strains have also been shown to induce ISR (Table 5.1). Infiltration of *Penicillum janczewskii* conidia or its culture filtrate into melon and cotton leaves induced systemic resistance and protected the lower part of the stem against *Rhizoctonia solani*. Increased level of peroxidase and PAL activity, PR genes, was observed (Madi and Katan 1998). Aqueous extract of *Penicillium chrysogenum* was effective against powdery (*Uncinula necator*) and downy mildew (*Plasmopora viticola*) in grapevine, scab

Table 5.1 List of nonpathogenic fungal species, inducing ISR in crop plants

	Fungal species	Phytopathogen	Plant species	Reference
1.	*Acremonium alternatum*	*Plasmodiophora brassicae*	*Brassica rapa* (Chinese cabbage)	Doan et al. 2008
			Arabidopsis	
2.	*Aspergillus ustus*	*Botrytis cinerea*	*Arabidopsis*	Salas-Marina et al. 2011
		Pseudomonas syringae DC3000		
3.	*Binucleate Rhizoctonia*	*Rhizoctonia solani*	Potato	Escande and Echandi 1991, Jabaji-Jabaji-Hare et al. (1999)
			Bean	
4.	*Fusarium oxysporum* strain Fo47	*Pythium ultimum*	Cucumber	Benhamou et al. 2002
5.	*Fusarium oxysporum* strain 162	*Meloidogyne incognita* (nematode)		Dababat and Sikora (2007)
6.	*Fusarium equiseti GF19-1*	*Pseudomonas syringae* pv. *tomato* DC3000	*Arabidopsis*	Kojima et al. (2013)
7.	*Glomus mosseae (mycorhizza)*	*Phytophthora parasitica*	Tomato	Cordier et al. (1998), Pozo et al. (2002)
8.	*Glomus intraradices (mycorhizza)*	*Colletotrichum orbiculare*	Cucumber	Lee et al. (2005)
9.	*Heteroconium chaetospira*	*Pseudomonas syringae* pv. *Maculicola*	Chinese cabbage	Morita et al. (2003)
		Alternaria brassicae		
10.	*Heteroconium chaetospira*	*Plasmodiophora brassicae*	Canola	Lahlali et al. (2014)
11.	*Penicillium chrysogenum*	*Plasmopara viticola*	Grapevine	Tamma et al. (2011)
		Uncinula necator	Apple	
		Venturia inaequalis	Onion	
		Peronospora destructor	Tomato	
		Phytophthora infestans		
12.	*Penicillum janczewskii*	*Rhizoctonia solani*	Melon, cotton	Madi and Katan (1998)
13.	*Penicillium simplicissimum* GP17-2	*Pseudomonas syringae* pv. *tomato* DC3000	*Arabidopsis*	Hossain et al. (2007)
14.	*Penicillium* sp. GP16-2	*Pseudomonas syringae* pv. *tomato* DC3000	*Arabidopsis*	Hossain et al. (2008)
15.	*Phoma* sp. GS8-3	*Pseudomonas syringae* pv. *tomato* DC3000	*Arabidopsis*	Sultana et al. (2009)
16.	*Piriformospora indica*	*Fusarium culmorum*	Barley	Waller et al. (2005)
		Blumeria graminis		
17.	*Piriformospora indica*	*Verticillium dahliae*	*Arabidopsis*	Sun et al. (2014)
18.	*Piriformospora indica*	*Golovinomyces orontii*	*Arabidopsis*	Stein et al. (2008)
19.	*Pseudozyma aphidis*	*B. cinerea*	Tomato	Buxdorf et al. (2013)
20.	*Pythium oligandrum*	*Ralstonia solanaceraum*	Tomato	Kawamura et al. (2009)
21.	*Trichoderma asperellum* T203	*Pseudomonas syringae pv. lachrymans*	Cucumber	Shoresh et al. (2005)
22.	*Trichoderma asperellum* T34	*P. syringae pv. lachrymans*	Cucumber	Segarra et al. (2007)

(continued)

Table 5.1 (continued)

	Fungal species	Phytopathogen	Plant species	Reference
23.	*Trichoderma asperellum* T34	*Pseudomonas syringae pv. Tomato, Hyaloperonospora parasitica, Plectosphaerella cucumerina*	*Arabidopsis*	Segarra et al. (2009)
24.	*Trichoderma asperellum* SKT-1	*Pseudomonas syringae pv. tomato* DC3000	*Arabidopsis*	Yoshioka et al. (2012)
25.	*T. harzianum* T39	*Botrytis cinerea*	Tomato, lettuce, pepper, bean and tobacco	De Meyer et al. (1998)
26.	*Trichoderma harzianum*	*Phytophtora capsici*	*Capsicum annum* (pepper)	Ahmed et al. (2000)
27.	*T. harzianum P1*	*Magnaporthe grisea*	Rice	Ngueko et al. (2002)
28.	*Trichoderma harzianum Rifai* T39	*Botrytis cinerea*	*Arabidopsis*	Korolev et al. (2008)
29.	*T. longibrachiatum*	*Phytophthora parasitica* var. *nicotianae*	Tobacco	Chang et al. (1997)
30.	*Trichoderma virens*	*Rhizoctonia solani*	Cotton	Howell et al. (2000)
31.	*T. viride*	*Pythium aphanidermatum*	Turmeric	Ushamalini et al. (2008)
32.	*Trichoderma* sp.	*Septoria tritici*	Wheat	Cordo et al. (2007)

(*Venturia inaequalis*) in apple, downy mildew (*Peronospora destructor*) in onion and late blight (*Phytophthora infestans*) in tomato (Tamma et al. 2011). *Penicillium simplicissimum* GP17-2 (Hossain et al. 2007) and *Phoma* sp. GS8-3 (Sultana et al. 2009), collected from the rhizosphere of zoysiagrass (*Zoysia tenuifolia*) has been shown to induce systemic defense responses in cucumber plants against several diseases. *Aspergillus ustus* induced systemic resistance against the necrotrophic fungus *Botrytis cinerea* and the hemibiotrophic bacterium *Pseudomonas syringae* DC3000 (Pst), through the induction of the camalexin (phytoalexin) and defense-related genes in *Arabidopsis* (Salas-Marina et al. 2011). Endophytic fungus, *Heteroconium chaetospira* suppressed clubroot (*Plasmodiophora brassicae*) on canola (Lahlali et al. 2014) and *Pseudomonas syringae* pv. *Maculicola* and *Alternaria brassicae* supresses disease on Chinese cabbage (Morita et al. 2003), it was observed that disease resistance was associated with an increase in PAL activity and several PR genes. The endophytic fungus *Piriformospora indica* isolated from Indian Thar desert has been shown to protect barley against fungal diseases (Waller et al. 2005). *Fusarium equiseti* GF183 had been shown to control the growth of *Fusarium oxysporum* f. sp. *spinaciae,* responsible for causing Fusarium wilt disease of spinach (Horinouchi et al. 2010). Arbuscular mycorrhizal fungi limit incidence of *Fusarium oxysporum* f. sp. *albedinis* on date palm seedlings by increasing nutrient contents, total phenols and peroxidase activities (Abohatem et al. 2011).

Induction of defense responses mediated by avirulent pathogenic fungi has also been described. Living spores and mycelia of non-pathogenic *Helminthosporium carbonum* and an incompatible race of *P. infestans* elicited the accumulation of rishitin and lubimin, a sesquiterpenoid phytoalexins, in potato tuber disks (Zook and Kuć 1987). Rice blast was suppressed when rice was pre-inoculated with a non-rice pathogen, *Bipolaris sorokiniana* and an avirulent rice pathogen, *Pyricularia oryzae* (Manandhar et al. 1998). Strawberry plants exposed to an avirulent isolate of *Colletotrichum fragariae* acquired resistance against a virulent strain of *C. acutatum*, as well as against *B. cinerea* (Salazar et al. 2013), whereas nonpathogenic *Verticillium dahlia* controlled wilt in strawberry plants (Diehl

et al. 2013). Nonpathogenic *F. oxysporum* had been shown to provide protection against Fusarium wilt on watermelon, tomato and cucumber (Benhamou et al. 2002). It has also shown to control root-knot nematode, *Meloidogyne incognita*, in tomato plants (Dababat and Sikora 2007). Systemic acquired resistance in Cavendish banana was also induced by infection with an incompatible strain of *F. oxysporum* f. sp. *cubense* (Wua et al. 2013).

5.4 Signaling Pathways Involved in ISR Triggered by Nonpathogenic Fungal Strains

To elucidate the signaling pathways, involved in induced resistance, researchers have applied several methods, including measuring hormone levels, the effect of specific inhibitors, studying the expression of inducible genes and using pathway-specific mutants or transgenic plants. Three major signal molecules were found to be involved in systemic defense responses of plants: salicylic acid (SA), commonly shown to be involved in SAR, induced by pathogens, whereas jasmonic acid (JA) and ethylene (ET) were shown to be involved in ISR, activated by beneficial microorganisms. Although the majority of studies on beneficial microbe-induced resistance points towards the role for JAs and ET in the regulation of the induced immune response (Van der Ent et al. 2009), several examples of SA-dependent SAR response, as well as multiple signaling pathway involving unknown signals, have been documented.

5.4.1 JA and ET Dependent Pathway

T. asperellum-203 induced resistance in cucumber plant *via* JA and ET signaling pathway (Shoresh et al. 2005). Treatment with an inhibitor of ET and JA strongly inhibited the protective effect of *Trichoderma* on plants, thus indicating that these hormonal signals are required. Further investigation in the involvement of these hormones in *Trichoderma*-mediated ISR was studied by analyzing the expression pattern of several defense-related genes regulated by these hormones, which include LOX, ETR1, *CTR1* and PAL. Their analysis revealed that *T. asperellum* modulates the expression of genes involved in the JA/ET signaling pathways of ISR. Similar to *T. asperellum*-203, another *Trichoderma* strain, T34, was shown to induce ISR in *Arabidopsis* plant *via* JA and ET dependent defense signaling pathway against *P. syringae* (Segarra et al. 2009). The study was conducted by testing SA biosynthesis mutants, sid2-1 and npr1-1, which were disrupted in SAR and both SAR and ISR, respectively. Mutant sid2-1 developed a similar level of resistance against the pathogen upon colonization of the roots by T34, indicating that T34-ISR functions independently of SA. However mutant npr1-1 was blocked in its ability to mount T34-ISR, indicating that the regulatory protein NPR1 is required for expression of this type of *Trichoderma*-induced resistance. Role of another regulatory protein, a root-specific transcription factor MYB72, was further demonstrated in the study of Segarra et al. 2009.

Examples from other PGPF include the endophytic fungus, *Heteroconium chaetospira*, which had been shown to upregulate several genes involved in the JA and ET pathways (Lahlali et al. 2014). Colonization of barley roots by an arbuscular mycorrhizal fungus, *Glomus intraradices*, leads to elevated levels JA biosynthesis enzyme (allene oxide synthase) and a jasmonate-induced protein (JIP23), followed by an increase in endogenous jasmonic acid levels (Hause et al. 2002). The nonpathogenic biocontrol agent *Pythium oligandrum* was also shown to activate JA and ET dependent signaling pathways in tomato; JA-responsive gene (*PDF1.2* and *JR2*) expression was upregulated (Kawamura et al. 2009).

5.4.2 SA-Dependent Pathway

Fusarium GF19-1 induced resistance in JA and ET mutant plants, jar1 and etr1 respectively, in *Arabidopsis* (Kojima et al. 2013); however, SA

biosynthesis mutant, NahG and mutant npr1, defective in regulatory protein NPR1, did not show induced protection against Pst, thus indicating that GF19-1 mediates systemic resistance *via* SA signaling pathway and NPR1 regulatory protein is required for the action. This mechanism of defense reaction was similar to SAR and was confirmed by observing accumulation of SAR markers PR-1, PR-2 and PR-5 in the leaves of *Arabidopsis* plants by GF19-1. Similar induction of SAR marker genes was also described for nonpathogenic *F. oxyporum*-mediated resistance against fusarium wilt in tomato (Kojima et al. 2013), indicating that nonpathogenic *Fusarium* isolates function as inducer of SAR.

5.4.3 Multiple Signaling Pathway

In *Arabidopsis*, root colonizing PGPF *Penicillium* sp. GP16-2 requires JA and ET as well as NPR1 regulatory protein, while its culture filtrate mediates ISR through SA, JA, ET and NPR1-dependent signaling pathways (Hossain et al. 2008). Study on another strain of *Penicillum* (*P. simplicissimum* GP17-2) and its culture filtrate suggests the possible contribution of additional signaling pathways as they are also found to control the expression of genes involved in both the SA and JA/ET signaling pathways (Hossain et al. 2007). Interaction between cucumber plant roots and *T. asperellum* strain T34, showed changes in both the SA and JA levels in the cotyledons to different degrees depending on the applied concentration of the fungi (Segarra et al. 2007). Cellulose extract of *Trichoderma longibrachiatum* has also shown to activate multiple signaling pathways, involving SA as well as JA/ET (Martinez et al. 2001). The epiphytic fungus *Pseudozyma aphidis* has also shown to induce JA, SA and NPR1-independent local and systemic resistance (Buxdorf et al. 2013). An aqueous extract of the mycelium of *Penicillium chrysogenum* has shown to induce resistance by some unidentified signaling pathways (Thuerig et al. 2006).

5.4.4 Priming of Plants against Pathogens

Apart from direct activation of defense responses in pathogen-infected plants, as in case of SAR or by nonpathogenic microorganisms as for ISR, characteristic of induced resistance is also associated with a sensitized state in which the plant responds more rapidly or more robustly against exposure to a pathogen. This state of enhanced capacity to activate stress-induced defense responses has been called the "primed" state of the plant (Conrath 2009).

Certain PR proteins are known to disrupt the pathogen cell wall and can be induced by pathogen attack, characteristic of SAR-mediated response. It was observed that *T. asperellum*-203 inoculated plants failed to induce a PR protein, β-1,3-glucanase indicating that SAR is not involved; however, the levels of PR gene expressions coding for β-1,3-glucanase, chitinase and peroxidase were increased when the Trichoderma-treated plants were further challenged by the pathogen (Yedidia et al. 2003), indicating that *Trichoderma* prepares the plant for subsequent pathogen infection. Priming of the plant parts for subsequent pathogen attack is also associated with T34 strain of *Trichoderma*. Treatment of *Arabidopsis* roots with T34 did not cause a direct transcriptional activation of SA- and JA-regulated genes, but with further pathogen attack resulted in increased lipoxygenase (LOX2) gene expression and formation of callose-containing papillae (Segarra et al. 2009). Similarly, the level of SA was raised after infection of *Fusarium* GF19-1 pretreated *Arabidopsis* with Pst, compared with the level of SA in plants exclusively infected with Pst (Kojima et al. 2013), indicating priming of the plant by GF19-1 treatment.

5.5 Elicitors/Signaling Molecules from Nonpathogenic Fungal Strains

Induction of a plant-mediated ISR response starts with the recognition of the microorganism. It is well documented that pathogenic and beneficial

microorganisms are specifically recognized by the plant through microbial signals called elicitors. Elicitors are designated Pathogen-Associated Molecular Patterns (PAMPs) when isolated from infectious agents or MAMPs (Microbe-Associated Molecular Patterns) from nonpathogenic microorganisms. MAMPs/PAMPs are essential structures for the microbes and owing to this they are conserved among pathogens, nonpathogenic and saprophytic microorganisms. MAMPs are recognized by pattern recognition receptors (PRRs), which are localized on the surface of plant cells (Bittel and Robatzek 2007; Newman et al. 2013). The recognition of these elicitor signals trigger a broad array of reactions, which leads to the activation of defense mechanisms.

Elicitors involved in systemic resistance triggered by fungal species are not so well characterized as compared to bacterial strains. Most of the elicitors have been isolated from pathogenic fungi or are present as common MAMPS in all groups of fungal species. Very few literature reports the presence of elicitors derived from nonpathogenic fungal strains, particularly species of *Trichoderma*. Summarized below is the list of some important elicitors, covering general as well as unique molecules reported from nonpathogenic fungal strains.

5.5.1 Chitin

Chitin, a polymer of N-acetyl-D-glucosamine, is a major component of fungal cell walls and has been recognized as a general elicitor of plant defense responses in both monocot and dicot plants for many years (Wan et al. 2008). In crop plants such as rice, wheat and tomato, chitin had been extensively shown to induce defense responses and protect them from pathogens (Shibuya and Minami 2001). During fungal infection, plant cells secrete chitinases that release chitin fragments, called chitooligosaccharides or chitin oligomers, from fungal cell walls, which can act as an elicitor to induce plant resistance mechanisms against the invading pathogen (Wan et al. 2008). Pretreatment of plants with chitooligosaccharides, either through seed treatment or foliar spray, has also been found to enhance plant resistance against various pathogens by regulating plant gene expressions. Plant receptors, CEBiP and CERK1, have been identified to recognize fungal chitin (Kaku et al. 2006; Wan et al. 2008), the extracellular domains of which consist of leucine-rich repeats (LRRs).

5.5.2 Chitosan

Chitosan, a deacetylated chitin derivative, also behaves like a general elicitor, inducing resistance against pathogens (Shibuya and Minami 2001). Putative receptors for chitosan are a chitosan-binding protein, possibly CEBiP, the chitin elicitor-binding protein (Iriti and Faoro 2009). Biological activity of chitosan depends on its physicochemical properties, such as deacetylation degree, molecular weight and viscosity. There are numerous reports of the protective effects of chitosan against pathogen infection in a range of crops, for chitosan seed treatment as well as foliar spray, e.g., has been shown to protect tomato plants from crown rot and root rot caused by *F. oxysporum* (Benhamou et al. 1994) and induction of defense mechanism in parsley, tomato and pea (Shibuya and Minami 2001).

5.5.3 Enzymes

Some of the enzymes present in the fungal strains have shown to trigger defense responses in plants, irrespective of their enzymatic activity. The most important one is Endo-β-1,4-xylanases that has been isolated and characterized from a variety of different plant pathogenic and nonpathogenic fungi (Enkerli et al. 1999). The xylanase from nonpathogenic *T. viride,* a 22-kD protein, has been extensively studied for their elicitor activity. They have shown to induce defense responses in tomato and tobacco plants (Hanania and Avni 1997; Enkerli et al. 1999). In suspension-cultured cells of tobacco and tomato they induce rapid medium alkalinization, oxidative burst, and ethylene biosynthesis. Chemical crosslinking of this

xylanase to microsomal membranes from *Nicotiana tobacum* revealed a 66-kDa protein complex, which may function as the receptor of xylanases (Hanania and Avni 1997).

From *T. virens* six peptides ranging from 6.2 to 42 kDa had been isolated and shown to have elicitor activity, causing activation of peroxidase as well as terpenoid phytoalexin biosynthesis in cotton. A 18-kDa protein was found to have sequence similarity with a serine proteinase of *Fusarium sporotichioides,* while another one was crossreactive with xylanase (Hanson and Howell 2004). Another class of enzyme, endopolygalacturonases from *Trichoderma*, have shown to generate ISR response in *Arabidopsis* (Zhang et al. 2014). Endopolygalacturonase are a type of pectinases that hydrolyze the homogalacturonan domain of pectic polysaccharides, causing cell-wall decomposition and tissue maceration (Boudart et al. 2003). *Trichoderma* activated and heat-denatured cellulases were found to elicit defense responses in melon through the activation of the SA and ET signaling pathways (Martinez et al. 2001).

5.5.4 Ergosterol

Ergosterol is a MAMP which triggers lipid-based signaling pathways. It is a 5,7-diene oxysterol, found commonly in all fungal cell membranes (Klemptner et al. 2014). Plants either possess an ergosterol receptor or ergosterol uptake leads to perturbations of a lipid raft structure because of the ability of this sterol to form very stable microdomains. They act as a MAMP molecule in tobacco and tomato plants, eliciting the synthesis of phytoalexins. Five sesquiterpenoid phytoalexins (capsidiol, lubimin, phytuberin, rishitin and solavetivone) induced by ergosterol had been identified, indicating activation of the terpenoid pathway by this molecule (Klemptner et al. 2014).

5.5.5 Peptaibols

Peptaibols are a class of linear, short-chain-length (≤20 residues) peptides of fungal origin (Mukherjee et al. 2011), containing an α-amino isobutyric acid, acetylated N-terminus and an amino alcohol at the C-terminus. There are a few reports indicating that peptaibols may also represent a novel class of plant elicitors. Exogenous application of the 20-residue peptaibol alamethicin, produced by *T. viride*, has been shown to induce defense responses in *Phaseolus lunatus* (lima bean) (Engelberth et al. 2001) and *A. thaliana* (Viterbo et al. 2007), by synthesizing volatile compounds and salicylate. Chrysospermin, a 19-residue peptaibol from *Apiocrea chrosospermin*, protected *N. tabacum* from tobacco mosaic virus infection (Kim et al. 2000). The 18mer peptaibols from *T. virens* elicited plant defense responses in cucumber against the leaf pathogen *P. syringae pv. lachrymans* by upregulating hydroxyperoxide lyase, phenylalanine ammonia lyase and peroxidase gene expression (Viterbo et al. 2007).

5.5.6 Avr Homologues

The protein products of *Avr* genes have been identified in a variety of avirulent fungal and bacterial plant pathogens. They usually function as race-specific elicitors that are capable of inducing defense reactions in plants. *Trichoderma*-specific *avr* genes has been investigated by proteome analysis and several putative proteins having corresponding avr function have being isolated and tested (Chinnasamy 2006). In *Trichderma* T-22, two proteins were identified that are homologues of Avr4 and Avr9 identified in *Cladosporium fulvum* (Chinnasamy 2006).

5.5.7 Cerato-Platanins

Cerato-platanins are small, secreted, cysteine-rich proteins that have been correlated in virulence of certain plant pathogenic fungi (Hermosa et al. 2012). These proteins have been identified in *Trichoderma* sp., Sm1 from *T. virens* and Epl1 from *T. atroviride*. The hydrophobin-like elicitor Sm1 isolated from T. virens Gv29-8 was shown to induce ISR in maize and cotton (Djonović

et al. 2006, 2007). Both the monocot and the dicot plant species generated enhanced levels of resistance against *Colletotrichum graminicola*. The resistance mechanism was further proved by creating a Sm1 deletion mutant, which did not protect maize plants against *C. graminicola*, while overexpression of Sm1 enhanced the resistance-inducing capacity of the fungus. In maize, it was demonstrated that Sm1 activates defense mechanisms through JA and green leafy volatile (GLV) signaling pathways and increases the expression profiles of the marker genes.

5.5.8 Elicitins

Elicitins, which are small peptides isolated from mycelia of several pathogenic fungal species, elicit defense responses in plants (Mohamed et al. 2007). The cell-wall protein fraction isolated from nonpathogenic fungus, *Pythium oligandrum* was shown to be made of two glycoproteins, POD-1 and POD-2, which were structurally similar to class III elicitins. In tomato plants, this fraction activates JA and ET dependent signaling pathways and provides resistance against *Ralstonia solanaceraum* (Kawamura et al. 2009).

5.5.9 Swollenin

Swollenin, expansin-like protein with a cellulose-binding domain, is involved in root colonization. Swollenin TasSwo, present in *T. asperelloides*, stimulates defense responses in cucumber roots and leaves providing protection against *B. cinerea* and *P. syringae* (Brotman et al. 2008).

5.5.10 Other Secondary Metabolites

Harzianolide isolated from *T. harzianum* strain SQR-T037 (Cai et al. 2013) protects tomato plants from the pathogen *Sclerotinia sclerotiorum*. It increases the activity of some defense-related enzymes and induces the expression of genes involved in the SA (PR1 and GLU) and JA/ ET (JERF3) signaling pathways (Cai et al. 2013). 6-pentyl-a-pyrone and harzianopyridone, isolated from *Trichodema* sp., activate plant defense mechanisms and regulate plant growth in pea and canola (Hermosa et al. 2012).

5.5.11 Volatile Organic Compounds

Volatile organic compounds (VOC) released from some PGPF have shown to induce defense mechanisms in plants. *Talaromyces wortmannii* FS2 emitted a terpenoid-like volatile compound, β-caryophyllene, which induced resistance against *Colletotrichum higginsianum* in *Brassica campestris* (Yamagiwa et al. 2011). VOC identified from PGPF, *Phoma* sp., *Cladosporium* sp. and *Ampelomyces* sp. (Naznin et al. 2014) have shown to protect *Arabidopsis* plants against Pst by inducing systemic defense mechanism. The most important compounds were m-cresol and methyl benzoate isolated from *Ampelomyces* sp. and *Cladosporium* sp.

5.5.12 Extracts or Extracellular Products

Extracts or extracelluar product of fungal strains had been shown to elicit defense responses against pathogens (Sultana et al. 2008, 2009). Addition of *T. viride* crude elicitor extract to grapevine cell cultures induced hypersensitive response and phytoalexin (resveratrol) production (Calderon et al. 1993). Heat stable extracts of *T. longibrachiatum* induced resistance in tobacco seedlings to the pathogen *Phytophthora parasitica* var. *nicotianae*, followed by expression of pathogenesis-related genes (Chang et al. 1997). Culture filtrate of *Penicillium* sp. (Hossain et al. 2007) and *Phoma* sp. (Sultana et al. 2009) had also shown to induce defense-related signaling pathways. A cell-wall extract from the endophytic fungus *P. indica* promotes growth of *Arabidopsis* seedlings and induces intracellular calcium elevation in roots (Vadassery et al. 2009). Yeast extracts sprayed on to barley leaves provided control over powdery mildew (Reglinski

et al. 1994). Metabolites from *Penicillium janczewskii* culture filtrate elicit resistance to stem rot in melon and cotton (Madi and Katan 1998).

5.6 Conclusion

With the discovery of disease resistance inducers, that induce a localized or systemic resistance in susceptible plants, an alternative to synthetic chemicals in plant protection has been obtained. This mechanism could be considered as one of the most beneficial strategies to control plant diseases, because it is triggered only upon activation, thus lowering the burden of constitutive production of defensive chemicals in plants. With the observation of defense mechanisms triggered by phytopathogens, many potential microbial strains had been screened and tested for this purpose. Based on those studies several elicitor molecules have been isolated and are currently utilized in agriculture. Among these microorganisms, nonpathogenic fungi have received little attention as potential inducers of resistance as compared to their similar group of bacterial strains. However, recently some beneficial fungi such as *Trichoderma* sp. mycorrhizal strains, *Phoma* sp., etc. are used as potential biological control agents and have led to the proposal that besides their recognized antagonistic properties, they could also act as elicitors of plant defense reactions. Therefore integration of the formulations of these ISR eliciting beneficial fungal strains in disease management programs are important and will help in long run. Further work is needed to identify more ISR-inducing fungal strains present in the environment and evaluate their mode of action. Elicitors from these microbes need to be identified and isolated, so that they can be directly applied in the crop fields for better production.

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Stress Management Practices in Plants by Microbes

6

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Abstract

Plants are constantly subjected to biotic and abiotic stress factors, from their planting time up to the harvesting, transport, storage and consumption of plant products. These stresses exert deleterious harmful effects on crop health as well as cause huge losses to their production worldwide. To combat these stress factors, researchers all around the globe are involved in procuring management practices ranging from traditional genetics and breeding techniques to present day available novel biotechnological tools. Use of microorganisms is one such method by which both abiotic and biotic stress can be tackled in an economical, ecofriendly and successful manner. Plant growth-promoting rhizobacteria (PGPR) are the bacteria living in rhizosphere region and promoting plant growth and suppressing stress components as well. Different microorganisms acquire different mechanisms to fight with these plant stresses. In this chapter, an effort has been made to impart the knowledge about the abiotic and biotic stress factors, their management in an efficient and novel way.

Keywords

Biotic stress • Abiotic stress • PGPR • Microbes • Bioagents • Bacteria

6.1 Introduction

Stress is a physiological condition caused by factors that affect the equilibrium process (Gaspar et al. 2002). The pliability of normal processes develops reaction to the environmental fluctuations that can be predicted over daily and seasonal cycles, which means every change in a component from its normal range is not likely to cause stress. Stress affects the normal metabolic

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_6

processes resulting in injury, disease or physiological changes. Plants are influenced by different environmental stresses like drought, low temperature, salt, flooding, heat, oxidative stress and heavy metal toxicity during their cultivation (Jaleel et al. 2009).

Agriculture is one of the highly unprotected sectors to climate deviation. Enhanced affect of abiotic and biotic stresses has evolved as an important cause for static crop production. There is considerable evidence of yield reductions of wheat and paddy in many regions of South Asia due to enhanced water stress, decreased number of rainy days and increased air temperature. The average temperature has increased by 0.57 °C in the last 100 years in the Indian sub-continent and it is expected to rise to a maximum of 2.5 °C by 2050 and 5.8 °C by 2100. Also the irrigation requirement in arid and semi-arid areas is estimated to rise by 10 % with every 1 °C increase in temperature. Besides high temperature, droughts, high CO_2, increased rainfall, floods, cold and heat waves, and cyclones are the natural calamities that result in economic depletion and are consequences of global warming. These factors affect crops quantitatively and qualitatively and also put critical pressure on land and water resources (Grover et al. 2011).

Stresses caused by various environmental factors including light, UV, temperature extremes, freezing, drought, salinity, heavy metals and hypoxia result in substantial crop losses worldwide (Boyer 1982; Mahaian and Tuteja 2005; Mittler 2006). These abiotic stresses might increase in the near future owing to the global climate change. Plant growth and development is affected by the various environmental factors (Wahid et al. 2012). Abiotic stresses, including temperature, extremes salinity and drought, are serious intimidation to the sustainability and productivity of economic plants. Current climatic model predicts that global air temperature may increase by 1.1–6.4 °C with doubling of atmospheric CO_2 (Kim et al. 2007; Lobell and Field 2007).

Around the world, abiotic and biotic stresses are largely affecting crop productivity. Due to imbalance in environmental conditions, stresses like drought, rains, floods, heat waves and frost damages can increase in future. To combat with these stresses wide range of modification plans are required. By well-planned use of available resources and crop improvement practices for producing better varieties, we can fight with abiotic stresses up to some level. But such strategies are time consuming and costly. We should formulate simple, effective and low-cost biological methods for managing abiotic stresses. Microorganisms possess qualities like endurance to extreme conditions, ubiquity, genetic diversity, relationship with plants and thus can play a pivotal role in this aspect. Through various modes of action like induction of osmoprotectants and heat shock proteins etc. in plant cells, microbes can affect plants' response to abiotic stresses. Use of these microorganisms can diminish plant stresses and they can also be used as important models for becoming aware of stress tolerance, adaptation and response mechanisms that can be transferred into plants to combat with climate change because of plant stresses (Grover et al. 2011).

Plants exposed to various climatic factors, in order to sustain, have developed different mechanisms (Rejeb et al. 2014). Physiological changes in plants are due to exposure to many types of biotic and abiotic stresses (Heil et al. 2002, Swarbrick et al. 2006; Bolton 2009; Massad et al. 2012) which finally causes reduction in plant yield (Shao et al. 2008). Abiotic stress effects plant health and causes heavy losses. Biotic stress means harmful effects due to pathogen infection in plants (Strauss and Zangerl 2002; Maron and Crone 2006; Maron and Kauffman 2006; Mordecai 2011). Growth stage of plants (Zhang et al. 2013) and climatic factors (Liu et al. 2008) play an important role in plant's reaction to abiotic and biotic stresses. Depending on the nature of abiotic stress and pathogen, defense mechanism gets altered in plants. Moreover, signaling compounds are increased when plants are exposed to both abiotic and biotic stress simultaneously, e.g. cross-tolerance.

6.2 Plant Stress

Plants' sensitivity towards abiotic and biotic stresses causes yield loss and plants devise many kinds of modifications to adapt in stressed conditions (Rejeb et al. 2014).

6.2.1 Abiotic Stresses in Plants

Plants' exposure to abiotic factors results in abiotic stresses reducing crop productivity (Heil and Bostock 2002) but it also affects ecological distribution of plants (Chaves et al. 2003). Abiotic stress examples are fluctuations in water, temperature, soil nutrients, toxic substances, light and soil texture (Versulues et al. 2006). Intergovernmental Panel on Climate Change 2012 (IPCC 2012) has predicted that various abiotic stresses like temperature extremes, drought, floods, climatic conditions and land-decline can cause huge losses in agriculture sector in many parts of developing countries (Field et al. 2012).

Among the various environmental conditions, cold, drought and salinity are most severely affecting plants resulting in heavy economic losses (Beck et al. 2007). Primary and secondary stresses are the result of primary and secondary damages; for example secondary stress and damage caused by ROS (reactive oxygen species, Allen 1995) is the consequence of electron transport rate fluctuations and the metabolic consumer activity of the reductive power. Similarly, the secondary stress occurs from primary stressors such as cold or excess of light energy (Huner et al. 1998).

The impact of natural and man-made issues (Eitzinger et al. 2010) can be seen in the form of average global temperature increase by 2–4 °C at the last of twenty-first century (IPCC 2007). One of the important causes of this temperature rise is the release of green house gases (GHG) (Maraseni et al. 2009; Smith and Olesen 2010). Due to this temperature fluctuation, various crops at different developmental stages are exposed to heat stress (Watanabe and Kume 2009).

6.2.2 Biotic Stresses in Plants

Apart from abiotic stress factors, plants are exposed to many kinds of pathogens including fungi, bacteria, viruses and nematodes and herbivores (Atkinson and Urwin 2012). The environmental conditions are likely to affect the habitual place of pests and pathogens. For instance, dispersal of pathogens is increased due to temperature extremes (Bale et al. 2002; Luck et al. 2011; Madgwick et al. 2011; Nicol et al. 2011). It is also reported that abiotic stress factors decrease the defense potential of plants and induce proneness to pathogen attack (Amtmann et al. 2008; Goel et al. 2008; Mittler and Blumwald 2010; Atkinson and Urwin 2012). In coming times, it is estimated that both abiotic and biotic stresses alone and in combination will attack crop plants with more power (Suzuki et al. 2014).

Biotic stress is a result of damage caused to the plants by other living organisms including bacteria, viruses, fungi, parasites, beneficial and harmful insects and weeds. Plants are under constant assault by biotic agents, including viral, bacterial and fungal pathogens, parasitic plants and insect herbivores, with enormous economic and ecological impact (Pimentel 2002). Biotic stress affects plant population dynamics and ecosystem nutrient cycling as well. Fungi, insects, viruses, bacteria and parasitic weeds can cause enormous loss to crop production (Mehta et al. 2012). The impact of aerial fungal diseases on crop yield differs with time and cropping areas. Rusts, downy mildews and powdery mildews are the major foliar diseases that have deleterious effects on crop production. For instance, species belonging to rust fungi can infect grains, e.g. *Puccinia* species, like *Puccinia graminis* on wheat rust, *P. sorghi* on maize and forage legumes; *Uromyces* species, like *U. appendiculatus* on common bean, lentil and *U. vignae* on cowpea. Different methods can be used for managing this disease as resistance sources are not available (Ramteke et al. 2004).

Root rot, caused by *Aphanomyces euteiches, Rhizoctonia solani, Fusarium solani* and wilt,

caused by many formae speciales of *Fusarium oxysporum* are the most critical soil-borne diseases in pea, chickpea, lentil, fababean and lupin (Infantino et al. 2006). Damping-off, usually caused by either *Rhizoctonia solani* or *Pythium* spp., can cause about 80 % of plant demise (Wang et al. 2003). Fusarium root-rot (caused by *Fusarium* spp.) can too result in rigorous seedling fatalities particularly in tomato and lentils (Hamwieh et al. 2005). The production of tomato and lentil (Bayaa 1997) is majorly effected by Fusarium wilt (caused by *F. oxysporum*) where leaf chlorosis, wilting and death occurs at seedling and adult stage of plants. Similarly, southern stem rot (*Sclerotium rolfsii*) and white mold (*Sclerotinia sclerotiorum*) can result in seedling and pod rots in warm and cool climate respectively (Kolkman and Kelly 2003).

The co-evolution of plants and the pathogens results in development of defense mechanism in plants. Whenever plants are attacked by pathogens they have to balance between their developmental and defense requirements (Zangerl and Berenbaum 2003; Berger et al. 2007). With respect to food security, worldwide research focus is required to develop crops that can give sustainable yields along with the capability to survive harsh abiotic (Duque et al. 2013) as well as biotic stress situations.

6.3 Practices to Mitigate Plant Stresses

Diverse biotic and abiotic stresses are responsible for the badly affected production and yield of a number of crops. Massive financial fatalities are accountable globally due to these stresses. As biotic and abiotic stresses are affecting agriculture adversely, there is need to develop plants that can tolerate stress with high yields. For stress tolerant plant production, presently tissue culture based *in vitro* selection has been developed as an economic and effective method. Various substances like NaCl (for salt tolerance), PEG or mannitol (for drought tolerance) and pathogen culture filtrate, phytotoxin or pathogen itself (for disease resistance) are used in culture media for making stress tolerant plants. Stimulation of genetic distinction between cells, tissues or organs in cultured and regenerated plants is needed for *in vitro* selection. The selection of somaclonal variations appearing in the regenerated plants may be genetically established and useful in crop improvement. To endure under strain circumstances plants have developed numerous biochemical and molecular mechanisms such as ROS (reaction oxygen species) creation and elimination in plants (Rai et al. 2011).

Key for crop improvement is conventional breeding technologies and appropriate management practices. To stimulate stress tolerance in plants, traditional breeding programs are used to incorporate good genes of interest from inter crossing genera and species into the crops (Purohit et al. 1998).

6.3.1 Management Strategies to Reduce Abiotic Stress

Plants react to temperature changes at cellular, tissue and organ levels. The main survival responses to high temperature stress are photosynthetic acclimation to heat stress, production and buildup of primary and secondary metabolites, generation of stress proteins. Heat shock protein (hsp) genes, dehydrins (dhn), senescence-associated (sag) genes, stay green (sgr) genes are expressed in reaction to heat stress. Plants exhibit various adaptations like preservation of membrane strength, scavenging of ROS, production of enzymatic and non-enzymatic antioxidants and amendment of companionable solutes against heat stress. Mass screening and morphological and biochemical markers-assisted selection, recognition, and mapping of QTLs conferring heat resistance, conventional and molecular breeding, and exogenous use of osmoprotectants and stress-signaling agents can be used for heat tolerance in plants (Wahid et al. 2012). To overcome pH stress, it is significant to alter the nutrient accessibility as well as the soil properties to modify the pH of the soil. For example, pH of soil can be neutralized by addition of lime (calcium or magnesium carbonate) (Mehta et al. 2012).

In plants, drought stress causes changes like leaf size decrease, stems expansion and root propagation, disturbs plant water relations and reduces water-use effectiveness. CO_2 assimilation by leaves is decreased by closing of stomata, membrane spoilage and disturbed action of enzymes like those of CO_2 fixation and adenosine triphosphate synthesis. Plants exhibit a variety of mechanisms to endure drought stress, such as shortened water loss by amplified diffusive resistance, improved water uptake with plentiful and deep root systems and its efficient use, and smaller and tender leaves to lessen the transpirational loss. Nutrients are also helpful in this aspect, like potassium ions in osmotic regulation, silicon for improved root endodermal silicification and cell water equilibrium enhancement. Plant growth regulators like salicylic acid, auxins, gibberellins, cytokinin and abscisic acid can also adjust the plant reaction towards drought. Enzymes like polyamines, citrulline behave as antioxidants and lessen the undesirable effects of water scarcity. Drought-responsive genes and transcription factors like dehydration-responsive element-binding gene, aquaporin, late embryogenesis abundant proteins and dehydrins have been reported. Mass screening and breeding, marker-assisted selection and exogenous application of hormones and osmoprotectants to seed or plants are the methods for overcoming the problem of drought stress (Farooq et al. 2009).

6.3.2 Management Strategies to Reduce Biotic Stress

In biological control, antagonistic microbes are employed to improve plant healthiness. Persistent demonstration of connections amongst the plant, the pathogen, the biocontrol agent, the microbial population on and in the region of the plant, and the physical surroundings is exhibited through disease inhibition by biocontrol agents. The use of biocontrol agents such as bacteria viz., *Pseudomonas* and *Bacillus* and the fungi *Trichoderma* symbolize an array of existence approaches and means of disease inhibition.

To diminish the biotic stress, biotechnological advances are also used. Many molecular marker-related methods have been used for managing biotic stresses like Random Amplified Polymorphic DNA (RAPD), Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), and Simple Sequence Repeat (SSR). Because of these, genetic maps for several species were recognized in which impending resistance and/or tolerance loci or QTLs have been located. This also provides knowledge about the number, chromosomal location and individual or interactive effects of the QTLs involved that strengthens the genetic management of specific resistance and/or tolerance in many crops. These areas of expertise have recognized precise molecular markers, which may possibly be used in breeding plan through Marker-Assisted Selection (MAS) to augment biotic stress tolerance. Diers (2004) used the MAS for the breeding of resistant soybean to cyst nematode and similar markers have also been used by Mutlu et al. (2005), Yang et al. (2002) and Yang et al. (2004) for the resistance of pinto bean to common bacterial blight, resistant of narrow-leafed lupin (*Lupinus angustifolius* L.) to phomopsis stem blight and anthracnose. Besides, the gene pyramiding strategy aided by MAS can be a proficient technique when resistance is bestowed by single gene and/or easily conquered by novel pathogen races (Mehta et al. 2012).

Plant growth-promoting bacteria (PGPB) can encourage plant growth either directly or indirectly. Inhibition of plant disease (bioprotection), better nutrient accessibility (biofertilization), or construction of phytohormones (biostimulation) are numerous diverse strategies for promoting plant production (Saharan and Nehra 2011).

Directly these bacteria can regulate functioning of plants by mimicking production of plant hormones or those that make minerals and nitrogen further obtainable in the soil, e.g. the leguminous symbionts *Rhizobium* (Hirsch and Kapulnik 1998; Saharan and Nehra 2011). The siderophore production or volatiles (2, 3-butanediol and acetoin) or different antibiotic compounds, or induction of plant-mediated induced systemic resistance (ISR) are the indirect proponent of plant growth (Saharan and Nehra 2011).

6.3.3 Role and Mechanism of Microbes to Reduce/ Conquer the Stress

Productivity of agricultural crops as well as the microbial activity in soil is being hampered by these stresses. The change in climatic conditions such as prolonged drought, intense rains, flooding, high temperatures, frost and low temperatures, which are expected to escalate in future, will significantly affect plants and soil microorganisms. The different stress factors have a significant influence over the performance of microorganisms. Mycorrhizal and/or endophytic fungi can interact with many plant species and thereby significantly contribute to the adaptation of these plants to a number of environmental stresses (Rodriguez et al. 2008). These conditions include drought, heat, pathogens, herbivores, or limiting nutrients.

Extensive research has been carried out on occurrence and functional diversity of agriculturally important microbes in stressed environments as reviewed by several authors (Grahm 1992; Venkateswarlu et al. 2008). The occurrence of *Rhizobium, Bradyrhizobium, Azotobacter, Azospirillum, Pseudomonas* and *Bacillus* has been reported from desert ecosystems, acid soils, saline and alkaline areas and highly eroded hill slopes of India (Tilak et al. 2005; Selvakumar et al. 2009; Upadhyay et al. 2009). Microorganisms could play an important role in adaptation strategies and increase of tolerance to abiotic stresses in agricultural plants. The impact of abiotic stresses (drought, low temperature, salinity, metal toxicity, and high temperatures) on plants can be minimized through the production of exopolysaccharates and biofilm formation by plant growth-promoting rhizobacteria (PGPR) which remain associated with plant roots. Different mechanisms like induction of osmoprotectors and heat shock proteins are mediated through their rhizospheric microorganisms when plants are exposed to stress conditions.

A variety of mechanisms have been proposed behind microbial elicited stress tolerance in plants (Table 6.1). The production of indole acetic acid, gibberellins and some unknown determinants by PGPR helps to increase the root length, root surface area number of root tips, leading to enhanced uptake of nutrients resulting in improved plant health under stress conditions (Egamberdieva and Kucharova 2009). In addition to this, PGPRs also help to enhance plant growth under saline conditions (Glick et al. 1997; Yildirim and Taylor 2005; Barassi et al. 2006).

The synthesis of cytokinin and antioxidants by the strains of PGPR can cause the building up of abscissic acid (ABA) and decomposition of reactive oxygen species (ROS). Oxidative stress tolerance has been found associated with the enhanced level of antioxidant enzymes (Stajner et al. 1997). There is effect of ethylene on different processes of plants and ethylene synthesis in plants is dependent on environmental factors and on various biotic and abiotic stresses (Hardoim et al. 2008). In the biosynthetic pathway of ethylene, S-adenosylmethionine (S-AdoMet) is converted by 1-aminocyclopropane-1-carboxylate synthase (ACS) to 1-aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ethylene. The plant hormone such as ethylene which endogenously regulates plant homeostasis under stress conditions results in reduced root and shoots growth. Plants supplemented with nitrogen and energy are also prevented from harmful ethylene effect, improved plant stress due to the degeneration of ACC by ACC deaminase enzyme produced from bacterial cells (Glick 2007). Saleem et al. (2007) have reviewed the role of PGPR containing ACC deaminase, in stress agriculture. Inoculation with ACC deaminase containing bacteria induces longer roots which might be helpful in the uptake of relatively more water from deep soil under drought stress conditions, thus increasing water-use efficiency of the plants under drought conditions (Zahir et al. 2008).

The volatiles emitted by PGPR, down-regulate hkt1 (High Affinity K^+ Transporter 1) expression in roots but upregulates it in shoots, orchestring lower Na^+ levels and recirculation of Na^+ in the whole plant under salt conditions (Zhang et al. 2008). By synthesis of the metabolite 2R, 3R-butanediol, the inoculation of *Pseudomonas chlororaphis* O6 in *Arabidopsis thaliana* roots resulted in increased abiotic and biotic stress tol-

Table 6.1 Mechanism shown by microorganisms against abiotic stress tolerance in crop plants

Organism	Crop	Type of stress	Mechanism	References
Pantoea agglomerans	Wheat	Drought	Rhizosphere soil aggregation through EPS	Amellal et al. (1998)
Paenibacillus polymyxa	Arabiodopsis	Drought	Induction of stress resistant gene ERD 15	Timmusk and Wagner (1999)
Rhizobium sp.	Sunflower	Drought	Soil aggregation through EPS	Alami et al. (2000)
Pseudomonas putida, Enterobacter cloacae, P. putida	Tomato	Flooding	Synthesis of ACC-deaminase	Grichko and Glick (2001)
PGPR	Chickpea	Metal toxicity	Sequestration of metal ions	Gupta et al. (2004)
Azospirillum sp.	Wheat	Drought	Improved Water relations	Creus et al. (2004)
Achromobacter piechaudii	Tomato	Salt, drought	Synthesis of ACC-deaminase	Mayak et al. (2004a)
Variovorax paradoxus	Pea	Drought	Synthesis of ACC-deaminase	Dodd et al. (2005)
Piriformaspora indica	Barley	Salinity	Elevated antioxidative capacity	Waller et al. (2005)
AM Fungi	Sorghum	Drought, salinity	Improved Water relation	Cho et al. (2006)
B. amylolequifaciens, B. insolitus, Microbacterium sp., *P. syringae*	Wheat	Salinity	Restricted Na^+ influx	Ashraf et al. (2004)
Paraphaeosphaeria quadriseptata	Arabiodopsis	Drought	Induction of HSP	McLellan et al. (2007)
Scytonema	Rice	Coastal salinity	Gibberellic acid & extra cellular products	Rodriguez et al. (2006)
Burkholderia phytofirmans PsJN	Grapevine	Low temperature	Synthesis of ACC-deaminase	Ait Bakra et al. (2006)
AM fungi & *Bradyrhizobium*	Dragon blood	Flooding	Development of adv. roots, aerenchyma and hyper trophied lenticels	Fougnies et al. (2007)
Brome mosaic virus	Rice	Drought	Unknown	Marquez et al. (2007)
Methylobacterium oryzae, Burkholderia sp.	Tomato	Ni & Cd toxicity	Reduced uptake and translocation	Madhaiyan et al. (2007)
Pseudomonas fluorescens	Groundnut	Salinity	Synthesis of ACC-deaminase	Saravanakumar and Samiyappan (2007)
P. putida	Canola	Low temperature	Synthesis of ACC-deaminase	Chang et al. (2007)
P. polymyxa and *Rhizobium tropici*	Common bean	Drought	Change in hormone balance and stomatal conductance	Figueiredo et al. (2008)
Pseudomonas sp.	Pea	Drought	Decreased ethylene production	Arshad et al. (2008)
Pseudomonas mendocina and *Glomus intraradices*	Lettuce	Drought	Improved antioxidant status	Kohler et al. (2008)

(continued)

Table 6.1 (continued)

Organism	Crop	Type of stress	Mechanism	References
Pseudomonas sp. AMK-P6	Sorghum	Heat	Induction of heat shock proteins and improved plant biochemical status	Ali et al. (2009)
Pseudomonas putida P45	Sunflower	Drought	Improved soil aggregation due to EPS production	Sandhya et al. (2009a, b)
Bacillus megaterium and *Glomus* sp.	*Trifolium*	Drought	IAA and proline production	Marulanda et al. (2007)
Achromobacter piechaudii	Tomato	Salt	ACC-deaminase	Mayak et al. (2004b)
Azospirillum	Maize	Salt	Amino acid and proline production	Hamdia et al. (2004)
Arthrobacter sp., *Bacillus* sp.	Pepper	Osmotic stress	IAA and proline production	Sziderics et al. (2007)
Bacillus polymyxa, Mycobacterium phlei, Pseudomonas alcaligenes	Maize	Nutrient deficiency	Improved nutrient uptake	Egamberdiyeva (2007)

erance. Studies with Arabidopsis mutant lines indicated that induced drought tolerance requires salicylic acid (SA), ethylene and jasmonic acid-signaling pathways (Cho et al. 2008).

Arbuscular mycorrhizal (AM) fungi alleviate the effects of drought and salinity stresses through osmoregulation and proline accumulation. AM symbiosis plays an important role in increasing the plant resistance against water deficit and drought stress through the alteration of plant physiology and the expression of plant genes (Subramanian and Charest 1998; Ruiz-Lozano and Azcon 2000). There are reports of AM-induced increases in drought tolerance, involving both increased dehydration and dehydration tolerance (Allen and Boosalis 1983). The role of abscissic acid (ABA) had been suggested behind AM-mediated stress response of plants (Aroca et al. 2008). In non-AM plants, it was observed that ABA content in the shoots increased as well as there was more expression of certain stress marker genes by the use of external source of ABA. However in AM plants such use of exogenic ABA reduced the ABA content in their shoots and did not result in increased expression of stress genes. Co-inoculation of lettuce with PGPR *Pseudomonas mendocina* and *G. intraradices* or *G. mosseae* augmented an antioxidative catalase under severe drought conditions, suggesting that they could be used in inoculants to alleviate the oxidative damage (Kohler et al. 2008).

6.4 Advantages of Microbes over Other Practices

A group of beneficial microbes has been reported by the various/different researchers from different agro ecosystem in the past. Some of these microbes are playing an important role in stimulating the plant growth and increasing the crop yields during adverse environmental conditions. Plant growth-promoting bacteria (PGPB) are able to promote the plant growth, production and nutrient availability through various mechanisms. For example, certain bacteria can cause elevation of plant growth by increasing nutrient uptake from soil or by production of some substances similar to plant hormones. The PGPR can affect plant growth and development in direct, indirect or collective manner (Joseph et al. 2007; Yasmin et al. 2007). For instance, few PGPR are known to alleviate growth of *Arabidopsis thaliana* by

exudation of compounds like 2, 3-butanediol and acetoin (Ryu et al. 2003). The inoculation of diazotroph bacteria in cotton resulted in promotion of the seed cotton yield, plant height and population of soil microorganisms (Anjum et al. 2007). Similarly in apple, it has been found that the strength and quality of rooting is increased due to collective use of IBA, bacteria and carbohydrates (Karakurt et al. 2009).

Many bacteria present in rhizoplane are able to utilize root exudates efficiently. Increased fertilizer use efficiency and lower fertilizer rates can be achieved by using PGPRs alone or in combination with AMF (Adesemoye et al. 2009). In rice, increased growth was observed with the inoculation of PGPR isolates (Ashrafuzzaman et al. 2009). In chickpea also, better development and production occurred due to the use of PGPRs as biofertilizers (Rokhzadi et al. 2008). There are two different kinds (direct and indirect) of effect of PGPR on plant growth. Directly PGPR can make available their synthesized products to the plant or they can help plants in taking up nutrients (Glick 1995). Indirectly PGPR can reduce or block the attack of harmful plant pathogens and thus enhance the growth of plants. Bacteria like *Pseudomonas fluorescens* and *P. putida* produce siderophores, which bind iron and facilitate its transport from the environment into the microbial cell (Fig. 6.1).

Rice (*Oryza sativa*) is one of the important crops grown globally and specially in Asian continent as noted by Kumar et al. (2011). For the proper growth and development of rice, there is more need of nitrogen (Sahrawat 2000). In Vietnam, rise in growth rate and production of rice was observed with the use of PGPR-based commercial product BioGro (Nguyen et al. 2003; Nguyen 2008). Similarly in India, the commercial PGPR formulation Ecomonas was found to decrease the incidence of rice sheath blight caused by the fungus *Rhizoctonia solani* over the control treatment by 37.7 % and a significant increase in yield was also noticed. In chickpea an increase in plant height, dry weight, number of pods and nutrient content was reported by the inoculation of vesicular arbuscular mycorrhizal fungi (*Glomus mosseae, G. fasciculatum, Acaulospora laevis* and *Gigaspora gilmorei*) in India (Kumar et al. 2009).

In another study, to access the role of PGPRs on nutrient uptake two rhizospheric *Pseudomonas* spp. were taken and their bioassociative effect with root nodulating symbiotic nitrogen fixer *Rhizobium leguminosarum*-PR1 on plant growth and nutrients uptake by lentil (*Lens culinaris* L.), was studied under greenhouse conditions. In *Pseudomonas* treated plants, more vigorous vegetative growth with increase in nodulation, leghemoglobin content, physiologically available iron, total iron, chlorophyll content, P uptake and N uptake was observed. Co-inoculation of *Pseudomonas* with *R. leguminosarum* recorded maximum increase in the nodulation, leghemoglobin content, total iron, total chlorophyll content, N uptake and P uptake over the plants treated with *R. leguminosarum* alone suggesting a strong synergistic relationship between *Pseudomonas* sp. and *R. leguminosarum* (Mishra et al. 2011).

In another experiment, *Methylobacterium oryzae* and three AMF were evaluated for nutrient uptake on red pepper (*Capsicum annum* L.). The co-inoculation of *M. oryzae* and AMF significantly increased various plant growth parameters like root and shoot length, fresh and dry weight and chlorophyll content compared to uninoculated controls. Also nitrogen and phosphorus content of the plants increased; in addition, Zn, Cu, Fe and Mn content of the inoculated plants also increased by almost 1.5 times that of uninoculated control in most of the inoculation treatments. The results obtained suggest that apart from affecting plant growth and nutrient uptake individually, microorganisms can also form mutualistic relationships thereby benefiting the plant (Kim et al. 2010).

Therefore in natural systems, plant pathogens co-exist with host plants and other microorganisms; also biological control entails any reduction in the incidence and severity of the pathogen achieved through any biological mechanism.

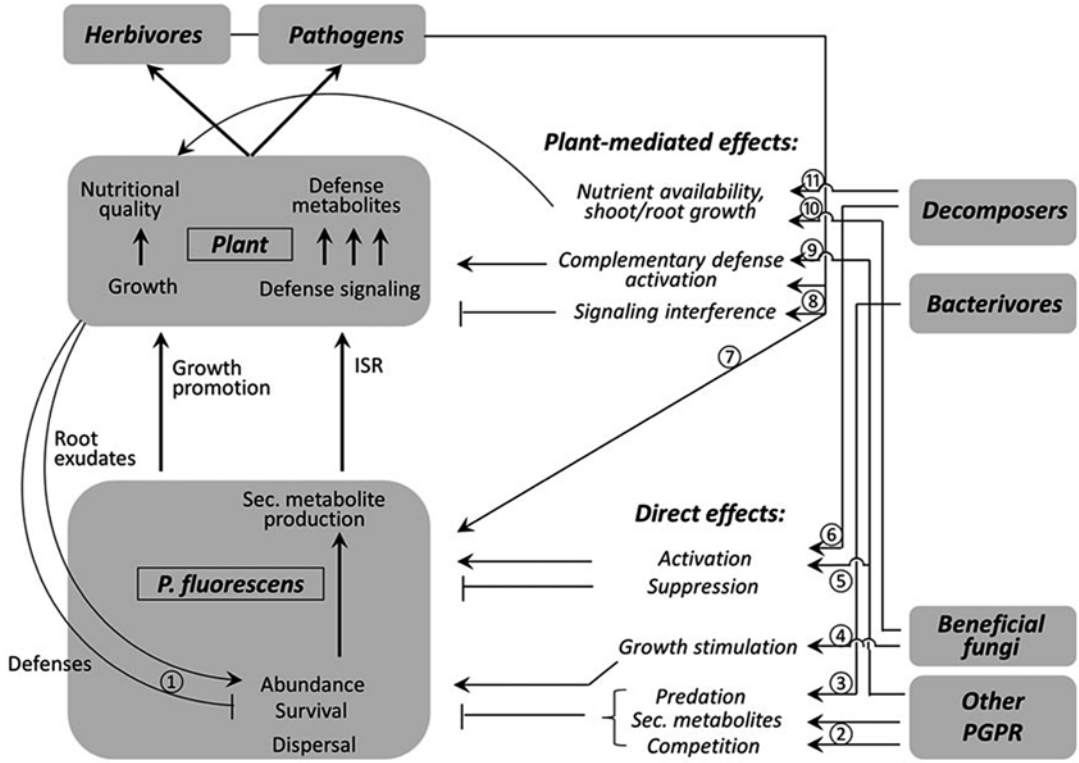

Fig. 6.1 Direct and indirect (plant-mediated) effects of rhizosphere- and plant-associated organisms on interactions between *Pseudomonas fluorescens* and host plant defenses (Adopted from Hol et al. 2013)

6.5 Conclusion

Successful management of plant stress requires a complex range of interactions. Understanding these interactlions between plants and microbes through different molecular and biochemical techniques will improve their stress management mechanism. Application of genetic analysis to microorganisms involved in stress management has resulted in significant advancement in understanding the microbial metabolites and regulatory genes involved in stress management. Ecological analyses have begun to describe the responses of microbial communities towards introduction of biocontrol agents. The integrated use of genetic, molecular and ecological approaches will form the basis for significant future advances in stress management research.

The development of stress tolerant crop varieties is a time-consuming effort, while microbial inoculation to manage stresses in plants could be a more economical and ecofriendly alternative which would be available in shorter time duration. In the future intensive research is required on field evaluation and application of potential microorganisms. Increasing concerns over environmental issues gives microbial biocontrol an exciting perspective. Therefore, by the application of naturally occurring soil microbes instead of deleterious chemicals can give a very promising substitute for plant stress management.

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Contribution of Microbial Inoculants to Soil Carbon Sequestration and Sustainable Agriculture

7

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Abstract

Soil is the incoherent matter on the earth's surface having organic and mineral content. It is subjected to environmental changes and hence shows effects of climate change as well as organisms over a period of time. Hence, it is the high time to find ways to increase the crop productivity in soil as green revolution cannot withstand this need. An alternative to this problem is the use of soil microorganism to increase the fertility of soil. Soil enzymes originate from soil microbes and regulate the nutrient cycle. Potential soil isolates can be used to increase nutrients in soil. In addition, these isolates can help in reducing the increase of carbon dioxide by sequestering carbon in soil. It is known that CO_2 is one of the major greenhouse gases that contributes to global warming and CO_2 fluxes are controlled by soil biota. Thus, soil act as buffer compartment to sequester carbon in relation to climate change. The sequestered soil carbon may further be utilized in agriculture and forestry and as a powerful option for global change mitigation. With this background, the present chapter aims to provide an insight into the contribution of microbial communities to soil carbon sequestration and its benefits to sustainable agriculture.

Keywords

Carbon sequestration • Climate change • Soil organic matter • Sustainable agriculture

7.1 Plant–Microbe Interaction

Symbiosis is a phenomenon in which two or more different organisms survive together for a long period of time (Ogle and Brown 1997; Douglas 1994). Generally, plants are dependent

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_7

upon soil, but plants and the soil microbes play a significant role in the formation or alteration of soil (Pate et al. 2001; Pate and Verboom 2009; Taylor et al. 2009). Since soil is the rudimentary foundation of food security, global economy and environmental quality, the soil quality is extensively monitored by soil organic matter (SOM) content. The carbon present in soil is principally obtained from plants either directly or indirectly. The occurrence of weather-beaten soil may be because of physico-chemical parameters mainly involving the plant itself, its roots or the activities of microorganisms that sustain root-derived carbon (Raven and Edwards 2001; Beerling and Berner 2005; Taylor et al. 2009).

Plant–microbe interactions take place in the rhizosphere. Rhizodeposition is a ubiquitous phenomenon by which carbon-containing composites are released from plant roots into the soil (Jones et al. 2004, 2009). Burgeoning of microbes inside the roots, on the surface and outside the roots takes place due to loss of carbon from root epidermis and cortical cells (Lambers et al. 2009).

7.2 Microbial Interaction and Carbon Storage

Soil structure plays an important regulatory role in microbe-mediated carbon storage and decomposition (Crawford et al. 2012). There are several groups of microbes that facilitate the formation and stabilization of microaggregates. They are also responsible for preferential stabilization of SOM. Aggregate stability increases linearly with carbon input (Woodward et al. 2009; Mummey et al. 2006; Lennon et al. 2012; Ward et al. 2009). The presence of microorganisms indicates soil microbial activity. These microbes are the source of soil enzymes that play a significant role in the deposition of organic matter in soil and regulation of nutrient cycle (Waldrop et al. 2004). Soil is an active pool containing carbon, nitrogen, phosphorus and other minerals. Microbial biomass C and N contribute a variable but significant pattern to this pool (Sicardi et al. 2004).

Soil organic carbon (SOC) plays multiple roles in ecological systems, and it is also known that microbial communities perform essential functions in land–atmosphere carbon exchange and deposition of soil carbon (Trivedi et al. 2013). Soil represents a massive reservoir of potentially volatile carbon. It is supposed to act both as a buffer against increased environmental CO_2 and as a possible store for extra carbon. This auxiliary carbon is thought to be dependent upon equilibrium between photosynthesis and respiration of microbes capable of decomposition and carbon stabilization in soil (Woodward et al. 2009; Lal 2004). It has been evaluated that by adopting efficient management practices the world agronomy and degenerated soils can store 0.4–1.4 Gt surplus carbon/year, which is considered to be comparable with 5–15 % of global fossil fuel releases (Lal 2004). In terrestrial ecological systems, the higher plants exhibit increased CO_2 uptake from the environment in terms of net primary production. However, at the same time, microbes also contribute to ecosystem carbon largely by functioning as plant symbiont, detrivores, etc. This in turn leads to modification in nutrient availability and significantly influences the carbon turnover and its maintenance in soil (Lal 2004). Carbon availability is a key determinant of the growth and activity of microbes, which establishes the close linkage between net primary production, activity in rhizosphere and litter substrate quality (Smith and Paul 1990) (Fig. 7.1).

The soil facilitates relatively quick decomposition of plant residues, and only a small amount of original plant residue carbon can be recognized that is retained in soil after a period of time. Hence, the prolonged cycling of microbial residues in soil is thought to be a major phenomenon that affects the changes in the amount of SOC. Lesser decomposition or more carbon inputs can support the carbon sequestration in soils (Li and Feng 2002). When the microorganisms decompose the biomass, there is reduction in the soil carbon level due to microbial respiration; however, a small amount of carbon remains in soil in the form of stable organic matter. If the

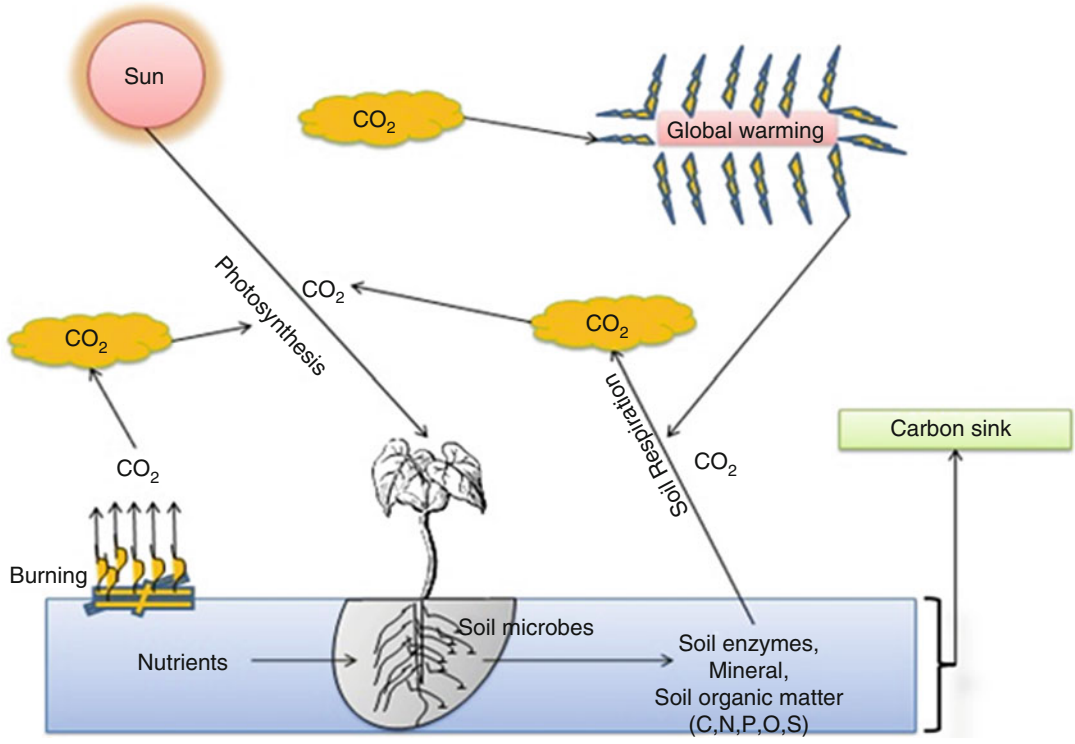

Fig. 7.1 Relation between different parameters involved in carbon sequestration

amount of carbon gained through photosynthesis surpasses the amount of carbon lost by soil respiration, then the SOC level rises over time giving net soil carbon storage/sinking (Schmidt et al. 2011; Reynaldo et al. 2012).

The breakdown of organic carbon in soil is primarily driven by the bacterial and fungal activities, whereas only 10–15 % of the soil carbon flux can be directly attributed to the actions of fauna (Hopkins and Gregorich 2005). It has been observed that fungal: bacterial ratio is associated with carbon sequestration; hence, a higher abundance of fungi in soil is related to higher soil carbon storage (Strickland and Rousk 2010). However, contrasting results have been observed and reported (Mulder and Elser 2009). Besides, it has been argued that fungi have a negative effect on carbon sinking due to their greater efficiency in breaking down recalcitrant litter (Baldrian et al. 2011; Cheng et al. 2012; Schneider et al. 2012). Generally, initial stages of breakdown (i.e. 14–25 days of adding substrate) can be attributed to bacterial-derived activity (Bastian et al. 2009). However, fungi preferably act on recalcitrant litter having high C:N ratio, thereby dominating the later decomposition stages (from 56 to 165 days) (Bastian et al. 2009).

The fungal:bacterial biomass ratio was shown to be dependent upon any kind of soil interference, with lesser ratios signifying augmented potency of cultivation (Bailey et al. 2002; Beare et al. 1992; Frey et al. 1999) and increased nitrogen fertilization inputs (Bardgett and McAlister 1999; Bardgett et al. 1996, 1999; Frey et al. 2004).

A distinct symbiotic relation exists between plant roots and mycorrhizal fungi, where fungi absorb soluble carbon from the plant (around 20 % of acquired carbon) to interchange for enhanced access to water and facilitate transportation of slightly soluble mineral and organic form of nutrients (Sylvia 2005). It is mostly governed by hyphal growth of fungi and concomitant bacteria in the neighbouring soil that significantly

enhances total surface area of root and potential depletion zone for principle nutrients (mainly phosphorus and nitrogen) as compared to non-mycorrhizal roots (Timonen and Marschner 2006; Powell and Klironomos 2007).

7.3 Effect of Nitrogen on Microbial Community and SOC

Nitrogen enrichment has a significant influence on the fungal:bacterial ratios, that is, nitrogen supplement decreases fungal and bacterial biomass and their ratios, and alters microbial community composition in the ecosystem (Treseder 2008; Farrer et al. 2013). Alteration in plant–microbe associations could also influence the success of plant species with nitrogen enrichment if there is a different effect of nitrogen on the benefits received by plant from microorganisms (Johnston et al. 2009). Similar mechanisms are involved in plant–microbe association independent of whether the microorganism is beneficial or pathogenic (Lugtenberg and Dekkers 1999; Chin-A-Woeng et al. 2000; Lugtenberg et al. 2001).

7.4 Global Warming and Soil Carbon

The phenomenon of global warming is one of the major concerns with respect to its impact on climate change. The greenhouse gases (GHGs) responsible for global warming include CO_2, CH_4 and NO_2. Since the Industrial Revolution, it has been observed that there is a dramatic rise in the amount of CO_2 and other GHGs. The rapid increase in CO_2 concentration in the atmosphere associated with other GHGs is an important consideration in reference to climate. The amount of CO_2 in the environment has increased from 280 to 387 ppmv from 1750 to 2007 and the rate of increase is 1.5 ppmv per year (Oren et al. 2001). The global surface temperature has significantly increased due to the combined effects of anthropogenic enhancement in atmospheric GHGs and cumulative radiative forces. Majority of atmospheric GHG enrichment is because of fossil fuel combustion and land management changes (Fig. 7.2).

Because of fossil fuel combustion and land-use changes, there has been an increase in environmental CO_2 by 38 % since 1750. Hence, it is necessary to identify the strategies that palliate the threat of global warming (Oren et al. 2001).

The diminution of SOC stock has significantly contributed to the atmosphere. Around a half or two thirds of original SOC is being lost from the cultivated soils giving a combined loss of approximately 30–40 Mg C/ha (Mg = Mega gram = 106 g = 1 ton). The reduction in soil carbon is highlighted by soil degradation and aggravated by land misuse and soil mismanagement. Hence, adopting land restoration and sustainable management practices on agronomical soils decreases the level of atmospheric CO_2 and arrests further enrichment, whereas it will have positive effects

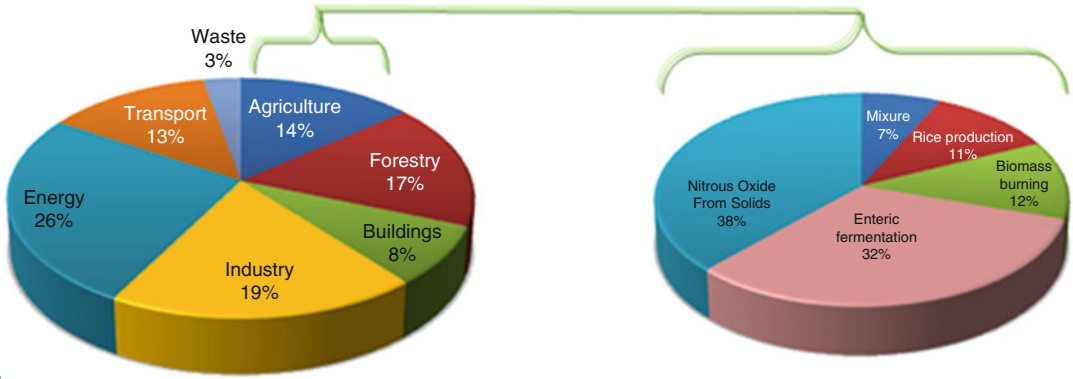

Fig. 7.2 Greenhouse gas emission by different sources (Source: IPCC 2007b; Smith et al. 2008)

on food security, quality and environment. The restoration of the depleted SOC pool can be achieved through conversion of the marginal lands into useful lands, adopting no-till practices with cover crops and mulch, incorporating systematic nutrient cycling by utilizing compost and manure and assessing the sustainable management of soil and water resources. With such sustainable management practices, the rate of soil carbon sequestration lies in the range of 50–1000 kg/ha/year. The turnover of soil carbon storage over 20–50 years can be 30–60 Pg (Peta gram = 1015 g = 1 billion ton; Oren et al. 2001). Thus, soil carbon sequestration can be considered as a method to restore damaged soil, increase biomass production, purify surface and ground waters and decrease the rate of enrichment of atmospheric CO_2 by reducing fossil fuel emissions.

The rate of soil carbon sequestration depends upon texture and structure of soil, temperature, agricultural system and management of soil system. The most widely adopted strategies to improve soil carbon pool include soil restitution and woodland restoration, reduced tilling, use of cover crops, improved grazing, enhanced agroforestry practices, crop rotation, etc. Besides enhancing food security, carbon sequestration has the potential to offset fossil fuel emissions by 0.4–1.2 Gt of carbon per year (Lal 2004).

The conversion of land to forests, grasslands or perennial crops by removing crops annually will enhance carbon sequestration, thereby mitigating the climate changes. However, there are associated indirect repercussions, such as land conversion under endemic vegetation, which negatively affect the benefits through CO_2 emission. Revegetation of degraded land can avoid this problem. Land revegetation is carried out by incorporating microbial inoculants in soil in order to combat climate changes by GHGs (Powlson et al. 2011). Addition of organic materials, namely, crop residues or manure so as to increase the level of SOC, usually does not promote release of atmospheric carbon into the soil. Increase in SOC due to reduced tillage now appears to be substantially less than previously claimed. Further, the elevated N_2O levels may also negate any enrichment of stored carbon (Powlson et al. 2011).

7.5 Carbon Sequestration

Soil is the rudimentary foundation of food security, global economy and environmental quality. The quality of soil is extensively monitored by change in SOM content. Land degradation, soil infertility and reduced productivity are the result of enhanced effects of global warming (Friedrich and Scanlon 2008). Hence, maintenance of soil quality and soil health can reduce these problems. Inappropriate agricultural practices can lead to severe soil loss. Soils represent one of the principal carbon sinks (atmospheric CO_2 and organic carbon) in the world as they contain about twofold carbon in comparison to atmosphere (Willey et al. 2009).

The continuous and rapid increase in atmospheric carbon dioxide and global warming contributed towards the awareness of carbon sequestration. Terrestrial carbon sequestration forms the basis of the overall carbon cycle, which is also being utilized to counter anthropogenic CO_2 emissions. A number of strategies have been espoused so far to alleviate global CO_2 releases as well as for carbon sequestration in the soil. For a given system, carbon sequestration is a network of biological activities at the spatial dimension of soil physical structure.

Carbon accumulates in soil when productivity, that is, addition of carbon-containing substrates, exceeds decomposition, thereby leading to increase in organic matter. Hence, the diminution of soil carbon pool is entailed by the decomposition rate. Soils contain carbon in both organic and inorganic forms. SOC is composed of a 'mixture of dead plant and animal residues, its decomposed product, the microbial products synthesized from the decayed products and the microbial and animal biomass of soil' (Schnitzer 1991). The inorganic carbon contributes to around 25 % of the global soil carbon inventory. The changes in SOC are greatly influenced by the current agricultural practices. The soil characteristics are

influenced by the percentage of SOC in soil. Soil quality is improved by increasing the SOC content, which eventually prevents soil erosion and degradation, improves surface water quality and enhances soil productivity (Li and Feng 2002). The overall carbon sequestration in soil, thus, enhances SOC content ultimately advantageous for environment and society.

7.6 Factors Affecting Carbon Sequestration

7.6.1 Temperature

Soil carbon accumulation is higher in warmer and medium-temperature sites as compared to colder regions. However, carbon sequestration is observed to be high in semi-humid sites than in their semiarid counterparts (Braimoh et al. 2012).

7.6.2 Soil Type

The type of soil also matters in case of carbon sequestration. Soils having more clay content sink carbon at a higher rate. The highest carbon sinking rates and variability are observed to be in inceptisols (comparatively young soils constituting around 9 % in tropics) in Africa and Latin America and in oxisols (soils of humid tropical zones under rainforests, savanna vegetation) in Asia (Braimoh et al. 2012).

There are some limitations with respect to efficiency of soil carbon sequestration in the context of climate change mitigation:

1. Finite quantity of secured carbon: Previous studies have proved that the SOC level increases until equilibrium is achieved, which suggests that the accumulation of SOC is a definite process (Johnston et al. 2009).
2. Reversible procedure: The maintenance of SOC level can be due to the prolonged carbon enrichment in soil or vegetation through alterations in land management practices (Freibauer et al. 2004). For instance, if a new forest is established, the carbon accumulated in trees and soil will be lost if the trees are felled (Saarsalmi et al. 2010). Similarly, with the inclusion of grasses and legume ley in arable crop network, it has been observed that the accumulated SOM is lost when ploughing is done in the next arable phases (Wu et al. 1998). However, an overall increase in SOC has been noted for long-term storage as compared to incessant arable cropping with the use of nonstop ley system (Johnston et al. 2009).
3. There may be either increase or decrease in the rates of strong GHGs such as N_2O and CH_4 because of alterations in land management techniques. N_2O and CH_4 have very high global warming potentials (GWP), that is, 298 and 25 times of the GWP of CO_2, respectively, in a 100-year time period scale (IPCC 2007b). Hence, it can be observed that a slight change in the rate of such gases has a greater influence on the total effect of climatic changes for particular land management changes.

7.7 Microbial Inoculants as Carbon-Sinking Agents

Soil microorganisms play a major role in nutrient cycling and global effects of carbon dioxide, methane and nitrogen. Microbial activities are responsible for the production and consumption of GHGs in soil (Allison et al. 2010). These gases have multitudinous functions in the metabolism of microbes.

Soil microbe activities frequently depend upon environmental parameters such as temperature, moisture and nutrient availability, all of which are affected by climate change (IPCC 2007a). The major uncertainity in prediction of climate change is microbes' response to increasing temperature. Several studies have shown that elevated temperature accelerates the rate of microbial decomposition resulting in increased emission of CO_2 via soil respiration, thereby leading to huge soil carbon losses and increase of global warming (Allison et al. 2010). Increased CO_2 concentration in the atmosphere is thought to be mitigated in part by the ability of terrestrial

forests to sequester a large amount of CO_2 (Schlesinger and Lichter 2001). In other words, the extent to which GHGs are emitted and the processes that lead to such emissions must be reduced.

The association between the aboveground and underground biodiversity contributes greatly to the restoration of ecosystem and involved in the important biological reactions (Goenadi and Santi 2009). In contrast, the improved growth and yield of plant strictly depend upon the efficiency of plant roots to gain water and nutrients from the soils. Soil inhabits diverse groups of microorganisms and the microbial activities contribute greatly to the maintenance of a sustainable agricultural system and also improve soil fertility. The preservation of crop residues and SOM content augment soil biodiversity and stimulate microbial diversity. The restriction in the use of pesticides correlates with labour costs, which is an integral part of management farming.

A number of beneficial services have been provided by the soil microbial community, especially bacteria and fungi, including regulation of nutrient cycle, transformation of SOM, soil carbon sequestration, bioremediation of toxic pollutants and providing beneficial nutrients for better plant growth, which are involved in the functioning and maintenance of a sustainable ecosystem (Bloemberg and Lugtenberg 2001). The application of biotechnology in the management of soil ecosystem provides an innovative approach which deals with such problems more efficiently. Modification of soil microbial community provides an improved and effective method to stabilize soil texture, enhance nutrient accumulation in plant, control soil-borne pathogen and catalyze the decomposition of organic wastes without increasing pollutant concentration in the environment. Enhancing microbial activity in the soils (EMAS) is an effective biofertilizer developed successfully using microbial consortia of *Azospirillum lipoferum, Azotobacter beijerinckii, Aeromonas punctata* and *Aspergillus niger* isolated from the native tropical soils (Goenadi et al. 2000). These processes enable conversion of nutrients during the symbiotic association of bacteria with the plant roots (Lodwig et al. 2003).

The crux of true soil conservation is carbon management. With proper and appropriate management of carbon in agricultural ecosystems, the following results are obtained:

- Reduced erosion and pollution
- Clean water
- Fresh air
- Healthy soil
- Increased fertility
- High yield and productivity
- More biodiversity and sustainability (Friedrich and Scanlon 2008)

SOM is both inherent, in the sense that it is related to particle size distribution, and dynamic, in the sense that it is related to the extent of organic matter input in soil. Soil carbon cycling with dynamic nature is directly related to 'biological carbon' cycle.

For significant carbon sequestration, carbon input should be maximized and carbon output should be minimized so that an economic balance is achieved (Friedrich and Scanlon 2008).

- Carbon outputs can be reduced by lowering the mechanical soil disturbance resulting in increased mineralization.
- Carbon inputs can be raised through increase in biomass production and retention of biomass as much as possible.

Significant carbon storage can be achieved by considering both elements together.

Carbon sequestration would be effective if the changes in land management practices cause a net supplementary transfer of carbon from environment CO_2 to terrestrial biosphere, which results in either decelerating or reversing the increase in atmospheric CO_2. This can be achieved by the following:

- Overall increase in photosynthesis (by planting more trees or grass)
- Slowing down the rate of SOC breakdown through land management practices

- Enhancing plant–microbe interaction that transfers more amount of carbon belowground, thereby increasing carbon sequestration in those conditions for a long period of time

SOM is derived either directly or indirectly from plant by photosynthesis. The environmental carbon dioxide thus converted into basic and composite organic carbon compounds facilitates plant growth and function along with other vital nutrients. Plant releases carbon dioxide into the atmosphere through respiration; however, most of the stabilized CO_2 is preserved and finally transported to the soil ecological system through a network of pathways.

Approximately 10–40 % of the total fixed carbon was obtained via photosynthesis from the plant root exudates and is mainly composed of a mixture of polysaccharides, amino acids, alcoholic sugars, organic acids and secondary metabolites (Bais et al. 2006). The microbial and faunal activities are particularly driven by root exudates present in soil. This is because of the fact that they are incorporated into the soil on a regular/semi-continuous basis, their comparatively greater bioavailability than aged plant detritus and their role in the regulation of bioavailability of nutrients (e.g., phosphorus) and phytotoxic compounds (e.g., aluminium) (Singh and Mukerji 2006; Neumann 2007).

The root exudates are responsible for enhancing the biological activity in plant roots by better accretion of soluble and organic soil nutrients fixed by the microorganism that provides an energy-rich carbon substrate beneficial for plant. These constitute the symbiotic relationship between plant roots and mycorrhizal fungi. The mycorrhizal fungi remain in a close association with the plant root cells and gain energy in the form of soluble carbon from the plant. These in turn provide the plant with improved access to water and also facilitate mobilization of slightly soluble mineral and organic forms of soil nutrients (Sylvia 2005).

Soil microorganisms play a crucial role in agro-ecosystem by maintaining the soil biochemical cycles (He et al. 2007). Microorganisms involved in the storage of soil carbon are completely related to the synthesis and degradation of microbial by-products. However, soil microbes indirectly influence the carbon cycling in soil by recovering soil clustering, which also defends SOM. Subsequently, carbon sequestration is regulated by the presence of microbial biomass, microbe-secreted by-products and microbe community and soil physiochemical properties such as soil texture, pore size distribution and clustering dynamics (Six et al. 2006). Agricultural practices such as crop rotation, organic farming and cover crops increase the total microbial biomass as well as microbial community for fungus, therefore increasing the deposition of Microbially derived Organic Matter (MOM).

The rhizospheric microorganisms have the ability to colonize plant roots and have multiple plant growth promotion properties. Therefore, most of the research is focused on the rhizosphere. The plants dynamically select microbial community by the process of rhizodeposition to enhance the availability of limited soil resources (Hamilton and Frank 2001). A plant community selects a microbial community, with a particular composition and functional diversity, which ensures supply of important carbon compounds (Rillig 2004; Wardle 2005). Generally, several plant communities show more productivity which results in the assimilation of carbon compounds from the environment. This in turn leads to the accumulation of soil carbon, nutrient retention and energy yield in soils due to increased microbe diversity and eventually changes the property of rhizodeposition and accelerates decomposition (Dang et al. 2005; Broughton and Gross 2000; Ekschmitt et al. 2001). SOM is directly associated with the microbial biomass, whereas biomass increases by changes in the organic matter content (Nannipieri et al. 2003; Plassart et al. 2008; Bastida et al. 2008).

Soil remains as an essential part of the environment, and the constant functioning of soil ecosystem is necessary for maintaining soil sustainability and productivity (reviewed by van-Elsas et al. 1997). Understanding the process that occurs in the soil ecosystem assists us in improving and managing the current agricultural practices and conservation methods.

Most of the microorganisms are able to survive and grow in the changing environment conditions such as increase in pollution and global warming. The soil system must be capable of supporting plant growth with a developing root system and maintaining a healthy ecosystem.

SOM is regulated and maintained by the soil microbial communities; it also maintains soil nutrient availability and modifies the composition and function of microbial community. In response to the other agricultural management practices, soil microbial communities play a key role in determining the rate at which carbon is lost from the soil (Six et al. 2006). These practices include enhanced C participation (Schnürer et al. 1985), less tilling (Beare et al. 1992; Doran 1987; Frey et al. 1999), preservation of crop residues despite removing it by burning (Gupta et al. 1994) and other farming practices that combine reduced tilling with more C inputs through organic amendments (Hassink et al. 1991).

Phytolith-occluded carbon (PhytOC) is considered as an essential part of SOC which is stored in the soil and significantly contributes to long-term terrestrial carbon sequestration. Some important agricultural crops such as barley, maize, rice, sorghum, sugarcane and wheat are abundant producers of phytolith and PhytOC. Approximately 87 million tonnes (Mt) of PhytOC is produced by these crops in India annually. Therefore, there is a huge potential to augment PhytOC acquisition in the soils of different agricultural ecosystems (Rajendiran et al. 2012).

7.8 Sustainable Land Management Practices for Carbon Sequestration

The factors affecting carbon storage include land management parameters that negate carbon sequestration by soil erosion, tilling, drainage, etc. The collective historical loss of carbon is commensurate with the potential carbon sequestration capacity. However, only 50–60 % of carbon sequestration can be achieved by adopting sustainable land management (SLM) practices (Braimoh et al. 2012).

Crop rotation plays a major role in enhancing soil carbon sequestration as compared to continuous crop management methods that involve fallow periods. By adopting and using more rigorous crop rotations, soil carbon input accelerates and microbe activity and biomass also increases (Six et al. 2006).

There are three different ways by which SLM delivers carbon benefits: First is conservation of carbon, that is, storage of ample amount of carbon in forests, wetlands, grasslands as carbon stocks. Preservation of terrestrial carbon can be taken as a 'least cost opportunity' with regard to the climatic changes, that is, modification and mitigation. This is considered important for enhancing the flexibility of agro-ecosystems. Second is carbon sequestration, where the microbes in the soil and natural biomass transfer atmospheric carbon into soil. Third is SLM practices which reduce GHG emissions emerging from agriculture production (Braimoh et al. 2012).

The alternative to conventional agricultural practices is SLM practices with respect to the three methods: conservation, sequestration and reductions in GHG emissions. Conventional practices involve biomass burns, drainage of wetlands, deforestation, land ploughing and some other types of soil imbalances which emit not only CO_2 to the environment but also NO_2 and CH_4 (major GHGs responsible for global warming; Braimoh et al. 2012). Carbon is mainly stored in soil rather than plant biomass or vegetation, and SOC accounts for about 81 % of the world's terrestrial carbon store. Global estimate of soil carbon stock (also known as pedologic pool) is at 2500 Gt for 2 m depth of soil, out of which SOC constitutes about 1550 Gt of the stock and the remaining 950 Gt is soil inorganic carbon (Batjes 1996). The soil carbon stock is more than three times that of the atmospheric store (760 Gt) and about 4.5 times that of the biotic store (560 Gt).

Attainable carbon sequestration is determined by factors which limit the input of carbon to the soil ecosystem. Net primary productivity (NPP), that is, the rate of photosynthesis minus autotrophic respiration, is the major factor affecting the attainable sequestration and is modified by

aboveground versus belowground distribution. Land management methods that accelerate carbon input by enhancing NPP tend to increase the attainable SOC storage to nearer to the potential level. Over a period of time, various workers have suggested a variety of land management practices that increase SOC. These are as follows:

1. Converting arable land into grassland or forest (Poulton et al. 2003)
2. Revegetation of degraded lands (Han et al. 2010)
3. Addition of organic materials into the soil (Angers and Carter 1996; Johnston et al. 2009)
4. Conversion of arable cropping systems to reduced tillage systems (Baker et al. 2007; Angers and Eriksen-Hamel 2008)
5. Application of fertilizers that increase crop yield (Glendining and Powlson 1995)
6. Carbon stabilization in sub-soil (Carter and Gregorich 2010)

7.9 Conclusion

Climate change is likely to have a significant effect on soil enzyme activities and microbial biomass, thereby affecting the soil microbial community. Different attenuation measures taken to limit global climate change have a significant impact on soil functioning and preservation. Microbial community of soil aids storage of carbon that has beneficial effects in terms of soil fertility, clean water, increased biodiversity and higher productivity. If the degradation of agricultural soil continues to be unchecked, then feeding a growing population would present serious problems. The challenges involve the measurement methods for belowground carbon storage. In addition, CO_2 emissions from transportation for decades in the form of biofuels, H_2 and CH_4 could be reduced by carbon sequestration. However, realizing this potential requires much more detailed knowledge regarding the concerned microbe and its mechanisms of C storage. The approaches involved at the molecular and ecosystem levels can be combined for bacterial and fungal activity observation and experimentation, enzyme activity distribution and microbial community structure and composition.

Acknowledgement The authors are thankful to the Director, MNNIT, Allahabad and Department of Biotechnology, Government of India for providing the necessary research facilities and financial support for this work.

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Remediation of Heavy Metal-Contaminated Agricultural Soils Using Microbes

8

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Abstract

Heavy metals are widely spread and accumulated in soil due to various inappropriate human activities, because of which metal pollution in soil has become one of the most serious environmental problems today. In this chapter, various microbial remediation mechanisms to remediate heavy metal-contaminated soils have been described. Microbial remediation, an emerging cost-effective, renewable, nonintrusive and aesthetically pleasing technology, uses the remarkable ability of microbes to remove and transform heavy metals from contaminated soils. The very limited understanding pertaining to heavy metal removal and transformation is hindering its effective application. Due to its great potential as a viable alternative to conventional contaminated soil remediation techniques, microbial remediation is currently being looked upon as an exciting area of basic and applied research.

Keywords

Microbial remediation • Heavy metals • Soil • Mechanisms

8.1 Introduction

Heavy metal soil pollution has become one of the most serious environmental problems today due to the rapid development of various industries, such as mining, fertilizer, pesticide and leather, which discharge the wastes containing heavy metals directly or indirectly into the soil (Wang and Chen 2006). These heavy metals are usually classified as the following three groups: (1) toxic metals (such as Hg, Cr, Pb, Zn, Cu, Ni, Cd, As, Co and Sn), (2) precious metals (such as Pd, Pt,

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_8

Ag, Au and Ru) and (3) radionuclides (such as U, Th, Ra and Am), whose specific weight is usually more than 5.0 g/cm^3. The toxic properties of heavy metals are as follows: (1) the toxicity can last for a long time in nature; (2) some heavy metals could even be altered from relevant lower toxic species into more toxic forms in a certain environment, for example, Hg; (3) the bioaccumulation and bioaugmentation of heavy metals in the food chain could damage normal physiological activity and finally jeopardize human life; (4) metals can only be changed and altered in valence and species but cannot be degraded using other methods, including biotreatment; and (5) heavy metals can be toxic even in low concentrations of about 1.0–10 mg/L. Some strong toxic metal ions, such as Hg and Cd, are very toxic even in lower concentrations of 0.001–0.1 mg/L (Alkorta et al. 2004; Wang and Chen 2006). Therefore, bioremediation of heavy metal-contaminated soil, using microbes such as fungi, bacteria, algae and yeast, is regarded as a cost-effective biotechnological approach (Ahluwalia and Goyal 2007). Microbial remediation of heavy metals can be defined as the process of using specific microorganisms to transform hazardous contaminants in soil to nonhazardous products (Thatoi et al. 2014). The process of microbial remediation mainly depends on microorganisms that attack the heavy metals and convert them to less hazardous products. As microbial remediation can be effective only where environmental, physical, chemical, biological and cultural conditions permit optimum microbial growth and activities, its application often involves the manipulation of the above-mentioned conditions to allow microbial growth and rapid degradation (Karigar and Rao 2011).

Many soil microbes are known to be effective in remediation of heavy metals only under in vitro conditions. Although microbes can exist in extreme environment, most of them prefer optimal conditions for growth, a situation that is difficult to achieve under in situ conditions. Thus, under in situ conditions, various factors play a role in governing the microbial growth, for example, pH, temperature, oxygen, soil structure, moisture and nutrients, bioavailability of heavy metals and presence of other toxic/xenobiotic compounds (Karigar and Rao 2011; Dua et al. 2002). Most microbial remediation systems operate under aerobic conditions, but anaerobic conditions may also permit microbial remediation of heavy metals.

8.2 Significance of Soil Microbes and Inherent Heavy Metal Resistance

Soil is an important habitat for a diverse group of microbes (e.g. fungi, actinobacteria, algae, protozoa and bacteria). These microbes can occur in association with the clay particles, organic matter, rhizosphere of plants and soil particle pores. Microbial remediation requires a good understanding of the physicochemical characteristics of the contaminated environment, as well as a detailed description of the microbial communities, which are involved in key physiological processes. More specifically, microbial communities require to be characterized in terms of structure, phenotypic potential, functionality and ecology (Rittmann et al. 2006; Stenuit et al. 2008). In quantitative terms, the microbes vary with the type of soil and their horizons, crops and stress (Vieira and Nahas 2005; Mishustin 1975). Torsvik et al. (1990) reported that the bacterial genetic diversity in the soil of a deciduous forest is tremendously high, with about 4,000 different genotypes, excluding those of unculturable bacteria. As 90–99 % of microbes living in natural environments are recalcitrant to conventional cultivation, the techniques to congregate information concerning the soil microbial diversity can be based on either culturable or unculturable ones (Kavamura and Esposito 2010; Stenuit et al. 2008). However, techniques based on culturable methods are known for their selectivity and do not represent the actual microbial diversity (Kavamura and Esposito 2010; Stenuit et al. 2008; Amann et al. 1995). Therefore, due to these limitations, it is necessary to use advanced molecular techniques of identification. In this way, a number of culture-independent molecular techniques are currently being used to study

complex microbial communities which are compatible with high-throughput setups such as fingerprinting techniques, real-time polymerase chain reaction (PCR), microarrays, metagenomics, metatranscriptomics, metaproteomics or metabolomics (Stenuit et al. 2008). These molecular tools have proved to be very useful tools for the qualitative and quantitative analysis of soil microbial communities.

Heavy metals play an important role in biochemical reactions, which are crucial for the growth and development of microbes and other living organisms. However, high concentrations of heavy metals can affect the soil microbes directly through the modification of the population size, diversity and activity through their cytotoxic effects. In the past few years, microbe–plant interactions in the rhizosphere have been mainly used as several kinds of bioremediation techniques. The rhizosphere is a volume of soil that is influenced by the plant roots where essential microbial activities are performed by the microbes which are known as rhizosphere microorganisms. The rhizosphere is composed of three components, the plant, the soil and the microorganisms, and has intense microbial activity due to the presence of organic matter that comprehends root exudates (Lynch and Moffat 2005; Bais et al. 2006). The rhizosphere microbes help the plant to absorb nutrients, thereby improving plant growth and soil fertility through the biogeochemical cycling of nutrients (Barea et al. 2002, 2005; Yang et al. 2009). Thus, they can be a good link between plants and the soil, changing metal availability and toxicity (Leyval et al. 1997; Kavamura and Esposito 2010). These microbes are important because they have several inherent mechanisms that result in the transformation (e.g. solubilization and reduction) of heavy metals (Gadd 2000; Gadd and Griffiths 1977). Various studies have demonstrated that certain soil microbes are capable of reducing and solubilizing metals such as Cr, Fe, Hg, Ag, Mn, Te and U, making them more or less available for plant absorption and minimizing their phytotoxicity (Giller et al. 1998; Lima de Silva et al. 2012; Watts and Lloyd 2012; Lasat 2002; Kashefi and Lovley 2000). Numerous studies have also reported that microbes were helpful in decreasing plant toxicity of Cd, Zn and Cr (Bennisse et al. 2004; Juwarkar et al. 2007; Khan 2005).

8.3 Microbial Remediation of Heavy Metal-Contaminated Soils

Heavy metal contamination in soils has received much attention in the recent years (Jing et al. 2007). Application of microbes for decontaminating the heavy metal-contaminated soils is a difficult task because heavy metals cannot be easily removed/decontaminated and thus persist in the soils ((A Review on Heavy Metals (As, Pb, and Hg) Uptake by Plants through Phytoremediation 2011; Hashim et al. 2011; Ma et al. 2010, 2011a, b; Khan et al. 2009a). Consecutively, for remediation of the heavy metal-contaminated soils, heavy metals should be removed/decontaminated by an appropriate technique. The recognized conventional techniques (e.g., thermal processes, physical separation, electrochemical methods, washing, stabilization/solidification and burial) for remediation of heavy metal-contaminated soils are generally too expensive and often harmful to soil health (Khan et al. 2009a; Rajkumar et al. 2012; Dermont et al. 2008; Akcil et al. 2015). Therefore, a promising, alternative approach to chemical amendments could be the application of microbe-mediated processes which is also being commonly referred to as ‘microbial remediation’. In this process, microbial metabolites/activities in the soil alter the mobility and bioavailability of heavy metals (Hietala and Roane 2009; Rajendran et al. 2003; Monachese et al. 2012; Umrania 2006; Wenzel 2009; Rajkumar et al. 2010; Miransari 2011; Yang et al. 2012; Zhu et al. 2015). It has thus been proposed as an alternative method to remediate heavy metals from soil since it does not affect soil health and fertility (Zhuang et al. 2007; Sessitsch et al. 2013). Microbial remediation is one of the key processes of removal/decontamination that involves the use of metal-resistant microbes to remove metals from soil by accumulation, assimilation, leaching,

sorption, transformation and precipitation (Colin et al. 2013; Esringü et al. 2014; Gadd 2000; Glassman and Casper 2012; Bolan et al. 2013, 2014; Bandara 2011; Mao et al. 2015). The success of heavy metal-contaminated soils is dependent on the potential of the microbes to produce high biomass, metabolites and biological activities under metal stress conditions (Ali et al. 2012; Pajuelo et al. 2014; Zaidi et al. 2006; Abou-Shanab et al. 2008; Esringü et al. 2014; Gullap et al. 2014; Braud et al. 2009; Cornu et al. 2014; Chompoothawat et al. 2010). There are several advantages associated with the use of microbes for the remediation of heavy metal-contaminated soils in comparison with chemical amendments because the microbial biomass, metabolites and biological activities are biocompatible in nature, and it is also possible to produce them under in situ conditions (Yu et al. 2014; Mani et al. 2015; Banni and Faituri 2013; Gaur and Adholeya 2004; He et al. 2009; Juwarkar and Singh 2010; Mani and Kumar 2014; Wang et al. 2014a). In addition, these microbes are also capable of producing plant growth-promoting substances, that is, organic acids, siderophores, plant growth hormones, 1-aminocyclopropane-1-carboxylic (ACC) acid deaminase and antimicrobial compounds, which are involved in the plant growth improvement in metal-contaminated soils (Wang et al. 2014a; Dimkpa et al. 2009; Glick et al. 2007; Göhre and Paszkowski 2006; Hayat et al. 2010; Khan et al. 2009b; Adediran et al. 2015; Ahemad and Kibret 2014; Zaidi et al. 2009; Burd et al. 2000; Wani et al. 2009). Heavy metal-contaminated soils have a diverse group of microbes (Bhatia and Malik 2011; Sowmya et al. 2014; Zhu et al. 2015; Del Val et al. 1999; Burd et al. 2000; Imran et al. 2011) that are capable of tolerating high concentration of heavy metals and thus provide a number of benefits to both the soil and plant. Among the microbes involved in heavy metal remediation, the rhizosphere bacteria received special attention because they can directly improve the heavy metal remediation process by changing the metal bioavailability through altering soil pH, release of chelators (e.g. organic acids, siderophores), biosurfactants, biomass production and oxidation/reduction reactions. (Zhuang et al. 2007; Wei et al. 2003; Watts and Lloyd 2012; Sivaruban et al. 2014; Barea et al. 2005; Khan 2005; Hietala and Roane 2009; Juwarkar et al. 2011; Pacwa-Plociniczak et al. 2011; Jing et al. 2014).

8.4 Mechanisms Involved in Heavy Metal Remediation by Microbes

8.4.1 Siderophore-Mediated Remediation

Most of the soil microbes (bacteria, fungi and algae) can produce iron-chelating compounds, well known as siderophores in response to low iron levels in the soil/rhizosphere. Siderophores are low-molecular mass (400–1,000 Da) compounds, which have high association constants for chelating iron, but can also chelate with other metals such as Al, Cd, Cu, Ga, In, Pb and Zn (Dimkpa et al. 2009; Glick and Bashan 1997; Schalk et al. 2011; Pattus and Abdallah 2000; Yakout et al. 2014). However, siderophores have been classified into four main classes (carboxylate, hydroxamates, phenol catecholates and pyoverdines; Beneduzi et al. 2012; Jeyanthi and Ganesh 2013). More than hundred types of siderophores have been identified, some of which are widely recognized and used by different microorganisms, while others are species specific (Beneduzi et al. 2012; Sandy and Butler 2009). Since siderophores solubilize unavailable forms of heavy metal-bearing minerals by chelation reaction, siderophore-producing microbes that inhabit the rhizosphere and soils are believed to play an important role in heavy metal remediation (Dimkpa et al. 2009; Gadd and Griffiths 1977; Rajkumar et al. 2010; Ma et al. 2011a; Schütze et al. 2014; Rojas-Tapias et al. 2014). For instance, production of pyoverdin and pyochelin by rhizosphere bacteria *Pseudomonas aeruginosa* increased the concentrations of bioavailable Cr and Pb in the rhizosphere, thus making them available for maize plant uptake (Braud et al. 2009). Similarly, inoculation of siderophore-producing *P. aeruginosa* strain KUCd1 stimulated

the growth of mustard and pumpkin plants in Cd-added soil through its establishment in the rhizosphere (Sinha and Mukherjee 2008). Likewise, Ni-resistant siderophore-producing *Pseudomonas* sp. inoculation increased the plant growth and reduced Ni uptake in chickpea plants. The results thus suggested and advocated the use of plant growth-promoting rhizobacteria (PGPR) to enhance plant growth in nickel-spiked land and remediate nickel from contaminated sites (Tank and Saraf 2009). Further, siderophores produced by *Streptomyces tendae* F4 significantly enhanced the uptake of Cd by sunflower plants (Dimkpa et al. 2009). The production of siderophores has also been demonstrated in some fungi (Haselwandter 2008; Rajkumar et al. 2012; Goodell et al. 1997; Renshaw et al. 2002). The ectomycorrhizal fungi (EMF), *Scleroderma verrucosum*, *Suillus luteus* and *Rhizopogon luteolus*, were isolated from fruiting bodies of *Pinus radiata* and shown to produce catecholate and hydroxamate siderophores under iron-deficient conditions (Machuca et al. 2007). Tolerance to Cd^{2+}, Cu^{2+} and Zn^{2+} ions and production of chelating compounds as a detoxification mechanism were evaluated in EMF collected from three uncontaminated sites. The fungi were grown in solid medium with Cd, Cu and Zn, and the tolerance index was determined. The metal-chelating compounds were determined by chrome azurol S (CAS) assay, and the chemical nature (hydroxamate or catecholate) of the compounds was analyzed. There was a clear inter- and intraspecific variation in the fungal responses at low and high metal concentrations. Some ecotypes of *Rhizopogon roseolus* and *Suillus luteus* were found to be more tolerant at 1 mM Cu and 10 mM Zn. The addition of Cu and Cd stimulated CAS-detected metal-chelating compounds and dark pigmentation production in all isolates. Hydroxamates and catecholates were detected only in some isolates, and catecholates were stimulated by Cd in *S. luteus* and *S. bellinii* (Machuca et al. 2014). The above studies suggested that the inoculation of the plants with siderophore-producing microbes removed the heavy metal from the soil through uptake by plants. However, some studies have also shown that siderophore-producing microbes do not always remove the heavy metals from soils and this may be attributed to the effect of biotic and abiotic factors (Kuffner et al. 2008, 2010; O'Brien et al. 2014; Dakora and Phillips 2002). Siderophore production by microbes is further regulated by various factors, including iron availability, pH, nutrient status of soils, type and concentration of heavy metals (Saha et al. 2013). The supplementation of heavy metals (Al, Cu, Ga, Mn Cr and Ni) in the presence and absence of iron induced pyoverdine and pyochelin production in the *P. aeruginosa*, which decreased the toxicity of metals; however, pyochelin increased the toxicity of vanadium in *P. aeruginosa* (Braud et al. 2010; Rajkumar et al. 2012). The fate and behaviour of siderophores in metal-contaminated soils may affect soil properties as well as environmental conditions for its inhabiting microbes. In particular, siderophore-producing soil microbes depend on the conditions of environment and edaphic factors for their nutritional requirements (Schütze et al. 2014). Thus, a further detailed study on the mechanistic aspects of siderophore biosynthesis and their role in remediation of the heavy metal-contaminated soil through the heavy metal mobilization is warranted.

8.4.2 Organic Acid-Mediated Remediation

Microbes possess the inherent ability to biosynthesize low-molecular-weight organic acids (LMWOAs), which are composed of CHO-containing compounds characterized by the presence of one or more carboxyl groups (Jones 1998; Ramachandran et al. 2006; Sauer et al. 2008; Muthukumar and Bagyaraj 2010). These LMWOAs have received much attention in the recent years because of their significant role in solubilization of heavy metals and mobilization of mineral nutrients in the rhizospheric zone (Rajkumar et al. 2012; Khan et al. 2009a; Bakshi et al. 2015). In general, organic acids can bind heavy metal ions in the soil solution through the complex formation, but the stability of organic

acid ligand–metal complexes is dependent on the following factors: (1) organic acids' nature (number of carboxylic groups and their position), (2) ligand–metal complex form type and (3) soil solution pH (Zaidi et al. 2006; Jones 1998; Ryan et al. 2001; Sultana et al. 2014). The organic acids biosynthesized by microbes play an important role in the complexation of toxic and essential metal ions and increase their mobility for plant uptake (Han et al. 2006; Sánchez-Marín and Beiras 2012; Fomina et al. 2004; Martino et al. 2003; Uroz et al. 2009; Topolska et al. 2014). The effect of the *Pseudomonas putida* inoculation on the solubility of pyromorphite $Pb_5(PO_4)_3Cl$ has been investigated in a set of batch solution experiments. Solubilization of pyromorphite was enhanced by the presence of *P. putida*, resulting in an elevated Pb concentration in the solution (Topolska et al. 2014). An endophytic bacterial strain JN27 isolated from roots of *Zea mays* displayed high tolerance and mobilization to Cd and was identified as *Rahnella* sp. based on 16S rDNA sequencing. The strain also exhibited multiple plant growth beneficial features including the production of indole-3-acetic acid, siderophore, ACC acid deaminase and solubilization of insoluble phosphate (Yuan et al. 2014). The bacterial strains JYX7 and JYX10 were isolated from rhizosphere soils of *Polygonum pubescens* grown in metal-polluted soil and showed high Cd, Pb and Zn tolerance and increased water-soluble Cd, Pb and Zn concentrations in a culture solution and metal-added soils. These strains produced plant growth-promoting substances such as indole acetic acid, siderophore, ACC deaminase and solubilized inorganic phosphate. Based upon their ability in metal tolerance and solubilization, two isolates were further studied for their effects on growth and accumulation of Cd, Pb and Zn in *Brassica napus* (rape) by pot experiments (Jing et al. 2014). A *Pseudomonas fluorescens* strain (JH 70-4) exhibiting plant growth-promoting characteristics (indole acetic acid production and 1ACC deaminase activity), as well as heavy metal(loid) (HM) tolerance and Pb precipitation, was isolated from HM-contaminated soil at an abandoned mine site. The JH 70-4 strain induced precipitation of Pb as PbS nanoparticles (NPs), which was confirmed by X-ray diffraction. Solution pH, incubation time and Pb concentration influenced removal and PbS formation. Inoculating contaminated soil with JH 70-4 decreased Pb availability; exchangeable Pb decreased while organic- and sulphide-bound Pb increased (Shim et al. 2014). A *Bacillus thuringiensis* strain GDB-1, isolated from the roots of *Pinus sylvestris*, had the capacity to remove heavy metals from mine tailing. The strain GDB-1 exhibited plant growth-promoting traits, including ACC deaminase activity, indole acetic acid and siderophore production and inorganic phosphate solubilization. The efficiency of GDB-1 to remove heavy metals was influenced by pH and initial metal concentration. Removal capacity (mg/L) was 77 % for Pb (100), 64 % for Zn (50), 34 % for As (50), 9 % for Cd (10), 8 % for Cu (10) and 8 % for Ni (10) during the active growth cycle in heavy metal-amended, mine tailing extract medium. Inoculating soil with GDB-1 significantly increased biomass, chlorophyll content, nodule number and heavy metals (As, Cu, Pb, Ni and Zn) accumulation in *Alnus firma* seedlings (Babu et al. 2013). The Zn-solubilizing [ZnO, $ZnCO_3$ or $Zn_3(PO_4)_2$] potential of *Gluconacetobacter diazotrophicus* strains under in vitro conditions by the production of a gluconic acid derivative, 5-ketogluconic acid, has been demonstrated (Saravanan et al. 2007). Similarly, *P. aeruginosa* strain CMG 823 isolated from a tannery air environment solubilizing insoluble ZnO or $Zn_3(PO_4)_2$ was found to solubilize large amounts of both ZnO and $Zn_3(PO_4)_2$ through the production of 2-gluconic acid (Fasim et al. 2002). Metal-resistant endophytic bacteria, *P. fluorescens* G10 and *Microbacterium* sp. G16, have also been reported to enhance the Pb accumulation in rape via secretion of organic acid (Sheng et al. 2008b). Likewise, inoculation of organic acid-producing *Pantoea* sp. and *Enterobacter* sp. increased P solubilization and Pb immobilization in soil (Park et al. 2011). Inoculation of soils with Cd/Zn-resistant bacteria significantly increased the mobilization of Zn and Cd due to the production of organic acids such as formic acid, acetic acid, tartaric acid,

succinic acid and oxalic acid (Li et al. 2010). The mobilization of Pb and Zn by the inoculation of three metal-resistant *Bacillus* strains, namely PSB 1, PSB 7 and PSB 10, have been demonstrated, and among them the *Bacillus* sp. PSB1 was found to solubilize a high amount of inorganic P via pH reduction with concurrent Pb and Zn mobilization (Wani et al. 2007). *Burkholderia caribensis* FeGL03 that has been isolated from Brazilian high-phosphorus iron ore significantly mobilized P and Fe from crushed iron ore. This FeGL03 produced gluconic acid and exopolysaccharides in good amount (Delvasto et al. 2009). The mycorrhizal fungi also have the ability for the biosynthesis of organic acids into the soil by which they can mobilize heavy metals through complexing them into the rhizosphere. Ericoid mycorrhizal fungi, *Oidiodendron maius*, have been identified to release ionic Zn from insoluble ZnO and $Zn_3(PO_4)_2$ through the production of Zn-chelating citric and malic acid (Martino et al. 2003). In support of this, *Beauveria caledonica*, a soil fungus was identified to solubilize cadmium, copper, lead and zinc minerals, converting them into oxalates via organic acid production (Fomina et al. 2004, 2005). An organic acid-producing fungi, *Aspergillus niger*, was able to mobilize large amounts of Pb and P from pyromorphite (Sayer et al. 1999). Different studies have also demonstrated that organic acids produced by soil microbes facilitate solubilization of metals into their respective ionic form, which are easily adsorbed/uptake by the plant root, that is, Pb and Cu (Sheng et al. 2008b; Chen et al. 2005). However, some studies show that organic acids either can have no effect or can negatively affect heavy metal mobilization. Inoculation of organic acid-producing bacteria *Bacillus subtilis* in metal-contaminated agriculture soils did not show any significant influence on the mobilization of Cr and Pb (Braud et al. 2006). An in-depth study on the factors that control the fate and behaviour of organic acids in soil is needed to identify the metal-specific mechanisms of microbes under the heavy metal-contaminated soils.

8.4.3 Biosurfactant-Mediated Remediation

Microbial biosurfactants have the potential to improve metal mobilization and remediation of heavy metal-contaminated soils (Braud et al. 2006; Singh and Cameotra 2013b; Mao et al. 2015; Franzetti et al. 2010). These biosurfactants are amphiphilic molecules consisting of a nonpolar (hydrophobic) tail and a polar/ionic (hydrophilic) head. A hydrophobic moiety usually contains saturated, unsaturated and hydroxylated fatty acids or fatty alcohols, and a hydrophilic group consists of mono-, oligo- or polysaccharides, peptides or proteins (Rajkumar et al. 2012; Müller et al. 2011). These biosurfactants produced by microbes form complexes with heavy metals at the soil interface, desorb metals from soil matrix and, therefore, enhance metal solubility and bioavailability in the soil solution. Interestingly, there is substantial evidence which suggests that the microbes producing surfactants increase the heavy metal mobilization in contaminated soils (Juwarkar et al. 2007; Sheng et al. 2008a; Venkatesh and Vedaraman 2012; Rajkumar et al. 2012; Mao et al. 2015). For instance, lipopeptide biosurfactant, consisting of surfactin and fengycin, was obtained from *B. subtilis* A21. Soil washing with biosurfactant solution removed significant amount of metals, namely Cd (44.2 %), Co (35.4 %), Pb (40.3 %), Ni (32.2 %), Cu (26.2 %) and Zn (32.07 %). Parameters like surfactant concentration, temperature, agitation condition and pH of the washing solution influenced the removal ability of the pollutant by the use of a biosurfactant mixture (Singh and Cameotra 2013a). The biosurfactant-producing *P. aeruginosa* strain A11 demonstrated resistance against all the metals detected in rhizosphere except Hg and Ni (Singh and Cameotra 2013b). Similarly, the potential of rhamnolipids produced by *P. aeruginosa* to mobilize Cu in contaminated soils was found as 2 % rhamnolipids removed 71 and 74 % of Cu from soil with initial concentrations of 474 and 4,484 mg/kg, respectively (Venkatesh and Vedaraman 2012). The

removal of Cd^{2+} increased with increased ligand concentration, particularly in solutions containing biosurfactants produced by the bacterial strains *B. subtilis* LBBMA155 (lipopeptide) and *Flavobacterium* sp. LBBMA168 (mixture of flavolipids; Lima et al. 2011). The Cd and Pb metal compounds' mobilization potential of *P. aeruginosa* BS2 under in vitro column experiments (artificial metal-contaminated soil) have been reported through the production of a dirhamnolipid biosurfactant (Juwarkar et al. 2007). The biosurfactants produced by microbes also show promise for enhancing metal uptake by plants, a desirable parameter for plants to be used for phytoextraction. For example, biosurfactant-producing bacterial strain *Bacillus* sp. J119 promoted Cd uptake by rape, maize, sudangrass and tomato in soil artificially contaminated with different levels of Cd (0 and 50 mg/kg). The study revealed that the inoculation of live bacterium *Bacillus* sp. J119 to soils significantly increased the plant Cd uptake when compared with the dead bacterial biomass-inoculated control (Park et al. 2011). Rhamnolipid biosurfactants produced by *Pseudomonas* species have been reported to remove toxic metals from soil (Herman et al. 1995). Several studies have been reported on the potential properties of biosurfactants produced by *Pseudomonas* sp., *Bacillus* sp. and *Acinetobacter* sp. for removal of heavy metals from contaminated soil and even acceleration of biodegradation of pesticides (Pacwa-Plociniczak et al. 2011; Kassab and Roane 2006; Sachdev and Cameotra 2013). Further, biosurfactants such as rhamnolipid and surfactin are known to remove heavy metals such as Ni, Cd, Mg, Mn, Ca, Ba, Li, Cu and Zn (ions) from soil with a new method of foaming-surfactant technology (Neilson et al. 2003; Mulligan et al. 2001). Therefore, in-depth studies on the interaction of biosurfactant-producing microbes and heavy metal mobilization and their consequences will improve our understanding of the role of biosurfactant-producing microbes for the heavy metal-contaminated soil remediation.

8.4.4 Biomass and Biological Macromolecule-Mediated Remediation

Biosorption can be defined as the removal of a metal or metalloid species, compounds and particulates from solution by the use of biological materials. Large quantities of metals can be accumulated by a variety of processes dependent on and independent of metabolism. Both living and dead microbial biomass as well as cellular products such as polysaccharides can be used for metal removal (Gadd 1993; Ma et al. 2011b; Javanbakht et al. 2014; Mudhoo et al. 2012). Therefore, biological materials of microbial origin have received increasing attention for heavy metal removal and recovery due to their good performance, low cost and large available quantities (Mudhoo et al. 2012; Javanbakht et al. 2014; Gaur et al. 2014). Metal biosorption by microbial biomass mainly depends on the components of the cell surface and the spatial structure of the cell wall, that is, bacteria (peptidoglycan, teichoic acids and lipoteichoic acids) and fungi/algae (polysaccharides, including cellulose, chitin, alginate, glycan etc.) have been proved to play a very important role in metal binding. Various microbial proteins have been also proved to be involved in metal binding for certain kinds of biomasses (Wang and Chen 2009). Bacteria are being used as biosorbents because of their small size, ubiquity, ability to grow under controlled conditions and their resilience to a wide range of environmental situations (Mishra and Malik 2012; Wang and Chen 2009). Bacterial species such as *Bacillus, Pseudomonas*, *Streptomyces*, *Escherichia* and *Micrococcus* have been tested and found to be effective for biosorption of heavy metals. These bacterial species either may possess the capacity for biosorption of many metals or, depending on the species, may be metal specific (Wang and Chen 2009). Although fungi are a very diverse group of eukaryotic microorganisms, among them, filamentous fungi and yeasts have been observed in

many instances to bind with metallic elements. In the field of biosorption, the moulds (filamentous fungi) and yeast (unicellular fungi) are areas of interests. The yeast biomass has been successfully used as biosorbent for removal of Ag, Au, Cd, Co, Cr, Cu, Ni, Pb, U, Th and Zn from aqueous solution. Yeasts of genera *Saccharomyces*, *Candida*, *Pichia* are efficient biosorbents for heavy metal ions and can absorb a wide range of metal ions (Podgorskii et al. 2004; Gadd 1993). Inoculation of metal-binding *Magnaporthe oryzae* and *Burkholderia* sp. reduced Ni and Cd accumulation in roots and shoots of tomato (Madhaiyan et al. 2007). Similarly, inoculation of *Trifolium repens* with *Brevibacillus* sp B-I decreased the concentration of Zn in shoot tissues compared with the respective uninoculated control (Vivas et al. 2003). This effect was due to the increased Zn biosorption by *Brevibacillus* sp. B-I. The pine seedling inoculation with the mycorrhizal fungi, such as *Scleroderma citrinum*, *Amanita muscaria* and *Lactarius rufus*, revealed reduced translocation of Zn, Cd or Pb from roots to shoots compared with the controls. This effect was attributed to the increased metal biosorption by outer and inner components of the mycelium (Krupa and Kozdrój 2007). The fungal cell wall components (e.g. chitin and extracellular slime) and intracellular compounds (e.g. metallothioneins and P-rich amorphous material) may also immobilize/arrest the metals in the interior of plant roots (Meharg 2003). Polymeric substances and glycoprotein can be defined as the removal of a metal or metalloid species, compounds and particulates from solution by biological material (Gadd 1993). Large quantities of metals can be accumulated by a variety of processes dependent on and independent of metabolism. The production of extracellular polymeric substances (EPS), mucopolysaccarides and proteins by microbes can also play an important role in complexing heavy metals and in decreasing their mobility in the soils. The inoculation of EPS-producing *Azotobacter* spp. to the metal-contaminated soils decreased Cd (−0.5) and Cr (−0.4) uptake by *Triticum aestivum* (Joshi and Juwarkar 2009). The arbuscular mycorrhizal fungi (AMF) produced insoluble glycoprotein, glomalin to form complexes with heavy metals and found that up to 4.3 mg Cu, 1.1 mg Pb and 0.1 mg Cd/g of glomalin (González-Chávez et al. 2004). The *B. subtilis* 38 (B38), a mutant species produced by UV irradiation, was found to be a good biosorbent for the adsorption of multiple heavy metals (cadmium, chromium, mercury and lead). Simultaneous application of B38 and NovoGro (organic fertilizer) exhibited a synergistic effect on the immobilization of heavy metals in soil (Wang et al. 2014b). Owing to the presence of a large number of negative charges on the external cell layers, EPS-producing cyanobacteria have been considered very promising as chelating agents for the removal of positively charged heavy metal, and an increasing number of studies on their use in metal biosorption have been published in recent years (De Philippis et al. 2011).

8.4.5 Metal Reduction and Oxidization-Mediated Remediation

Reactivities and mobilities of various elements including heavy metals in biological system depend upon the redox reaction conditions. Metal bioavailability is also influenced by redox potential (Eh), as generally aerobes require a substrate having a positive Eh, meaning that aerobic microorganisms grow rapidly under a high oxidation–reduction potential. For anaerobes, the substrate having a negative Eh seems to be beneficial (Singh et al. 2011). Microbes have the capability to increase the mobility of heavy metals through redox reactions and play an important role in the remediation of contaminated soils (Rajkumar et al. 2012). Changes in redox conditions are known to occur in soils during growth of bacterial cultures due to various biochemical reactions and metabolites formed. Sulphur-oxidizing rhizosphere bacteria have been reported to enhance Cu mobilization in metal-contaminated soils through the decrease of the rhizosphere pH, which facilitate the conversion of reduced sulphur to sulphates (Shi et al. 2011). Similarly, Fe/S-oxidizing bacteria have been

found to have potential to enhance metal bioavailability in the soils through acidification reaction (Chen and Lin 2001). The soil microbes can also immobilize the heavy metals in the rhizosphere through metal reduction reactions.

Microbes that are widespread in nature, under anaerobic conditions, are reported to utilize insoluble forms of variable-valence metal oxides as terminal electron acceptors in respiratory processes. These processes, referred to as dissimilatory metal reduction (DMR), have enormous biotechnological potential for the bioremediation of heavy metals in contaminated soils (Tikhonova and Popov 2015). Thermophilic microorganisms can reduce Fe(III), Mn(IV), Cr(VI), U(VI), Tc(VII), Co(III), Mo(VI), Au(I, III) and Hg(II). Ferric iron and Mn(IV) can be used as electron acceptors during growth (Slobodkin 2005). Priming of Cr-resistant bacteria *(Cellulosimicrobium cellulans)* to seeds of green chilli grown in Cr (VI)-contaminated soils decreased Cr uptake into the shoot by 37 % and root by 56 % compared with the control. This was possibly due to reduction of mobile and toxic Cr(VI) to nontoxic and immobile Cr(III) by bacteria (Chatterjee et al. 2009). The synergistic interaction of metal-oxidizing and metal-reducing microbes on heavy metal mobilization in contaminated soils has also been studied. Inoculation of Fe-reducing bacteria and Fe/S--oxidizing bacteria together significantly increased the mobility of Cu, Cd, Hg and Zn by 90 % and the researchers attributed this effect to the coupled and synergistic metabolism of oxidizing and reducing capability of the microbes (Beolchini et al. 2009). Many bacteria have the ability to reduce selenite [Se(IV)] and/or selenate Se(VI)] to red elemental selenium that is less toxic. An aerobic bacterium, *Comamonas testosteroni* S44, previously isolated from the metal(loid)-contaminated soil in southern China, reduced Se(IV) to red selenium nanoparticles (SeNPs) with sizes ranging from 100 to 200 nm (Zheng et al. 2014). Similarly, *Stenotrophomonas maltophilia* isolated from the rhizosphere of *Astragalus bisulcatus* had the capability to reduce Se [Se(IV) to insoluble and unavailable Se(0)] (Di Gregorio et al. 2005). Bacterial activity was identified as the major mechanism for the interconversion between As(V) and As(III), as well as for the production of methylated arsenic species in river sediments (Gorny et al. 2015).

The potential of the nanostructured materials (NSMs) has been utilized to create novel and effective decontamination of groundwater due to their high catalytic activity, large surface area and solubility. In a study, the effectiveness of Cr(VI) reduction and immobilization using NSMs and metal-reducing bacteria (MRB) was assessed for the remediation of Cr(VI) under batch and column conditions (Seo et al. 2013). Similarly, another bacteria *Thermoanaerobacter* sp. X513 has been utilized for the extracellular biosynthesis of Cu nanoparticles (CuNPs) under anaerobic conditions after 3 days of incubation. This bacterial strain not only nucleated NPs outer surface of the cell but also controlled the Cu^{2+} reduction rate to form CuNPs with an average diameter of 1.75 ± 0.46 μm (Jang et al. 2015). Likewise, the responses of ammonia-oxidizing bacteria and archaea for metal reduction were investigated for 10 weeks under two different acidic alfisols (Rayka and Hangzhou), spiked with different concentrations of As, Cu and As heavy metals. The data revealed that ammonia-oxidizing archaea were more abundant than ammonia-oxidizing bacteria in all the treatments (Subrahmanyam et al. 2014). In another recent report, *P. aeruginosa* strain SRD chr3 also exhibited the inherent capability of chromium removal from soil (Shukla et al. 2014). A novel strain of *Serratia proteamaculans* isolated from a chromium-contaminated soil also showed Cr(VI) reduction via the production of membrane-bound enzymatic proteins (Tahri Joutey et al. 2014). The potential of Cr(VI)-reducing bacteria has also been efficiently utilized to improve growth and yield of okra (*Hibiscus esculentus* L.) under in situ condition (Cr contaminated soil) (Maqbool et al. 2015).

8.5 Significance of Endophytic Microbes in Phytoremediation

Phytoremediation has emerged as an economic and sustainable alternative technique to conventional remediation techniques (Vangronsveld et al. 2009). In general, phytoremediation is contemplated to be an economic, eco-friendly and sustainable technology that offers the possibility of economic stability and in many cases provides economic valorization potential (Vangronsveld et al. 2009; Vassilev et al. 2004; Weyens et al. 2013). However, this technique comes with certain drawbacks such as heavy metal availability, uptake and phytotoxicity, which are the main limiting factors for a large-scale application. In addition, this technique may not be efficiently used to remediate all the heavy metals from contaminated soils (Weyens et al. 2009). Therefore, optimization of phytoremediation is the need of the hour which requires efforts from researchers around the globe. For optimization, various strategies have been already applied, that is, genetic manipulation of plants, associated rhizosphere microbial communities and addition of soil conditioners (Lebeau et al. 2008; Kuffner et al. 2008).

Metal-resistant endophytic microbes are reported to be present in various heavy metal hyperaccumulator plants growing under contaminated soils, which play an important role in successful survival and growth of such plants. These endophytic microbes reside within plant hosts without causing disease symptoms. In recent times, the inoculations of endophytic bacteria with plants for increased remediation of toxic metal from contaminated soils have been successfully tried. In addition, the heavy metal-resistant endophytic microbes are reported to promote plant growth by various mechanisms, such as nitrogen fixation, solubilization of minerals, production of phytohormones, siderophores, utilization of ACC as a sole N source and transformation of nutrient (Weyens et al. 2013). For example, the *ncc-nre* (nickel resistance) genes of *Ralstonia metallidurans* 31A was efficiently expressed in *Burkholderia cepacia* L.S.2.4 and *Herbaspirillum seropedicae* LMG2284. These endophytic bacterial strains showed removal of nickel up to 35 and 15 % under in vitro condition, respectively. These genetically modified strains have successfully acquired the capability to remove nickel via sequestration or bioprecipitation processes and consequently lowered the free nickel concentration at places of contamination. Therefore, in the near future, identification of such potent endophytic strains could offer interesting benefits for both host plants and contaminated sites (Lodewyckx et al. 2001).

8.6 Conclusions

The importance of microbes for the remediation of heavy metal-contaminated soils is now well appreciated by the scientific community. Microbial remediation techniques for the treatment of soils contaminated with heavy metals could offer cost-effective, sustainable alternatives for ecological reconstruction of contaminated soils. Microbes can play a significant role in the management of soils polluted with heavy metals. Numerous studies have demonstrated that microbes and their biomolecules are the essential determinants of heavy metal decontamination and thus can provide a sustainable method for remediation of heavy metal-contaminated soils. However, the survival of these microbes greatly influences the metal decontamination in soils, because of the unfavourable physico-chemical-biological properties of soils which reduce the survival and biological activity of the inoculated microbes. Thus, in-depth studies on microbes with edaphic factors in heavy metal-contaminated soils are needed.

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Role of Microbial Inoculants in Nutrient Use Efficiency

9

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Abstract

Since microbial inoculants have the ability to promote plant growth, nutrient enrichment, uptake, and support plant health, they are designated as a promising part of integrated solutions to agro-environmental problems. Inoculations with microbial consortia or plant-growth-promoting bacteria have been shown to enhance nutrient use efficiency, that is, mainly phosphorus, nitrogen, and carbon. It is generally believed that the huge diversity of the microbial communities associated with the rhizoplane in the rhizosphere and phylloplane helps plants to acquire minerals, organic substances, and many other small-molecule metabolites including amino acids, phytohormones, etc., to improve plant productivity. The interaction between microbes and plants has been shown to improve plant growth and impart biological control against biotic and abiotic stresses and work silently to improve the biogeochemical cycle in the natural ecosystem. Enhanced nutrient use efficiency benefits the plant by induction in seed germination, plant yield, and more uptake of nutrients along with enhancement in plant height and effective biocontrol. In this chapter, the effect of microbes and microbial inoculants in the enhancement of nutrient use efficiency is elaborated.

Keywords

Nutrients • Management • Microbes • Plant uptake • Rhizosphere

9.1 Introduction

A great diversity of microbial resources in the soil and water, plays a critical role in the uptake and acquisition of the nutrients by the plants. Microbial inoculants are promising and encouraging constituents in relation to agro-environmental

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_9

problems that provide integrated solutions because inoculants possess the capacity to enhance nutrient uptake and availability and also promote plant growth and favour plant health (Barea et al. 1998; Dobbelaere et al. 2001; Hodge et al. 2001; Bonfante 2003; Vessey 2003; Kloepper et al. 2004; Han and Lee 2005; Weller 2007; Adesemoye et al. 2008). Microbial inoculants include mainly arbuscular mycorrhiza fungi (AMF), cyanobacteria, and plant-growth-promoting rhizobacteria (PGPR).

It is generally believed that the large diversified microbial communities associated with rhizosphere and phylloplane help plants in acquiring minerals, organic substances, and different small molecule metabolites including phytohormones, amino acids, vitamins, etc. to ensure better plant productivity. The important role of the microbes as nitrogen fixers in the soils (Bashan et al. 2004), in siderophore production for sequestration of iron (Bakker et al. 2006), phosphate solubilization (Rodriguez and Fraga 1999), as potassium solubilizers and mobilizers (Basak and Biswas 2009), phytohormone producers (Prasanna et al. 2011), and 1-aminocyclopropane-1-carboxylate (ACC) deaminase producers that alleviate and reduce biotic stresses in the plants (Adesemoye and Kloepper 2009) has been emphasized. The interaction between plants and microbes has been shown to improve plant growth and impart biological control against biotic and abiotic stresses and several studies are available related to these aspects (Bhardwaj et al. 2014). The fundamental concept of organic farming has its base in the role of indigenous microbial abundance that work silently to improve and to make a better biogeochemical cycle in the natural ecosystem. The arbuscular mycorrhizal fungi (AMF) and PGPRs are well known to improve and enhance nutrient conditions and amounts in the soils that will eventually be available to the crop plants (Adesemoye and Kloepper 2009). Microbial inoculants and their formulations applied as seed treatment or soil inoculants are believed to multiply many fold in the soils and to benefit crop improvement, participating in the nutrient cycling (Singh et al. 2011). Nutrient use efficiency of fertilizers has been shown to be enhanced by the inoculations with PGPR and AMF (Bhardwaj et al. 2014). Numerous combinations of chemical fertilizers with PGPR and AMF have affected fertilizer usage and soil management (Han and Lee 2005; Adesemoye et al. 2008). A significant increase in N accumulation in wheat shoots or grains was observed by the application of soybean residues with *Azospirillum brasilense* with or without inorganic N fertilizer in poor fertility sandy soils. It is reported that the application of rock phosphate with farmyard manure (FYM) or vermicompost (1:2 ratio) along with phosphate-solubilizing bacteria (PSB; Manjunath et al. 2006) improves phosphorus (P)-use efficiency in French bean. The available phosphorus (Setiawati and Handayanto 2010) significantly improved with the application of PSB in acidic soil (Oxisol). To improve early-season P acquisition in crops, mycorrhizal association may help and work efficiently. In general, the AM endophytes are not host specific; moreover, preferential associations are reported with some host plants (Jansa et al. 2013).

Although tripartite interactions of plant–PGPR–AMF may facilitate and expedite the nutrient uptake by the plants (Barea et al. 1998), such interaction appears to be promising even if the alliance between PGPR and AMF may become interdependent or antagonistic. Therefore, there is a great demand for investigations to be conducted in this direction (Adesemoye and Kloepper 2009). The mechanisms behind plant–microbe (PGPR or AMF) interactions involve a complex phenomenon of numerous direct and indirect biochemical and molecular cellular processes (Berg 2009), but a clearer understanding of such procedures will definitely help in the identification of the mechanisms of interactions that benefit nutrient uptake by the plants. The use of microbial inoculants is protecting the environment from nutrient run-off. Increment of plant parts, that is, their height, weight and root, and overall shoot length with biocontrol, is generally achieved by the enhanced nutrient use efficiency as a result of plant–microbe interactions (Mahaffee and Kloepper 1994; Raaijmakers et al. 1997; Bashan et al. 2004; Yang et al. 2009).

9.2 Microbes and Nitrogen Use Efficiency

Microbial inoculants are elements that encourage agricultural management systems. The efficiency of fertilizers can be enhanced by the use of microbial inoculants, including PGPRs and AMF. With the application of chemical fertilizers and green manures, plant uptake was improved leading to increased nutrient use efficiency as reported earlier (Adesemoye et al. 2008). Environmental problems are generated by the continuous use of fertilizers. Low efficiency in the uptake of fertilizer, being a major factor, results in negative environmental effects (Barlog and Grzebisz 2004). Over 50 % of the N applied to the field can be lost from agricultural systems in the form of N_2, trace gases, or leached nitrate (Vitousek et al. 1997; Tilman 1998), and they can result in a long-term impact (Vitousek et al. 1997; Rabalais et al. 1998). Apart from chemical fertilizers, modifications such as compost extract, organic manure, compost, and compost tea are also used around the world to enhance crop production and to restrict plant pathogens. A 3-year study conducted with field corn hypothesized that plant growth can be increased by microbial inoculants, and this yield can promote nutrient uptake, thereby removing more nutrients, especially N, P, and K from the field as a component of an integrated nutrient management system. Soil analysis showed that in comparison to the data obtained during the initial year, the amount of nitrogen in the field increased at the end of the study (Adesemoye et al. 2008). Development of genetic varieties with refined and upgraded nitrogen use efficiency (NiUE) is essential for sustainable agriculture. Characterization of genes related to nitrogen assimilation was done and identified using whole genome transcriptional profiling approach along with the development of growth system for rice in which nitrogen was a limiting factor (Bi et al. 2009). Nutrient content of plants can be increased by the implication of microbial inoculants and therefore can increase overall plant growth. For example, increased N per gram of seed and N uptake per plot were observed when treated with inoculants. Improvement in N uptake efficiency and potential reduction in nitrate leaching can be achieved with the use of microbial inoculants that enhanced N uptake (Adesemoye et al. 2008). Proper nutrient cycling, where cyanobacteria constitute the major participating group of microbes, results in the proper utilization of the nutrients by the crop plants and augmentation of NiUE particularly in the form of organic fertilizers (Song et al. 2005; Wagner 2011; Fattah 2005; Herrero et al. 2001). Cyanobacterial nitrogen fixation process reveals the generation of hydrogen gas (H_2) at the same time, and there is recycling of 40 % of the evolved H_2 with the help of hydrogen uptake gene (*hup* gene; Margheri et al. 1991), while the rest 60 % can be used as a source of green fuel (Dutta et al. 2005). There was an investigation on the performance of some selected bacterial strains such as *Brevundimonas* sp., *Providencia* sp., and *Ochrobacterium* sp. in amalgamation with two species of *Anabaena* and one *Calothrix* sp. with rice variety Pusa-1460 in a pot experiment encompasses recommended fertilizer as control with 51 treatments (Prasanna et al. 2011). The soil nitrogen content increased by 13–14 % under field conditions by the addition of cyanobacteria; the cyanobacteria-amended soil released nearly 50 % of its ammonium nitrogen at 50 days of flooding (Syiem 2005). The rate of cyanobacteria-released nitrogen was recorded to be 12 and 35 % after 7 and 35 days of flooding in the field, respectively. An enhancement in the release of inorganic nitrogen into the soil was recorded by *Nostoc muscorum, Nostoc commune*, and *Anabaena* sp., apart from *Aulosira*. The soil cyanobacteria N content was found to be higher (due to N gain from cyanobacteria) when exposed to light in comparison to unexposed soil. A significant elevation in the phosphorus and NiUE in wheat crop was observed when the seed was inoculated with *Azotobacter* and *Azospirillum* strains (Kivi et al. 2014). In the rhizosphere, carbon use efficiency (CUE) is directly or indirectly affected by the changes in C and N balance through plant–microbe interactions (Blagodatskaya et al. 2014). Another major source of N input in agriculture, besides chemical N fertilizers, is the biological conversion of

atmospheric N_2 to ammonium which is carried out by symbiotic bacteria (Xu et al. 2012).

There is a direct correlation between nitrogen uptake efficiency of legumes and other crops owing to the nitrogen fixation by legumes in symbiotic association with *Rhizobium*. Exudates can be taken as an energy source for associative N_2 fixers in nonlegumes; for example, as compared to the bulk soil, the density of *Azospirillum* sp. (free-living nitrogen fixer) is higher in the rhizosphere (Assmus et al. 1995). For nitrogen nutrition of plants, the relevance of associative N_2 fixation is not clear but may be beneficial in low-nutrient soils. It is observed that the nitrogen mineralization is more in the rhizosphere as compared to bulk soil because of the release of root exudates, which decompose easily, compared with native soil organic matter. An enrichment in the denitrification of soil was observed subjected to abundance of anaerobes in the rhizosphere due to microbial biomass and root respiration (Hawkesford and Barraclough 2011).

Biofilms are observed on the root surface of rice by the action of ammonia-oxidizing bacteria (Briones et al. 2002). Ammonia-oxidizing bacteria in contact with roots could play a crucial role in the nitrogen nutrition of plants. The nitrate produced in the biofilms could be taken up directly by roots. By attracting associative N_2 fixers, and possibly by releasing exudates, NiUE could be increased that can be utilized only by roots and therefore it may increase their competitiveness in the rhizosphere. NiUE would also increased by endophytic colonization of N_2 fixers like *Azospirillum*. With respect to the use of nitrogen from the soil, NiUE could be amplified by the enhancement of microbial activity and thus nitrogen mineralization. However, this would have to be achieved by the stimulation of microbial biomass turnover to enhance release of immobilized nitrogen.

9.3 Microbes and Phosphorus Use Efficiency

An important role in mediating the availability of phosphorus to plants is governed by microorganisms that are integral to the soil phosphorus (P) cycle. Over many decades, understanding of the microbial contribution to plant P availability, and opportunities for the manipulation of specific microorganisms to enhance P nutrient in soil ecosystem have therefore been of considerable interest. This interest is accentuated by P deficiency being very common in tropical and weathered soils throughout the world and rising costs of P fertilizers. Although an abundant amount of P is available in soil, P-use efficiency for plants from soil and fertilizers may be diminished because very small amount is available to plants. Therefore, microbial inoculants in association with several beneficial microbes are promising substitute to increase available P in soil. It is quite common in both developed and developing countries to make agriculture sustainable. Use of mineral fertilizers is restricted and limited in developing countries (Sánchez 2010). The use of microbial inoculants for the increasing P-use efficiency is very common. Soil bacterial community enhances the P content of plants (Gerretsen 1948) by the solubilization of precipitated calcium phosphates. P solubilization and improved nutrient efficiency that accompany microbial inoculants in rhizosphere should be the focus of investigation (Richardson and Simpson 2011).

By using inoculants, the fate of nutrients solubilized in the soil is yet to be convincingly proven in the literature. For example, the correlation between microbial phosphate solubilization and plant uptake of the solubilized P in practice is not yet clear. Different studies related to nutrient use efficiency explain that P from insoluble form is available through the action of microorganisms (Peix et al. 2001; Idriss et al. 2002; Ivanova et al. 2006). The proportion of phosphate solubilization and available P to plants is not well documented, and there is no indication on how much amount is taken by the plants. There are various possible factors that could affect P-use efficiency of plants, namely the amount of P solubilized, P needed for bacteria, root exudation from the plants, and favourable soil conditions (including soil P status, P absorption capacity, and pH). Additional focus should be given to studies on similar points with other components and the molecular aspects of the microbial impact on plant-associated nutrients and fertility manage-

ment which will facilitate our understanding of using microbial inoculants to minimize adverse effects of fertilizers (Adesemoye et al. 2008). Co-inoculation of rock phosphate with FYM or vermicompost with PSB enhances the P-use efficiency in French bean (Manjunath et al. 2006). It is also shown that there was a significant increase in the available phosphorus in an acidic soil (Oxisol) after the application of PSB (Setiawati and Handayanto 2010). Mycorrhizal associations generally magnify the improvement of early season P acquisition in crops.

There is an increasing need for the better management of P fertilizer in agricultural systems (Tunney et al. 1997) to minimize any adverse environmental effects owing to P losses. Through microbial associations, P-use efficiency would be improved with considerable economical and environmental benefit. Mycorrhiza signifies its contribution in phosphate acquisition in agriculture. Majengo et al. (2011) determined that vesicular arbuscular mycorrhiza (VAM) and rhizobial inoculants have variable effectiveness on plants.

In common beans, nutrient use efficiency along with growth, phosphorus acquisition, nitrogen availability, and nodulation ability was observed with the co-inoculation of *Rhizobium tropici* CIAT899 and *Glomus intraradices* under phosphorus-limited and phosphorus-sufficient conditions. In all growth-related parameters, there was a significant change as a result of this co- inoculation. However, a maximum P-use efficiency was observed in the presence of mycorrhiza. This enhanced P-use efficiency was achieved with the improvement of symbiotic nitrogen fixation when rhizobia and mycorrhiza together were applied in P-deficient conditions (Tajini et al. 2011). A recent investigation showed seeds inoculated with PGPRs, *Azotobacter* and *Azospirillum*, may enhance nitrogen and phosphorus-use efficiency of spring wheat (*Triticum aestivum* L.; Kivi et al. 2014). While it is assumed that the mycorrhizal fungi generally increase phosphorus-uptake efficiency by the crop plants, it has also been observed that in comparison to non-mycorrhizal plants the phosphorus concentration of leaves of mycorrhizal plants is higher (Treseder 2013). A contradictory situation is also observed which reflects that the mycorrhizal symbiosis increase the nutrient use efficiency but to a smaller extent reduces other nutrients as the phosphorus amount is inversely related to carbon concentration and N content demand is higher for fungal communities in soil (Hodge and Fitter 2010).

9.4 Microbes and Carbon Use Efficiency

A fundamental parameter for ecological models is carbon use efficiency (CUE) that is based on the physiology of microorganisms. Rates of ecosystem carbon storage, conversion of plant-produced carbon into microbial products, and material flows along with energy to higher trophic levels are determined by CUE. Biosynthetic process-associated microbial fragment is considered as CUE (Steinweg et al. 2008; Manzoni et al. 2012). Consequently, abundant microbial biomass resulted in more CUE, and, when respired, more amount of assimilated substrate was observed in the cells. Microbial CUE is generally assumed to decline with increasing temperature in new microbial–biogeochemical models. Based on this assumption, under warm conditions, soil carbon losses are small because of the decline in microbial biomass (Allison 2014).

Two theoretical models relating to CUE and microbial uptake rate were explained. Under warm conditions, microbes and microbial inoculants minimize enzyme resources and nutrient uptake system (Allison 2014). The net primary production of the biosphere is mediated through decomposer food webs (Cebrian and Lartigue 2004) during mineralization. The microbial biomass production from the catabolism of detrital organic matter is the trophic base of these food webs. The efficiency of this conversion is predominantly termed as the CUE and mostly governs and controls the conversion of plant-produced carbon into microbial products, rates of ecosystem carbon storage rate to ecosystem, and flow of energy and materials to higher trophic levels (Six et al. 2006; Miltner et al. 2012).

Stable soil organic matter is mostly derived from microbial compounds; the C storage potential in soils is determined by the partitioning of C uptake by microbial inoculants into growth and respiration. The carbon substrate proportion, which is infused into new microbial biomass (i.e. microbial growth), is often compared and correlated to the substrate carbon fraction which is respired as CO_2 and designated as CUE or substrate use efficiency (SUE). The CUE of microbes is strongly governed by the nutrient availability such as nitrogen (N) as indicated by the stoichiometric theory. Therefore, microbial inoculants respire excess C (low SUE) when deficient in nutrients, while conversely excess N is mineralized when C is deficient (high SUE; Takriti et al. 2014).

Considered as the elementary and basic characteristic of microbial metabolism, C and N balance in the rhizosphere is altered and affected by the plant–microbial interactions affecting microbial CUE as a consequence. From dormancy to activity, researchers must estimate CUE in microbial assemblages due to change in microbial physiology (Blagodatskaya et al. 2014). Carbon substrate glucose was taken for the measurement of induced microbial growth in root-free and rhizosphere soil under steady-state environment. Due to variation in respiration burst and DNA increment, there was a large amount of variation in the microbial CUE in root-free soil and rhizosphere. A stagnant constant CUE in rhizosphere represents the balanced growth in a log-phase growing culture. At the end of the log phase, enhanced CUE was observed more in root-free soil as compared to rhizosphere. Plants with the equilibrium of physiological processes affect microbial CUE and differ in root-free soil (Blagodatskaya et al. 2014). Total rhizosphere microbial biomass was 14–31 % higher as compared to the root-free soil, while the active part of microbial biomass was 45–83 % higher (Blagodatskaya et al. 2014).

The management of crop residues has become increasingly important in sustaining long-term fertility in cropping systems. Integration of crop residues can vary with the microbial processes, which affect the availability of nutrient and crop yields. During rice straw decomposition, CUE by soil microbial communities was determined in a rice paddy soil, under aerobic and anaerobic (flooded) conditions at varying temperatures (5, 15, and 25 °C). Elevated CH_4 production and flooding condition can result in diminished CUE; however, with decreasing temperature, CH_4 is considered to be negligible. The waste product of fermentation was used by anaerobic bacteria and longer incubation led to lesser net CUE in flooded conditions as compared to without flood condition. Finally, almost similar microbial products were obtained by either aerobe or anaerobe implication (Devevre and Horwath 2000).

While microbial NiUE has not been studied in depth, microbial CUE has been the focus of numerous investigations in soil biogeochemistry, and this has been established as a prompt factor for determining growth of microbes, nutrient immobilization, and ultimately sequestration of soil C (Manzoni et al. 2012; Six et al. 2006). Microbial C metabolism is considered as a highly regulated interplay between anabolic and catabolic processes (Shimizu 2013). Apart from C:N resource control, there are several different factors controlling CUE and NUE. In this sequence, the first factor is the limitation of microorganisms associated with any ecosystem by a particular nutrient other than C and N, resulting in the minimization of CUE and NiUE. Second, both C and N abundances and their use efficiencies involve enzyme production, but more amount of N is needed as compared to C for enzyme production than production of biomass (Schimel and Weintraub 2003). Third, a large fraction of metabolically inactive microbes are present in soils (Lennon and Jones 2011; Blagodatskaya and Kuzyakov 2013). This microbial dormancy promotes the reduced CUE and NiUE of microbes that consequently diminished the NUE of plants. Fourth, as a result of microbial responses against abiotic and biotic stress, both carbon and NiUE are affected with the production of osmolytes and other osmoprotectants by microbes (Schimel et al. 2007). A number of factors affecting these use efficiencies are illustrated in relation to microbial perspectives in C and N cycling (Mooshammer et al. 2014).

Because of varying and improper farming practices, agricultural lands have been subjected to degradation worldwide as wind and water lead to soil erosion, depletion in nutrients, and loss of soil organic matter, all of which have contributed to a major, serious decline in soil fertility, soil carbon, and productivity (Parr et al. 1994). With better production, a restored degraded soil with good C content can be achieved after the addition of microbial inoculants with numerous organic fertilizers. The restoration and rehabilitation of these degraded soils can be obtained, leading to better productivity and increasing the soil–carbon content, by proper and regular additions of various organic fertilizers along with microbial inoculants. Microbiologists have long known about indigenous populations of microorganisms associated with several organic wastes and residues, including animal manures, crop residues, green manures, and municipal wastes (both raw and composted), which have major physiological capabilities. Organic fertilizers added to the soil are associated with the long-term sustainable agriculture. Their implication is limited to the fields and restricted by the farmers because of their physical and chemical nature. Microbial consortia or inoculants of mixed cultures of beneficial microorganisms have considerable potential to control the soil microbiological equilibrium and therefore provides a more favourable environment for plant growth and protection (Parr et al. 1994).

Based on the physiology of microorganisms, CUE is a fundamental and basic parameter for ecological models. Biomass composition, environmental factors, and stoichiometric constraints are responsible for microbial CUE (Sinsabaugh et al. 2013). In future, under higher CO_2 environment, microbes will play crucial roles in the enhancement of plant productivity and C/N nutrient use efficiencies. Plant production and yield can be increased by PGPRs, and microbial respiratory C loss is decreased under elevated atmospheric CO_2. A plant-growth-promoting bacteria, *Pseudomonas fluorescence*, was subjected to rhizosphere to observe the effect on C and N cycling under higher CO_2 condition. This microbial community was involved in the enhancement of plant productivity. Soil microbial decomposition in elevated CO_2 condition was alleviated by the incorporation of *P. fluorescence* into the soils and the competition between soil microbes and plants for the acquisition of nutrients increases (Nie et al. 2014).

9.5 Conclusion

A wide range of microbial inoculants and communities are associated with plants and present in soil, directly or indirectly, enhancing nutrient use efficiency. The enhanced nutrient use efficiency therefore gives a significant improvement in plant growth. The degraded soils could be restored and rehabilitated to an optimum level of productivity by proper and regular additions of various organic fertilizers along with microbial inoculants, increasing the soil–carbon sequestration value. A number of bacterial communities, mycorrhiza, and cyanobacterial abundances influence the uptake of phosphorus, carbon, and nitrogen efficiently and make available to the plants. Earlier investigation therefore indicates that the application of crop residues with microbial inoculants such as *Pseudomonas, A. brasilense*, cyanobacteria, and mycorrhiza with or without inorganic N fertilizer in poor fertility sandy soils showed a significant increase in nutrient accumulation in crops. *Rhizobia* and mycorrhiza provide good examples on the use of microbial inoculants to enhance nutrient use efficiency. These microbial inoculants were shown to increase plant productivity and accelerate soil decomposition in relation to N cycling. A better nutrient use efficiency in an ecosystem leads to an improvement in the biogeochemical cycling that ultimately improves the plant productivity and yields.

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Nutrient Management Strategies Based on Microbial Functions

10

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Abstract

There is a common misconception that nutrient deficiency can only be managed by the application of required fertilizers into the field, but most of the times even after applying fertilizers, plants are not able to attain proper growth. The major cause is unavailability or inadequate availability of nutrients to the plants. Therefore, there is a need to understand different nutrient management practices of the field. Millions of microbes are present in the soil, but still only a fraction of this microbial population is known to researchers. Therefore, the specific role of these microbes present in the soil cannot be denied. So far, researchers identified few microbial populations that are characterized for their significant role in nutrient management of the soil, but the information about the characterization and mechanism of these beneficial microbes has not been documented. In this chapter, an attempt has been made to explain the plant-required nutrients, their deficiency, and the role of different beneficial microbes that can manage the nutrient requirement of plants.

Keywords

Microbes • Functions • Nutrient • Management • Micronutrients • Macronutrients

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10.1 Introduction

Plant nutrients, which are readily available in soil, play an important role in crop growth and productivity. Availability of nutrients to the plants depends on their presence or abundance in the soil. The external source for enriching soil nutrients is mainly chemical and biological fertilizers

D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_10

such as urea, diammonium phosphate (DAP), superphosphate, composts, and biofertilizers. The most important factors that affect the availability of these nutrients to the plants are timing of fertilizer application and their proper quantity. Sometimes, just after the harvesting of the first crop, without knowing the requirement of nutrient to the soil, the farmer applies additional fertilizer into the soil. This increased amount of nutrient into the soil is not always beneficial to the plant. At times, it shows adverse effects on the crop, such as higher concentration of nitrogen, which can increase the plant growth but somehow reduce the availability of other nutrients to the plant; similarly, overapplication of phosphorus (P) can result in P runoff causing eutrophication of surface water (Barlog and Grzebisz 2004). Therefore, nutrient management of the soil is required. Most of the nutrient management practices are controlled by human activities, but microbes that are present in the soil also play an important role in nutrient management.

In a given set of environmental conditions, the unpredictable nature and biosynthetic capabilities of microbes have made them important candidates for resolving nutrient-related issues in rhizosphere (the area around the roots). Soil microbes can transform organic molecules into mineral elements that are readily available to plants and they also help to maintain soil structure by producing cementing compounds. Most of the bacterial communities have a mucilaginous sheath that helps to bind small soil aggregates; similarly, fungal communities have a hyphal structure that spread all over the soil and, because of this, small soil particles are trapped in between these hyphal structure that helps to hold soil aggregates. During decomposition, soil microbes (mainly mesophilic and thermophilic) convert raw organic material into humus (Mehta et al. 2014). This conversion starts with the breakdown of complex molecules into simpler molecules. These simpler molecules, in the form of essential minerals, are released into the soil and help plants growing in nearby areas (Fig. 10.1). In addition to increased nutrient availability, these microbes also help in reducing the disease and nutrient loss, as well as help in degrading toxic elements

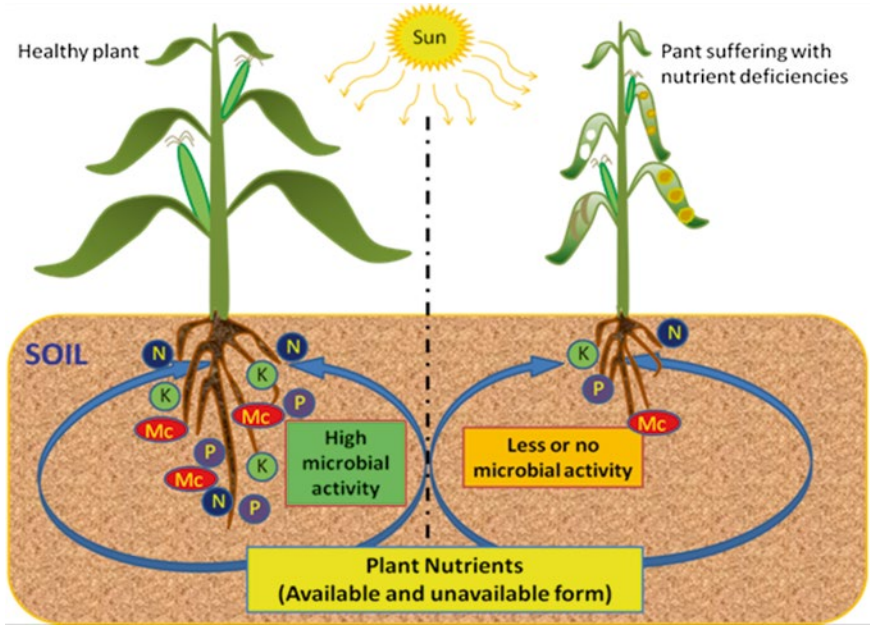

Fig. 10.1 Potential role of soil microbes in nutrient management (*K* potassium, *P* phosphorus, *N* nitrogen, *Mc* other nutrients)

present in the soil. Plant health totally depends on the microbial community present in the rhizospheric soil. In healthy rhizosphere, beneficial microbes work as a mediator between plant life and soil life that helps produce healthy crops, whereas, in unhealthy rhizosphere, the soil is dominated by different soil-borne plant pathogens that can attack on the crops and restrict their physiological as well as morphological activities. Therefore, there is a greater need for better understanding of microbe-mediated nutrient management practices.

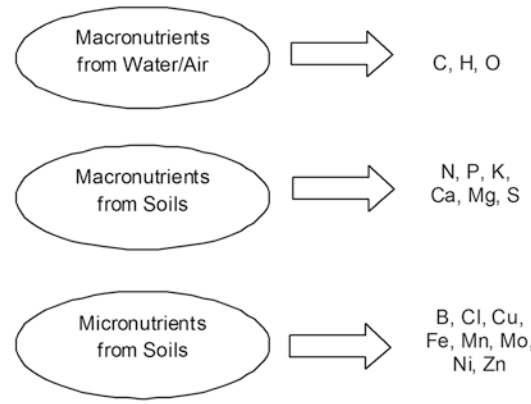

Fig. 10.2 Essential plant nutrients required for plant growth

10.2 Role of Nutrient in Plant Health

Since centuries it is known that plants obtain nourishment from the soil. During the first half of the nineteenth century, it was found that plants require certain nutrients known as essential nutrients and that nutrients are taken up by the roots in the form of inorganic ions. Nutrients are indispensable as plant constituents, for biochemical reactions, and for the production of organic materials referred to as photosynthates (carbohydrates, proteins, fats, vitamins, etc.) by photosynthesis. In crop production, adequate mineral nutrition is important to produce healthy crops with high and good quality. The balanced plant nutrient is a pivotal factor, which helps crops to give the desired yield potential. Plants can get their required nutrients from fertilizers, organic manures, the atmosphere, etc.

Balanced nutrients are necessary for plant structures and for all physiological processes; for example, nitrogen and magnesium are a fundamental part of the chlorophyll required in photosynthesis process. On the other hand, phosphorus stimulates energy production and its storage. In addition, nitrogen is necessary for nucleic acid synthesis, and potassium is required for osmotic maintenance and enzyme activation (Waraich et al. 2011). Currently, there are 17 essential plant nutrients. Some of them (carbon and oxygen) are taken by the plant from air, others including water can be taken up from the soil. To produce a healthy plant, the following mineral nutrients should be supplied to growing media (Allen and Pilbeam 2007):

Essential plant nutrients include *macronutrients and micronutrients.* In *macronutrients*, nitrogen (N), phosphorus (P), and potassium (K) are *primary nutrients*. Those nutrients are usually less in soil because plants use them in large quantity and therefore they supply to the soil at higher rates compared to secondary nutrients and micronutrients. Another group of s*econdary nutrients* includes calcium (Ca), magnesium (Mg), and sulphur (S), and they are supplied in smaller amounts compared to primary nutrients. *Micronutrients* include iron (Fe), chlorine (Cl), manganese (Mn), zinc (Zn), copper (Cu), boron (B), molybdenum (Mo), and nickel (Ni): they are required even in smaller amounts compared to secondary nutrients (Fig. 10.2).

10.2.1 Essential Plant Nutrients

A total of 17 elements are essential for the growth and full development of higher green plants according to the criteria laid down by Arnon and Stout (1939). These criteria are as follows:

1. The element must be essential for supporting normal growth and reproduction, and the plant cannot complete its life cycle or set the seeds if the element is absent.

2. The element is specific and its function must not be replaced by another.
3. The element must be directly implied in plant metabolism.

The basis of most plant micronutrients was initiated from 1922 to 1954. In 1987, Brown et al. established the essentiality of nickel (Ni), though there is no agreement whether Ni is essential or beneficial nutrient. However, this list may not be considered as final and it is probable that more elements may prove to be essential in future. The chronology discoveries, form absorbed, and the concentration in plant dry matter of nutrient essentiality are summarized in Table 10.1.

Essential nutrients can also be categorized into four broad categories depending on their functions. These categories are as follows:

1. Essential nutrients that are biomolecules and enhance cell structure (e.g. carbon, oxygen, hydrogen, and nitrogen).
2. Essential nutrients that are chemical energy-related compounds in plants (e.g. magnesium in chlorophyll and phosphorus in adenosine triphosphate (ATP)).
3. Essential nutrients that activate or inhibit enzymes.
4. Essential nutrients can change the movement of water molecules within a cell.

10.2.1.1 Macronutrients

Macronutrients required in plants can be categorized into two groups: primary nutrients and secondary nutrients.

10.2.1.1.1 Primary Nutrients

The primary nutrients are nitrogen, phosphorus, and potassium. For most crops, these three mineral nutrients are needed in large amounts than other nutrients.

(a) Role of Nitrogen

Nitrogen is one of the three nutrients most important for plant growth and it is required in large quantity. It stimulates fast vegetative growth, enhances the maturity of the crops, and boosts the development of seeds. It is essential for most metabolic processes that take place in the plant as a constituent of amino acids that are necessary for proteins and other product synthesis. Nitrogen

Table 10.1 Essential plant nutrients, forms taken up, and their typical concentration in plants (Roy et al. 2006)

Nutrient (symbol)	Essentiality established by	Forms absorbed	Typical concentration in plant dry matter
Macronutrients			
Nitrogen (N)	de Saussure	NH^{4+}, NO3	1.5 %
Phosphorus (P, P_2O_5)	Sprengel	H_2PO^{4-}, HPO_4^{2-}	0.1–0.4 %
Potassium (K,K_2O)	Sprengel	K^+	1–5 %
Sulphur (S)	Salm-Horstmann	SO_4^{2-}	0.1–0.4 %
Calcium (Ca)	Prengel	Ca^{2+}	0.2–1.0 %
Magnesium (Mg)	Sprengel	Mg^{2+}	0.1–0.4 %
Micronutrients			
Boron (B)	Warington	H_3BO_3, $H_2BO_3^-$	6–60 μg/g (ppm)
Iron (Fe)	Gris	Fe^{2+}	50–250 μg/g (ppm)
Manganese (Mn)	McHargue	Mn^{2+}	20–500 μg/g (ppm)
Copper (Cu)	Sommer, Lipman	Cu^+, Cu^{2+}	5–20 μg/g (ppm)
Zinc (Zn)	Sommer, Lipman	Zn^{2+}	21–150 μg/g (ppm)
Molybdenum (Mo)	Arnon and Stout	MoO_4^{2-}	Below 1 μg/g (ppm)
Chlorine (Cl)	Broyer and others	Cl^-	0.2–2 %
Nickel (Ni)	Brown and others	Ni^{2+}	–

is considered as a fundamental component of the green pigment known as chlorophyll, necessary in photosynthesis process. Photosynthesis may be defined as a process by which green plants utilize sunlight to synthesize their own nutrients (carbohydrates) from atmospheric carbon and water in the presence of the green pigment known as chlorophyll. The required energy for growth and development is taken from the synthesized carbohydrates (sugars). Here is a summary of the chemical equation of this complex process:

$$6CO_2 + 12H_2O + 672\,\text{Kcal light energy} \rightarrow C_6H_{12}O_6 + 6H_2O + 6CO_2$$

Nitrogen plays an essential role in temperature stabilization. High temperature is proportional to the light intensity and it can have negative effects on mineral nutrient uptake and plant growth. Among mineral nutrients, it has an important role in sun radiation use and metabolism of carbon during photosynthesis process (Kato et al. 2003; Huang et al. 2004).

(b) Role of Phosphorus

Phosphorus is an essential element considered as fundamental blocks of life, the ribonucleic acid (RNA), deoxyribonucleic acid (DNA), phospholipids, coenzymes, nicotinamide adenine dinucleotide phosphate (NADP), and most importantly ATP; it is also needed for various biochemical and physiological processes such as transfer of energy, protein synthesis, and other functions (Prabhu et al. 2007).

(c) Role of Potassium

Plant nutrients play a critical role and enhance plant resistance (Marschner 1995). Potassium (K) is required for the protection of crop plants from unfavourable situations. Also, it is necessary for photosynthesis, translocation of photosynthesis products from source organs to sink organs, turgidity keeping and activation of enzymes to metabolize carbohydrates for the manufacture of amino acids and proteins, under stress conditions, hasten cell multiplication and growth by stimulating the transfer of starches and sugars between cell components, improve stalks and stem rigidity, and increase disease resistance as well as drought tolerance and control of osmotic potential (e.g. opening and closing of stomata); it is also responsible for firmness, texture, size, and colour of fruit crops, and is essential for oil content of oil crops (Marschner 1995; Mengel and Kirkby 2001).

10.2.1.1.2 Secondary Nutrients

The secondary nutrients are calcium, magnesium and sulphur. For most crops, these three are needed in lesser amounts than the primary nutrients.

(a) Role of Calcium

Various plant physiological processes are moderated by calcium and its action occurs basically at tissue, cellular, and molecular levels that can affect growth and plant responses to environmental stresses in plant. Calcium is immobile and persists in the older tissue of the plant. It has the ability to neutralize organic acids produced during the growth process and to participate in carbohydrate transport and absorption of nitrogen (Waraich et al. 2011). Calcium supply induces stomatal closure, when temperature is low, and it stimulates the elasticity and expansion of cell walls, which in turn prevent plant-growing regions to become rigid and brittle. It has also been shown that Ca^{2+} mediates *abscisic acid* (ABA) that controls stomatal closure and releases in internal guard cell stores or the apoplast

(Wilkinson et al. 2001). Calcium plays an important role in regulating cold temperature stresses and recovery from injury, and it allows good performance of plants during cold stress periods (Palta 2000). Calcium plays a very prominent role in the maintenance of cell structure and is involved in the production of new growing points and root tips. It is responsible for the plasma membrane enzyme activation such as ATPase that is required for the pump-back of nutrients lost during cell membrane damage and helps the plant recover from cold injury. It also acts as calmodulin that regulates plant metabolism and expedites plant growth under cold environment. In addition, it is considered as a fundamental brick in the plant because it is necessary for the manufacture and development of a cell (Waraich et al. 2011).

(b) Role of Magnesium

Magnesium (Mg) participates in different physiological and biochemical processes that can influence plant growth and its development (Waraich et al. 2011). It is important for photosynthesis process and many other metabolic processes. Small fluctuation in magnesium levels can strongly affect the main chlorophyll enzymes (Shaul 2002). Many findings confirmed that Mg plays an essential role in electron transport chain of chloroplast. Mg transfers energy from photosystem II to $NADP^+$ and protects thylakoid membrane by reducing accumulation excitation energy and oxidative damage (Halliwell 1987). It has been reported that, magnesium promots antioxidative enzymes and antioxidant molecule concentration in bean (Cakmak and Marschner 1992; Cakmak 1994), *Mentha pulegium* (Candan and Tarhan 2003), maize (Tewari et al. 2004), pepper (Anza et al. 2005), and mulberry (Tewari et al. 2006). Magnesium plays a major role in water and nutrient uptake by increasing the root growth and root surface area. As a chlorophyll component, Mg enhances sucrose production and its translocation for further use (Waraich et al. 2011). It stimulates the transfer of carbohydrates across phloem and reduces the production of reactive oxygen species (ROS). Under high- or low-temperature stress, Mg protects chloroplast from photooxidative damage. Chloroplast structure maintenance by Mg nutrition stimulates photosynthesis activities under extreme temperature, thereby increasing plant productivity (Waraich et al. 2011).

(c) Role of Sulphur

Sulphur plays an important role in amino acid synthesis that results in protein production. It is also needed in chlorophyll production and uses phosphorus as well as other essential nutrients. It is considered as nitrogen for crop yield and quality giving. Sulphur enhances the quality of crop grains and improves nitrogen use efficiency during protein synthesis in crops that require a high amount of nitrogen. It is also important for yield and protein quality of forage and grain crops as well as quality of fibre crops (Reddy 2012).

10.2.1.2 Micronutrients

Out of 17 essential plant nutrients, eight are micronutrients because plants need them in relatively small amounts. They include chlorine (Cl), manganese (Mn), boron (B), copper (Cu), iron (Fe), molybdenum (Mo), zinc (Zn), and nickel (Ni). Their roles in plant health are narrated subsequently.

(a) Role of Chlorine

Chlorine is essential in photosynthesis, where it is involved in the evolution of oxygen. It increases cell osmotic pressure and the water content of plant tissues. It is found in many bacteria and fungi, and it reduces the severity of certain fungal diseases (Reddy 2002).

(b) Role of Manganese

Manganese is an essential nutrient involved in photosynthesis and nitrogen metabolism, as well as to form other compounds required for plant metabolism. Manganese is essential for regulation of adverse temperature conditions by promoting photosynthesis activity and metabolism of nitrogen within the plant body. Manganese is

necessary to prevent chlorosis between veins and necrotic brown spots on old leaves, and it decreases the shedding of premature leaves. It is known as an enzyme activator in plant body, mostly in oxidation–reduction, decarboxylation, and hydrolytic reactions, and hence intervenes in ROS detoxification (Marschner 1995). Recent findings confirm that manganese has the ability to inhibit the production of oxygen-free radicals and enhances antioxidative compounds and enzymatic activities under temperature stress (Aktas et al. 2005; Turhan et al. 2006; Aloni et al. 2008).

(c) Role of Boron

Boron can intervene in various physiological and biochemical processes during plant growth and development such as cell elongation, cell multiplication, cell wall biosynthesis, membrane function, nitrogen metabolism, photosynthesis, and uracil synthesis (Marschner 1995). It can promote the antioxidant activities of the plant and prevent the damage that can be induced by temperature stress. Boron supply can improve the transport of sugars within the plant and results in seed germination and grain formation (Waraich et al. 2011). Boron application enhances carbohydrates and reduces phenolic compounds in leaves. This stimulates photosynthetic rate by inhibiting the production of ROS species (Waraich et al. 2011).

(d) Role of Copper

Copper (Cu) is an essential redox-active transition metal and it is involved in many physiological processes in plants such as chlorophyll formation, although its specific role is still unclear. Under physiological conditions, Cu exists as Cu^{2+} and Cu^{+}. Cu acts as a structural element in regulatory proteins and participates in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses, cell wall metabolism, and hormone signalling; it is also thought to be involved in protein synthesis. It intensifies colour, improves the flavour of fruits and vegetables, increases sugar content, and plays a major role in reproductive stages (Marschner 1995; Raven et al. 1999).

(e) Role of Iron

Iron is more abundant, though its quantity is low and not available for plant and microorganism needs, due to the low solubility of its mineral that contains iron, particularly in arid zones with alkaline soils. Iron is an essential nutrient in crops, for enzymes such as cytochrome that is required in electron transfer chain. It synthesizes chlorophyll and maintains the chloroplast structure and enzyme activity (Mamatha 2007; Ziaeian and Malakouti 2001; Zaharieva and Abadia 2003; Welch 2002). In addition, iron is necessary for chlorophyll production. For instance, iron is a site activator of glutamyl-tRNA reductase, an enzyme necessary for 5-aminolevulinic acid, which is a progenitor of chlorophyll (Kumar and Soll 2000).

(f) Role of Molybdenum

Molybdenum (Mo) is needed in biological nitrogen fixation (nodulation) by legumes and it is involved in protein synthesis by reducing nitrates. For normal growth, the plant requires 0.1–2.5 ppm in its tissues. The recommended dose for Mo soil application ranges from 0.1 to 0.5 Ib Mo/acre (Reddy 2012).

(g) Role of Zinc

Zinc has a crucial role in plant enzymes and proteins for carbohydrate metabolism, protein biosynthesis, gene expression, plant hormone metabolism (auxin), formation of pollen and biological membrane support, photooxidative damage and temperature stress protection, and resistance to certain pathogen infections (Alloway 2008).

(h) Role of Nickel

Nickel is important for iron absorption and seed germination. Its application on crops prevents certain yield-limiting diseases, and hence results in the significant reduction of pesticide use and promotes crop yield as well. It can also be used as biocontrol for microbial pests, and acts as a key factor for secondary plant metabolites by promoting disease resistance (Wood and Reilly 2007).

10.3 Problems Associated with Nutrient Deficiency in Plants

In the early nineteenth century, Baron Justus von Liebig, a German chemist, showed the essentiality of nutrients for plants' life. He stated, 'We have determined that a number of elements are absolutely essential to plant life. They are essential because a plant deprived of any one of these elements would cease to exist'. He also established the fact that plants obtain their carbon from carbon dioxide in the air, and not from the soil. His theory of 'law of the minimum' states that 'plants will use essential elements only in proportion to each other, and the element that is in shortest supply in proportion to the rest will determine how well the plant uses the other nutrient elements' (Tucker 1999; Reddy 2002).

Generally, all plant problems do not arise because of pests or diseases. A healthy plant requires 16 essential elements to complete its life cycle. Nutrient deficiency usually occurs as leaf discoloration or distortion (Fig. 10.3), reducing flowering and poor fruiting in most of the genus. The goal of farming system is being able to identify these deficiencies.

The occurrence of nutrient deficiencies or toxicities is a result of soil, climatic, crop, and agronomic factors. Such knowledge of soil pH, farming background, and soil texture can be essential for nutrient deficiency predictions (Stevens et al. 2002; Reddy 2012). Moreover, a higher productivity also requires knowing fertilizer rate, application method and time of application, and interaction of these elements with edaphic and environmental factors. It should be kept in mind that many other factors generate similar nutrient deficiency indications, which hamper the visualization and diagnosis. Factors such as inherent plant senescence, aberrant weather (cold, drought), intense sunlight, soil condition (compactness, wet and dry conditions), and also fertilizer burn have resulted in similar indications. However, biotic (disease) stress tends to appear with an asymmetrical pattern, unlikely to nutrient deficiencies where symptoms are distributed or become aligned in a symmetrical pattern over the entire plant (Brown 2013; Wong 2005).

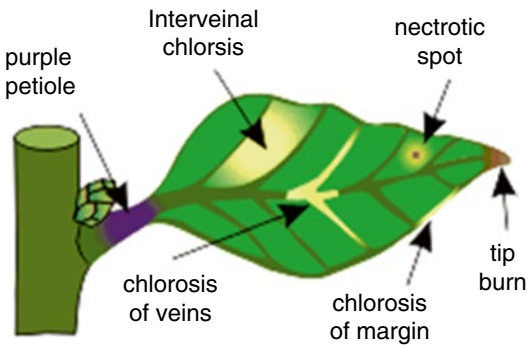

Fig. 10.3 Some common leaf abnormalities resulting from nutrient deficiencies (Reproduced from Flairform 2015)

10.3.1 How to Know a Deficiency?

Visual symptoms are the cheapest diagnostic technique in identifying nutrient stress. However, several other abiotic or biotic stresses hamper identifying features similar to nutrient disorders. There are several steps to identify symptom characterization caused by nutrient stress:

1. Observation of the growth and development pattern when the plant is healthy and has disorder variations.
2. Recognition of plant part affected (new leaves, old leaves, edge of leaf, veins, etc.).
3. Identification of the nature of symptoms: chlorotic, necrotic, or deformed.

Nutrient deficiency is mostly categorized on the basis of whether the symptoms occurred on plant's older leaves or on younger leaves. Any nutrient capable of translocation within plants, such as N, P, K, or Mg, and the symptoms emerged on older leaves is known as 'mobile nutrient'. Immobile nutrients (such as S, Ca, Fe, Cu, Mn, and Zn), which are restricted in movement, are not translocated to the growing region, so younger leaves or apical buds show their deficiency indications first (Reddy 2002).

Table 10.2 Nutrient deficiency and their indicator plants

Deficient nutrient	Indicator plant
Nitrogen	Cauliflower, cabbage, maize, sorghum
Phosphorus	Rapeseed, tomato, lucerne, duranta
Potassium	Potato, banana, cucurbits, cotton, lucerne
Calcium	Cauliflower, cabbage
Magnesium	Potato
Sulphur	Clover, tea, lucerne
Iron	Sugarbeet, gooseberry, acacia, eucalyptus
Manganese	Sugarbeet, oat, potato, citrus
Boron	Sugarbeet, coconut, guava
Zinc	Tomatoes, beans, citrus
Copper	Citrus
Molybdenum	Cauliflower, cabbage

Reproduced from Reddy (2012)

10.3.2 Nutrient Deficiency and Their Visual Symptoms

Plant nutrient deficiency can be observed by some visual symptoms. Most of the symptoms can directly represent the specific nutrient deficiency (Table 10.2).

10.3.2.1 Primary Nutrient Deficiency

10.3.2.1.1 Nitrogen Deficiency

Nitrogen deficiency generally appears in the oldest leaves and lower part and progresses if deficiency is not reversed (Uchida 2000). Low nitrogen content reduced tillering in many cereals and lowers the yield. The plant remains stunted and chlorotic (pale yellow leaves) (Brown 2013). In severe conditions, yellowing of leaf tips and spindly stalks were reported in corn and other small grain cereals. Poor root and secondary shoot development are further related disorders (Sawyer 2004). Nitrogen disorder is normally favoured under poor nitrogenous fertilization, sandy soil, and denitrification process, or in regions of excessive rainfall (Tucker 1999).

10.3.2.1.2 Phosphorus Deficiency

Phosphorus is readily mobilized in plants and their deficient symptoms exist first on older leaves. The plant remains darker green, growth-stunted with reddish purple leaf tips (Fig. 10.4) and margins (Uchida 2000). At temperate areas or whenever soil temperature is less than 60 °F due to heavy wetness or dryness, phosphorus deficiency is also commonly characterized in young plants. In corn hybrid cultivation, although soil is fertile, sometimes phosphorus deficiency may occur due to abrupt changes in soil temperature or moisture level (Sawyer 2004).

10.3.2.1.3 Potassium Deficiency

Lower leaves exhibit chlorosis (lack of greenness) at the margin (Fig. 10.4) and random chlorotic spots that turned into necrotic spots in severe cases (Uchida 2000; Reddy 2012). Poor branching and shoot stunting can also be caused by interaction with other nutrients. Poor grain size in grain crops, leaves scorching of cotton, uneven fruit ripening in tomatoes, and low quality of forage crops (Tucker 1999) are characteristics of phosphorus deficiency.

10.3.2.2 Secondary Nutrients and Plant Growth

10.3.2.2.1 Calcium Deficiency

Calcium deficiency starts from younger leaves, failure of terminal buds, and root tips. As severity occurs, new buds start to die and curl. New leaves turn into white and roots become distorted. 'Blossom-end rot' is the common term of failure of terminal bud observed in tomatoes and peppers. In groundnut, pod development is restricted with poor seed setting (Reddy 2002; Tucker 1999). Low soil pH and excessive soluble salts of aluminium and manganese are more likely causes of phosphorus deficiency.

10.3.2.2.2 Magnesium Deficiency

Interveinal chlorosis (leaves yellowing between the veins) is particularly the common symptom of magnesium deficiency (Fig. 10.5). The deficiency reported under sandy soil in rainfall season is known as 'sand drown' (Tucker 1999; Hosier and Bradley 1999). The symptoms start from older leaves and progress up the plant in severe cases. Older leaves turn into reddish

Fig. 10.4 Deficiency symptoms of phosphorus (**a**), potassium (**b**), and iron (**c**) in corn (Reproduced from Sawyer 2004)

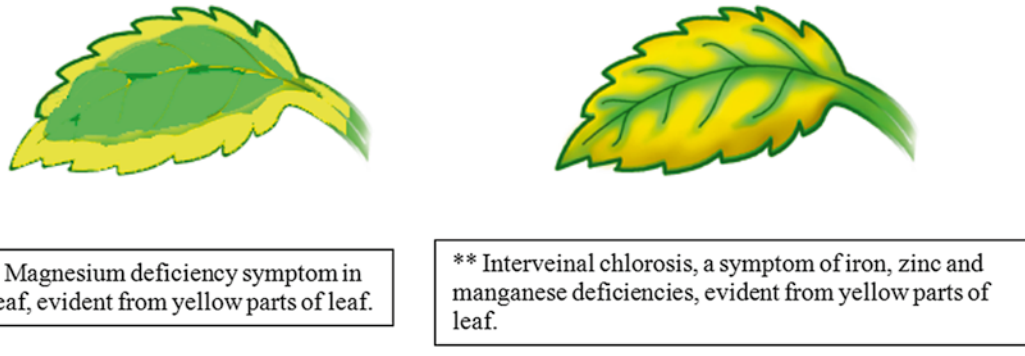

Fig. 10.5 Micronutrient deficiencies in leaves (Reproduced from Hosier and Bradley 1999)

colour and necrotic spots emerge (Stevens et al. 2002). Tobacco, corn, and forage crops commonly exhibit magnesium deficiency. Also, 'grass tetany' in ruminant animals is caused by magnesium deficiency (Tucker 1999).

10.3.2.2.3 Sulphur Deficiency

Sulphur deficiency symptom is characterized by general yellowing of foliage, similar to nitrogen deficiency. However, the yellowing of leaves begins in younger leaves because sulphur is highly

immobilized in plant tissues (Reddy 2012). Delayed maturity and stunted growth are other characteristics of deficiency. Interveinal chlorosis is commonly favoured under sandy or low organic content soil. At acute deficiency, entire plant chlorosis may also occur (Sawyer 2004).

10.3.2.3 Micronutrient Deficiency

10.3.2.3.1 Manganese Deficiency

Manganese (Mn) is relatively immobile in plants. The typical characteristic due to manganese deficiency is interveinal chlorosis in new leaves. Brown patches develop on the leaves of tobacco and reddening occurs in cotton leaves (Fig. 10.5). Yellow stripes run parallel to the leaf blade in the case of corn plants; however, greyish speck formation in the grain is termed as 'grey speck' especially in oat (Tucker 1999; Hosier and Bradley 1999). The problematic soils such as alkaline soils, poorly drained soils, sandy coastal soils, and soil rich in available Fe content can also induce Mn deficiency (Sawyer 2004).

10.3.2.3.2 Zinc Deficiency

Relatively, interveinal chlorosis is an obvious symptom of zinc deficiency (Hosier and Bradley 1999); also, stunted growth and affected plant parts give a rosette-like appearance. Leaves develop into small size, along with short internodes (Reddy 2012). In the acute case, white leaves become rusty brown in colour. In coarse cereal grains (corn and sorghum), whitish band formations occur at the side of the leaf midrib, which is known as 'white bud'. 'Little leaf' in cotton is also common due to zinc deficiency (Tucker 1999; Wong 2005). Zinc deficiency is also favoured by high pH and low soil organic matter, cool or wet soil, and high phosphorous fertilizer application in poor zinc availability of the soil (Wong 2005).

10.3.2.3.3 Iron Deficiency

Iron-deficient plant develops interveinal chlorosis (Figs. 10.4 and 10.5) in leaf growth (Hosier and Bradley 1999). Yellowing or bleaching of newly emerged leaves is quite common (Sawyer 2004; Donohue 2001). Corn rarely shows iron deficiency due to low requirement; however, high soil pH, poorly aerated soil, and calcareous soil favour the iron deficiency (Stevens et al. 2002).

10.3.2.3.4 Copper Deficiency

Chlorotic symptoms without wilting in leaves are considered as a common indicator of copper deficiency. New shoots will not emerge and the whole plant turns into pale green colour. Yellowing of younger leaves, prominent at the start followed by leaf curling, result in 'die-back' symptoms commonly found in small grains (Tucker 1999). In an acute situation, leaves twist and shrivel, and the plant dies prematurely. Oats are reported as the most sensitive crops to copper deficiency and result in 'leaf tip die-back' sickness. High pH soils, compact soils, and soils lacking in nitrogen also favoured copper deficiency (Wong 2005; Reddy 2012).

10.3.2.3.5 Boron Deficiency

The boron-deficient leaves are curled or thickened and have copper structure. Other prominent disorders are the death of growing tips where later shoots deform. Stunted root, poor to set flowers, and the presence of cracked or water-soaked condition in petioles and stems are also included (Reddy 2002). The initial symptoms start with dark rings near the petiole and further progression causes leaf deformation (Tucker 1999). Specific symptoms such as rotting of fruits, tubers or roots, and cork spot in crops such as beets, turnips and potatoes, and apples are also listed in boron deficiency. Twisted stem and poor boll formation also occurred in cotton in the severe absence of boron in the soil (Stevens et al. 2002). Low soil pH below 5.5 or above 6.8 and poor organic matter content especially in sandy soils also induced boron deficiency.

10.3.2.3.6 Molybdenum Deficiency

The plant symptoms of molybdenum deficiency, such as general yellowing, are quite similar to nitrogen deficiency. The whole plant remains pale green to yellow; whiptail leaf formation (top leaves deformed into a shape of whip-like structure) (Tucker 1999) occurs. Marginal chlorosis and mottling along with leaf cupping are other

molybdenum deficiency characteristics. Highly podzolized soils and well-drained calcareous soils are also associated with molybdenum deficiency (Stevens et al. 2002).

10.3.3 Indicator Plants

Some plants are more sensitive to certain element content in the soil and can also be used as a diagnostic tool for plant nutrient deficiencies. These plants are commonly termed as 'indicator plants'.

10.4 Nutrient Management Practices by Microbes

Nutrient management practices promote low chemical input into the soil and increase nutrient use efficiency of crops to improve their growth and productivity. Free-living microbes present in the soil have a great impact on nutrient management practices. The major microbial communities that have a significant impact on nutrient management practices are plant growth-promoting rhizobacteria (PGPR), plant growth-promoting fungi (PGPF), actinomycetes, protozoan, and nematodes.

10.4.1 Plant Growth-Promoting Rhizobacteria

PGPR can affect plant growth in either direct or indirect ways. In the direct way, PGPR increase the availability of different nutrients such as P, K, and N, which are essential for plant growth (Glick et al. 2007; Adesemoye et al. 2008), whereas, in the indirect way of plant growth promotion, PGPR prevent plants from harmful effects of one or more deleterious microorganisms. The major processes involved in the indirect way of plant growth promotion are through biocontrol or by antagonism against soil-borne plant pathogens. Specifically, colonization or biosynthesis of antibiotics (Fenton et al. 1999) and other secondary metabolites are considered as major mechanisms involved in the suppression of pathogens. However, the information about the beneficial effects of PGPR on crops is limited and the mechanisms used by PGPR are unclear (Glick 1995).

Inoculation of different PGPR strains such as *Pseudomonas* and *Acinetobacter* resulted in the increased uptake of Fe, Zn, Mg, Ca, K, and P by crop plants (Khan 2005). A significant impact of different PGPR (*P. mendocina* Palleroni) was observed on the uptake of N, P, Fe, Ca, and manganese (Mn) in lettuce (*Lactuca sativa* L. cv. Tafalla) under different water stress conditions (Kohler et al. 2008). Including nutrient management practices, PGPR have also shown an increase in seed germination rate, root growth, yield, leaf area, chlorophyll content, nutrient uptake, protein content, hydraulic activity, tolerance to abiotic stress, shoot and root weights, biocontrol, and delayed senescence (Mahaffee and Kloepper 1994; Raaijmakers et al. 1997; Bashan et al. 2004; Mantelin and Touraine 2004; Bakker et al. 2007; Yang et al. 2009).

While considering nutrient management practices of PGPR, it has been observed that PGPR enhance P availability to the plant, sequestering iron for plant with the help of siderophore production (Bakker et al. 2007). Only a portion of chemical fertilizers is taken up by plants, for example, after applying P into the soil it precipitates and becomes less available to the plants (Gyaneshwar et al. 2002). In 1948, Pikovskaya reported solubilization of insoluble P by microorganisms. Since the 1950s, phosphate-solubilizing bacteria (PSB) are being used as biofertilizer (Kudashev 1956; Krasilinikov 1957). The release of unavailable form of P to available form is an important aspect in terms of soil fertility and plant nutrient availability. There is strong evidence that soil bacteria can convert this unavailable form of P to available form by several mechanisms. As compared to fungi, bacteria are more effective phosphate solubilizers (Alam et al. 2002). Several strains of bacterial species such as *Pseudomonas* and *Bacillus* bacteria (Illmer and Schinner 1992) are reportedly known for their phosphate solubilization ability. Microorganisms enhance the P availability to plants by mineralizing organic P in the soil and

by solubilizing precipitated phosphates (Chen et al. 2006; Kang et al. 2002; Pradhan and Sukla 2005). Contribution of PSB among the whole microbial population in the soil is about 1–50 % (Chen et al. 2006). Strains from bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium*, and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi are the most powerful P solubilizers (Whitelaw 2000). *Bacillus megaterium*, *B. circulans*, *B. subtilis*, *B. polymyxa, B. sircalmous, P. striata*, and *Enterobacter* could be considered as the most important strains (Subbarao 1988; Kucey et al. 1989).

The major portion of P present in the soil is in unavailable organic and inorganic forms, but some bacterial species have mineralization and solubilization potential that can convert this unavailable form of P into bioavailable phosphorus (Hilda and Fraga 2000; Khiari and Parent 2005) (Fig. 10.6). Phosphate solubilization takes place through various microbial processes including organic acid production and proton extrusion (Dutton and Evans 1996; Nahas 1996). PSB secretes organic and inorganic acids that solubilize inorganic P by the action of hydroxyl and carboxyl groups of acids that chelate cations (Al, Fe, Ca) and decrease the pH in basic soils (Kpomblekou and Tabatabai 1994; Stevenson 2005). At the same pH conditions, inorganic acids, for example, hydrochloric acid, are less effective as compared to organic acids (Kim et al. 1997). Therefore, under certain conditions, phosphate solubilization is induced by phosphate starvation (Gyaneshwar et al. 1999). Some strains of *Pseudomonas, Bacillus*, *Enterobacter*, and *Burkholderia* present in the rhizospheric soil were found to produce siderophores and indolic compounds (ICs), which can solubilize phosphate (Ambrosini et al. 2012).

Soil microbes also influence the availability of nitrogen in the soil. For many years, a limited number of bacterial species were believed to be nitrogen fixers (Postgate 1981), but in the last 30 years nitrogen fixation has been shown to be a property with representatives in most of the phyla of Bacteria and also in methanogenic Archaea (Young 1992). Two major families of soil bacteria, namely *Rhizobium* and *Frankia*, are associated with soil N fixation. Another important group of nitrogen-fixing bacteria is cyanobacteria, found in association with a large variety of higher and lower plants, fungi, and algae (Meeks and Elhai 2002). A study on the effect of different strains of *Azotobacter*, *Azospirillum*, *Phosphobacter*, and *Rhizobacter* showed enhanced nitrogen availability to *Helianthus annus* plants, which resulted in increased plant height, number of leaves, stem diameter, seed filling, and seed dry weight (Dhanasekar and Dhandapani 2012). Similarly, potassium-solubilizing bacteria (KSB) such as genera *Bacillus* and *Clostridium* are helpful for the solu-

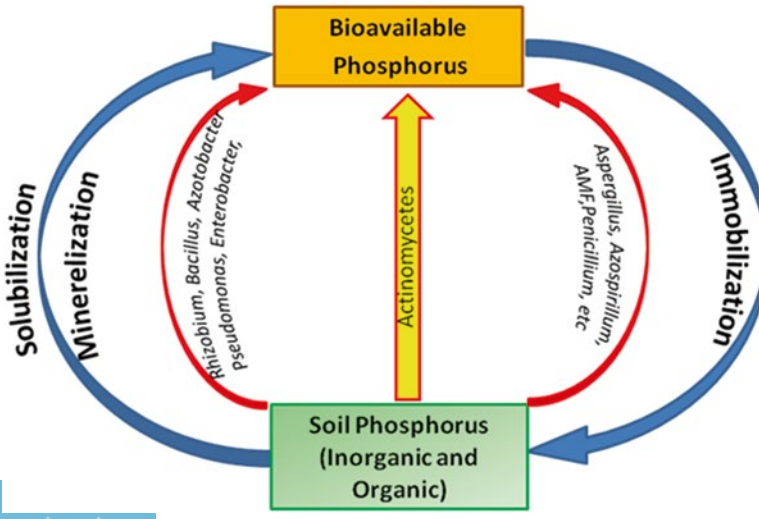

Fig. 10.6 Mobilization and immobilization of phosphorus present in the soil by different soil microbes

bilization and mobilization of potassium from soil to different crops (Mohammadi and Yousef Sohrabi 2012). It has also been reported that including increased availability of P and N, PGPR such as *Pseudomonas* and *Acinetobacter* have a significant impact on the enhanced uptake of Fe, Zn, Mg, Ca, and K (Khan 2005).

10.4.2 Plant Growth-Promoting Fungi

In the last few decades, most studies have focused on the role and interaction of different rhizobacteria, but still the role and mechanism of PGPF are not very well known (Murali et al. 2012). The beneficial effects of certain rhizosphere fungi in terms of plant growth promotion and biological control have been reported by many researchers (Windham et al. 1986; Narita and Suzuki 1991). PGPF are mainly nonpathogenic saprophytes known for their plant growth-promoting property and also for their suppressiveness property against different pathogenic fungi and bacteria of a number of crop plants (Shivanna et al. 1996; Chandanie et al. 2006). The most commonly known PGPF are *Trichoderma* spp. and arbuscular mycorrhizal fungi (AMF).

Trichoderma spp. is most commonly known for its biocontrol potential where it protects plants from different pathogen populations under different soil conditions. In recent years, these fungi have been widely commercially marketed as biopesticides, biofertilizers, and soil amendments. *Trichoderma* spp. also produces numerous biologically active compounds, such as cell wall-degrading enzymes and secondary metabolites (Vinale et al. 2008). The study reports that, after amendment of *T. herzianum* to the soil, a significant improvement in seed germination along with a significant increase in the concentration of Cu, P, Fe, Zn, Mn, and Na was observed in inoculated roots (Yedidia et al. 2001). Another species of *Trichoderma* known for its increased nutrient availability and plant growth-promoting property is *T. viridi* (Srivastava et al. 2006). In recent years, a lot of work has been done to isolate, identify, and characterize different strains of *Trichoderma* spp. to check their availability as PGPF.

PGPF may also improve plant growth indirectly, via alterations to the structure of rhizosphere soil, which benefit the plant. Different fungal strains, namely *Penicillium* sp., *Trichoderma* sp., *Rhizoctonia* sp., and *Pythium* sp., have been reported for their suppressive nature against *S. graminicola.* Pathogen control by PGPF may also occur via niche exclusion, antibiosis, predation, mycoparasitism, and induced systemic resistance (ISR) induction (Murali et al. 2012). Therefore, there is a direct relation of pathogen suppression with plant growth promotion. If there is less pathogen attack in the plant, it will directly improve the plant nutrient availability.

Phosphate solubilization mainly occurs in two ways in soil system: first, by direct solubilization process (Rodriguez and Fraga 1999) and second by the accumulation of P in the form of biomass of microorganisms (Oehl et al. 2001). There are two ways in microbial P solubilization: by solubilization processes and from P accumulation in the biomass of microorganism. The important genera of PSF are *Aspergillus* (Vassilev et al. 2007) and some species of *Penicillium* (Oliveira et al. 2009). *Penicillium oxalicum* isolated from the rhizosphere of rock phosphate mine showed a significant impact in solubilizing rock phosphate rather than promote the growth of wheat and maize. The most important feature of PSF is that they do not lose their activity during subculturing under laboratory conditions (Kucey 1983). Therefore, these microbes can be isolated from any source and grown under laboratory conditions for further application in the field for phosphate solubilization. The solubilization of P in soil depends on the availability of rock P in the soil. If higher concentration of rock P occurs in the soil, it increases the solubilization process.

Trichoderma spp. is known for its high activity for the solubilization of inorganic-bound phosphate into available form. The mechanism so far discussed for this solubilization process is that this unavailable form of phosphorus might accumulate inside fungal body for cellular processes and this sequestration of P in fungal

mycelium results in the depletion of P in nearby areas, but after the lysis of mycelium with age, this phosphorus is released into the soil and readily available to the plants (Kapri and Tewari 2010). Ejikeme and Anyanwu (2013) reported that the efficiency of solubilization of tricalcium phosphate (TCP) by PSF is related to reduction in pH due to the secretion of organic acids excreted by PSF (Sharma et al. 2012). Including phosphate solubilization, *A. niger* and *P. glaucum* are also known for nitrogen fixation in the soil. The investigations of Jodin and Hallie carried out as early as the 1960s led them to believe that fungi possessed the power to fix nitrogen.

In the scientific world, AMF are known as one of the most promising fungi in terms of increased nutrient uptake by plants and for increasing soil fertility. The arbuscular mycorrhizal (AM) symbiosis between fungi and plant roots is the most common type of interaction in the rhizosphere (Smith and Read 1997). AM is one of the oldest symbioses formed by plants. Phosphate absorption by plants is explained under two different pathways: the "direct" uptake pathway at the plant–soil interface through root epidermis and root hairs, and the "mycorrhizal" uptake pathway via fungal mycelium (Smith et al. 2003). Much of the inorganic phosphate applied to soil as a fertilizer is rapidly converted to unavailable forms with low solubility. Soluble P is released from insoluble phosphates by a variety of solubilization reactions involving rhizosphere microorganisms (Kapoor et al. 1989).

Mycorrhizal plants can take up more phosphorus than non-mycorrhizal plants, mainly from the same soluble phosphate pool (Fig. 10.7). Soluble phosphate released by the activity of phosphate-solubilizing microorganisms (PSM) can be actively taken up by mycorrhizal roots (Kapoor et al. 1989).

Mycorrhiza is known for its functioning in phosphorus uptake and it encodes a phosphate transporter gene that plays a key role in this mechanism. The process of phosphate transport from the mycorrhiza to the plant has been studied previously by identifying a complementary DNA (cDNA) that encodes a transmembrane phosphate transporter termed GvPT from *G. versiforme* (Harrison and Van Buuren 1995). In recent years, several phosphate transporter genes have been identified and characterized for their involvement in different uptake pathways. Shin et al. (2004) reported that two Pht1 transporters, which are normally expressed at the root periphery, after loss of function in *Arabidopsis*, exhibited a strong reduction of phosphate uptake by 75 %.

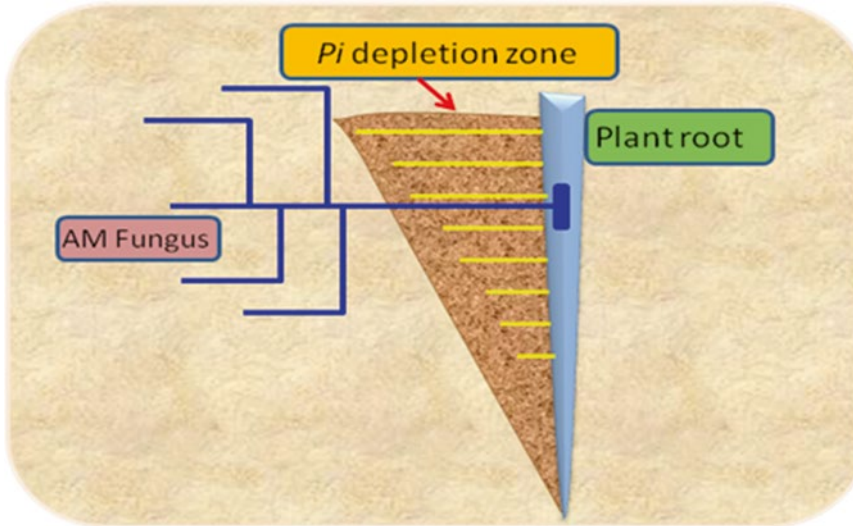

Fig. 10.7 Phosphate depletion zone in growing plant and its management by AMF (Reproduced from Karandashov and Bucher 2005)

In the AM symbiosis, firstly, fungal hyphae interact with plant roots through aspersorium followed by phosphate uptake by the fungus from the soil and then transfer to the root. Two phosphate transporter genes, namely GvPT and GiPT from *G. versiforme* and *G. intraradices*, respectively, are predominantly expressed in the extraradical fungal mycelium that encodes proteins, which are likely to participate in phosphate uptake at the fungus–soil interface (Harrison and Van Buuren 1995; Maldonado-Mendoza et al. 2001). The mechanisms involved in the release of phosphate from the fungus to colonized plant cells are presently unknown, but it is believed that phosphate ions pass through periarbuscular membrane (PAM) inside plant roots and probably because of concentration gradient their transfer through the membrane could be facilitated by ion-specific carriers, pumps, or channels (Karandashov and Bucher 2005).

In mycorrhizal plants, P uptake per unit root length is two to three times higher than in non-mycorrhizal plants (Tinker et al. 1992). However, as soil available P levels increase, benefits to plant growth decrease because the plant can directly take P from the soil without the need of mycorrhizae mycelia. Few reports on mycorrhiza and its role in increased Zn and Cu uptake by both maize (Kothari et al. 1990) and soybean (Lambert and Weidensaul 1991) are also present. Therefore, AMF has a significant role in rhizospheric soil and it shows a positive impact on plant nutrition in soil systems where low plant available nutrient levels are present. The fungus supplies the plant with water and nutrients such as phosphate, while the plant provides fungus with photosynthetically produced carbohydrates.

10.4.3 Actinomycetes

Actinomycetes are known as the most successful microbial source for all types of bioactive metabolites, including the agroactive type. During 1988–1992, over 1000 secondary metabolites from actinomycetes were discovered. *Streptomyces* is reported as a major genus that produces these compounds. In the past 5 years, about 60 % of the new insecticides and herbicides originated from *Streptomyces* (Tanaka and Omura 1993). It is also estimated that as many as three-quarters of all *Streptomyces* species are capable of antibiotic production (Alexander 1977). Actinomycetes have antifungal, antitumour, and immunosuppressive activities. These activities are associated with the production of a variety of antibiotics with diverse chemical structures such as polyketides, b-lactams, and peptides in addition to a variety of other secondary metabolites produced by different species of actinomycetes (Behal 2000).

Despite the role of actinomycetes as a biocontrol agent, these microbes are also known for their capacity to enhance plant growth (Aldesuquy et al. 1998). Only few studies have been carried out on the species of genus *Streptomyces* investigating their potential as PGPR. This is surprising, as streptomycetes is generally present in abundance in soil microflora and effectively colonizes plant root system, but at the same time it is also able to endure unfavourable growth conditions by forming spores (Alexander 1977).

Only a few studies on plant growth-promoting role of streptomycetes have been reported so far. The study by Merriman et al. (1974) reported the use of a *S. griseus* (Krainsky). Waksman and Henrici isolate as a seed treatment of barley, oat, wheat, and carrot, in order to increase their growth. Marketable yields were increased over controls by 17 and 15 % in two separate field trials. Specifically, both trials also indicated an increased yield of large and very large grade carrots over controls (Merriman et al. 1974). These strains were isolated and screened for their biocontrol activity against *Rhizoctonia solani*, but, in addition to this, these isolates are also increasing the plant growth. Therefore, a correlation can be established between the biocontrol activity and plant growth promotion. This can be explained as an indirect correlation between biocontrol agents and plants. As an active biocontrol agent, these microbes reduce the activity of pathogen and, on the other hand, they also provide a suitable environment for the plant to increase their nutrient availability from rhizosphere soil.

10.4.4 Other Microbes

Including PGPR, PGPF, and actinomycetes, there are few other microbes such as protozoan and nematodes that are present in the soil in a smaller proportion, but having a significant impact on plant growth and plant protection from different harmful agents present in the soil.

10.4.4.1 Nematodes

Nematodes respond rapidly to the disturbance and enrichment of their environment. Increased microbial activity in the soil leads to changes in the proportion of opportunistic bacterial feeders in a community. Nematodes are important in mineralizing, or releasing, nutrients in plant-available forms. When nematodes eat bacteria or fungi, ammonium ($NH4^+$) is released because bacteria and fungi contain much more nitrogen than the nematodes require. Over time, the enrichment opportunists are followed by more general opportunists that include fungal feeders and different genera of bacterial feeders (Bongers and Ferris 1999). This succession of nematode species plays a significant role in the decomposition of soil organic matter, mineralization of plant nutrients, and nutrient cycling (Ingham et al. 1985; Hunt et al. 1987; Griffiths 1994).

The feeding habit of nematodes is dependent on the C:N ratio. The results concluded that bacteria- or fungi-feeding nematodes either have higher or on par C:N ratio than host (Ferris et al. 1997; Chen and Ferris 1998). Most of the carbon (nearly 40 %) in C:N ratio utilized for metabolic activities (Ingham et al. 1985) and the released by-products of consumption as ammonia in the soil (Rogers1969) is found to be beneficial to microbes and plant uptake. The rate of nutrient cycling such as nitrogen cycle considerably varies depending on the behavior of microbivorous nematodes.

Such nematodes are also considered as environmental purity indicators. Any changes in soil fertility and pollutants can be assessed by studying nematode activities. In addition, immediate changes in decomposition process or particular nutrient status have also shown considerable changes in nematode activities and work as different indices (Bongers and Ferris 1999).

10.4.4.2 Protozoa

Soil protozoan genera have an intensive role, deciding the nutrient mineralization especially of nitrogen availability. As compare to bacterial cell, protozoa having poor concentration of nitrogen in their body. However, because protozoa have a feeding habit similar to nematodes, a certain amount of nitrogen will be released in soil as ammonia that is utilized by soil microbes and plant uptakes. Bacterial growth and colonization are also regulated when such protozoa feed and stimulate their population. Hence, soil aggregation and organic decomposition are also facilitated. Protozoa are also considered as feed to other microfauna, which helps in the suppression of many diseases as competition to them.

10.5 Conclusion

So far, nutrient deficiency in the soil is made up by the direct application of fertilizer, but in recent years, researchers are focusing on soil nutrient management practices through different soil microbes, because most of the time nutrients are already present in the soil, but their availability to the plants is very less or none. Rhizospheric microbes can help to overcome the problem of nutrient unavailability or its deficiency to the plants. It is important to comprehend the aspects of useful microbes and implement its application to modern agricultural practices. The new technology developed using the powerful tool of molecular biotechnology can enhance the biological pathways of the production of phytohormones. If identified and transferred to the useful microbes, these technologies can help in relief from environmental stresses. However, there is lack of awareness among the farmers, ecologists, and agriculturists for the application of these beneficial microbes in the field. To fill this gap between research laboratories and field application of beneficial microbes, there is a need for a better understanding of these microbes, their mechanism, functioning, application, and their sustainability, so that it can ultimately reach the agricultural field.

Overall, plant nutrients play different roles and may reduce disease incidence in certain cases

or increase them in others, depending on particular nutrients, the host plant, and other factors. The role of beneficial microbes for the management of these nutrients in plants cannot be denied, and recent advancement in technologies helps us to understand the mechanism, functioning, and role of these microbes in nutrient management. An appropriate management of nutrients is essential to achieve healthy plants, and this is a significant benefit to the environment. Therefore, to achieve this important multidisciplinary goal, there is a need for joint research between different streams of science.

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Organic Acids in the Rhizosphere: Their Role in Phosphate Dissolution

11

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Abstract

Phosphorus is an essential plant nutrient that is made available to plants primarily from the soil phosphorus reserves. But its limited mobility in the soil and high fixation capabilities within in the soil matrix necessitate the use of fertilizer forms of phosphorus, which are again prone to fixation, thereby reducing the availability of this crucial element for plant nutrition. Soil microbes play a crucial role in mobilizing various forms of phosphorus (inorganic and organic) and making them available for plant nutrition. Microbe-mediated phosphorus mobilizing processes involve either organic acids that solubilize the inorganic forms of phosphorus or enzymes that mobilize the organic sources of phosphorus. The organic acids that play a crucial role in the dissolution of phosphates can be of plant and microbial origins and vary in their nature and properties depending on the soil, plant, and microbial species involved. Besides playing a crucial role in P cycling, they also perform assorted functions that have a direct bearing on the plant growth and development. This chapter attempts to capture the information on the nature, properties, and functions of organic acids in the rhizosphere.

Keywords

Organic acids • Rhizosphere • Microbes • Phosphate • Nutrients

11.1 Introduction

Phosphorus (P) is an essential macronutrient that often limits plant growth and development due to its reduced availability from the soil. This happens primarily due to its low solubility and fixation within the soil matrix (Marschner 1995). Therefore, the application of fertilizer forms of

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_11

phosphorus has become an imperative practice in modern-day agriculture. The main sources of phosphorous nutrition for plant growth are derived from phosphatic rocks (phosphorites) that contain one or more phosphatic minerals that are predominantly calcium phosphate forms. However, mineable rock phosphate (RP) reserves, which provide the base raw material for inorganic fertilizer production, are relatively small and finite and may only last for another 100–400 years (Van Kauwenbergh 2010; Cordell and White 2011). As there are no alternative forms for meeting the ever-growing phosphorus requirements of agriculture, several processes have been devised for the efficient utilization of inorganic phosphate forms. The role of microorganisms, which are an integral part of the soil P cycle and play an important role in the transfer of P between different pools of soil, becomes crucial, because they are a vital cog in this process. Phosphate-solubilizing microorganisms (PSMs) can be added to soil as individual strains or as a consortium of elite strains for solubilizing poorly soluble RPs and legacy soil P, a term coined to denote the P that has accumulated in soil as a result of past application of fertilizers and manures (Sharpley et al. 2013). In microbe-mediated mobilization of soil phosphorus, low molecular mass organic acids (OAs), secreted by soil microorganisms, play a crucial role in P solubilization/mobilization (Maliha et al. 2004; Khan et al. 2010; Marra et al. 2012). It is postulated that apart from mobilizing P, OAs secreted by rhizospheric microbes play a crucial role in zinc and other metal mobilization, alleviation of metal toxicity, and improving iron availability (Archana et al. 2012).

Though several studies related to PSMs and their role in sustainable agriculture have been conducted since the beginning of the last century, basic studies on the nature, properties, and behavior of OAs involved in phosphorus mobilization have been reported primarily from in vitro experiments, performed under conditions that are remarkably different from those that exist in vivo, and have therefore drawn a reasonable critique (Drever and Stillings 1997; Jones 1998; Parker and Pedler 1998). This could be mainly attributed to the absence of reliable methods for studying the nature and properties of OAs in the rhizosphere and partly to the ability to publish in vitro generated data with ease. Considering the pivotal role of OAs in soil processes, it can be rightly argued that there exists a critical knowledge gap in our understanding of the soil P dissolution processes. In this chapter, we attempt to highlight the nature, distribution, and behavior of rhizospheric OAs that are primarily involved in phosphate dissolution.

11.2 Significance of P in Plant Nutrition

Being a major nutrient, an adequate supply of phosphorus is required for optimum plant growth and reproduction. Phosphorus enters the plant through root hairs, root tips, and the outermost layers of root cells. It is taken up mostly as primary orthophosphates ($H_2PO_4^-$) and/or secondary orthophosphates (HPO_4^-) ion. Once it gains entry into the plant roots, P may be stored in the root or transported to the upper portions of the plant through various mechanisms (Schachtman et al. 1998). Phosphorus gets incorporated into organic compounds, including nucleic acids (DNA and RNA), phosphoproteins, phospholipids, sugar phosphates, enzymes, and energy-rich phosphate compounds such as adenosine triphosphate (ATP) (Turner et al. 2002; Condron et al. 2005). Through these organic forms and other inorganic forms, the phosphate ion moves throughout the plant, making it available for further reactions. Phosphorus plays a vital role in virtually every plant process that involves energy transfer. High-energy phosphate, which forms part of adenosine diphosphate (ADP) and ATP, drives numerous chemical reactions within the plant. When ADP and ATP transfer the high-energy phosphate to other molecules (phosphorylation), many essential processes occur (Bergman 1999).

Phosphorus is a vital component of the building blocks of genes and chromosomes, and hence it is an essential part of the process of transfer of the genetic information from one generation to the next, thereby providing the blueprint for plant growth and development. Large quantities of P are found in seeds and

fruit and are believed to be essential for seed formation and development. In cereal grains, phosphorus is mainly stored as phytin. It is observed that nearly 50 % of the total P in legume seeds and 60–70 % in cereal grains are stored as phytin or closely related compounds. Therefore, an inadequate supply of P can reduce seed size, seed number, and viability (Dibb et al. 1990).

Plant cells accumulate nutrients at much higher concentrations than concentrations present in the soil solution in the immediate root vicinity. As a result, plants have the ability to extract nutrients from the soil solution even at very low concentrations. As the mobility of nutrients within the plant system depends largely on transport through the cell membranes, it requires energy to oppose the forces of osmosis. This energy is provided by ATP and other high-energy P compounds. The most striking symptoms of P deficiency include reduction in leaf expansion, leaf surface area, number of leaves, and decreased shoot-to-root dry weight ratio. Root growth is also impaired by P deficiency, leading to an overall reduction in water and nutrient uptake from the soil (Marschner 1995). Insufficient P levels also retard the processes of carbohydrate utilization, though carbohydrate production through photosynthesis continues. As a result, sugars that accumulate in leaves turn reddish purple because of the accumulation of anthocyanin pigments.

11.3 Forms of P in Soils

Soils receive both organic and inorganic P reserves in the form of inorganic fertilizers and organic fertilizers (manures, composts, and sewage sludge). Inorganic P reserves are less sensitive to changes in the fertilization regime than organic P reserves. Therefore, the soil P reserves exist in either the organic or inorganic forms (Busman et al. 2009).

11.3.1 Organic P Pool

This contributes to around 50 % of the total P in soils and consists mainly of esters of orthophosphoric acid, such as inositol phosphates, phospholipids, and nucleic acids. The inositol phosphates represent a series of phosphate esters ranging from monophosphates to hexaphosphates. The total inositol phosphates content of soil may range from 10 to 50 %. Phospholipids are phosphorus-containing fatty compounds that are insoluble in water but are readily utilized and synthesized by soil microorganisms. As most common phospholipids are derivatives of glycerol, the rate of release of phospholipids from organic sources in soil is rapid. Phospholipids constitute 1–5 % of total organic P in soils. Nucleic acids occur in all living cells and are produced during the decomposition of residues by soil microorganisms. The distinct forms of nucleic acids, namely RNA and DNA, contribute 0.2–2.5 % of total organic P in soil (Yadav and Verma 2012).

11.3.2 Inorganic P Pool

The inorganic P pool comprises calcium-complexed phosphate or phosphates complexed with iron and aluminum ions. As calcium is the most dominant and controlling cation, apatite minerals are nearly insoluble and are found mostly in weathered soils, especially in their lower horizons. Iron and aluminum compounds include hydroxy phosphates such as strengite (iron phosphate) and variscite (aluminum phosphates). Iron and aluminum phosphates are usually abundant in acidic soils (Yadav and Verma 2012).

With regard to their availability to plants, the soil P fractions can be classified as solution P, active P, and fixed P. The solution P fraction is the smallest of all, containing only very small quantities of the orthophosphate form for plant uptake, and has a measurable mobility. The plant quickly depletes the P in the soluble P fraction and therefore requires continuous replenishment. The active P fraction present in the solid phase is rapidly released to the soil solution (the water surrounding soil particles). It comprises inorganic phosphates attached (or adsorbed) to small particles in the soil, phosphate that reacts with elements such as calcium or aluminum, and organic P that is easily mineralized. As a result of

continual plant uptake, the concentration of phosphates in the solution gets decreased and phosphate from the active P fraction is released into the solution P fraction. As the solution P fraction is very small, the active P fraction is the main source of available P for crops. The ability of the active P fraction to replenish the soil solution P fraction is crucial for the phosphorus fertility status of a soil. The fixed P pool contains inorganic phosphate compounds that are highly insoluble and organic compounds that are resistant to mineralization by microorganisms in the soil. Phosphates in this pool remain in soils for years without being made available to plants and may have very little impact on the fertility of a soil. The inorganic phosphate compounds in this fixed P pool are more crystalline in their structure and less soluble than those compounds considered being in the active P pool. Some slow conversion between the fixed P pool and the active P pool does occur in soils (Busman et al. 2002). The major soluble form of inorganic phosphate in soils is $H_2PO_4^-$, which usually occurs at low pH (Sharma et al. 2013). Under strong basic conditions, PO_4^{3-} dominates, while HPO_4^{2-} dominates in weak basic conditions. In weak acid conditions, H_2PO^{4-} dominates, while H_3PO_4 dominates in strong acid conditions (Uchida and Hue 2000).

11.4 P Transformations in Soils

Soil P undergoes a series of transformations that either enhance or diminish its plant availability. The following transformations occur commonly in soils.

(a) *Weathering of parent material*: Native P is released into soil solution by natural weathering of soil parent material by physical weathering (disintegration), chemical weathering (decomposition), and chemical transformations of primary minerals (Gardner 1990).

(b) *Sorption/desorption (interaction between P in solution and solid/mineral surfaces)*: P-sorption occurs when the orthophosphates $H_2PO_4^-$ and HPO_4^{2-} bind tightly to soil particles. As phosphate is an anion, particles that generate an anion exchange capacity form strong bonds with phosphates. Particles with strong anion exchange capacity include aluminum and iron oxides, highly weathered kaolin clays, and amorphous materials (under acidic conditions). In addition, in calcareous soils, P-sorption may occur as phosphates adsorb impurities such as aluminum and iron hydroxides or displace carbonates in calcium carbonate minerals under alkaline soils (Weil and Brady 2002).

(c) *Dissolution/precipitation*: Precipitation is a process in which phosphorus reacts with another substance to form a solid mineral, whereas dissolution occurs when a mineral dissolves and releases phosphorus, that is, dissolving a solid substance in a solvent to form a solution. The soil pH for optimum phosphorus availability is 6.5. At high or neutral pH, phosphates react with Ca to form minerals such as apatite that are poorly soluble. These insoluble forms are made available to plants by the action of OAs. Under acidic conditions, phosphorus reacts with Al and Fe to form minerals such as strengite and variscite. Under such conditions, OAs play a role in P transformations by chelating the Fe and Al oxides, thereby enhancing the availability of P for plant utilization (Khan et al. 2009).

(d) *Biological transformations*: Plant and animal remains (containing large quantity of organic P compounds) undergo decomposition by saprophytic microbes by the action of three groups of enzymes that release radical orthophosphate from the carbon structure of the molecule. The enzymes involved in this are nonspecific phosphatases, phytases, and phosphonatases. Apart from these, plant roots exude a variety of enzymes in response to P deficiency, and these enzymes play a dual role by catalyzing the P release from organic molecules and enhancing the organic matter transformation in soil. The enzymes identified in root exudates from different plant species are acid/alkaline phosphatases, invertases, amylases, and proteases (West

1939; Fries and Forsman 1951; Rovira and Harris 1961; Vancura 1964; Vancura and Hovadik 1965; Boutler et al. 1966; Rovira 1969; Gardner et al. 1983; Lipton et al. 1987; Fox and Comerford 1990; Ae et al. 1990; Ohwaki and Hirata 1992; Hoffland et al. 1992; Gagnon and Ibrahim 1998). Low P concentration in roots as a result of P deficiency induces de novo synthesis of extracellular and intracellular acid phosphatases, followed by release of the extracellular phosphatases into root exudates. They function by hydrolyzing and mobilizing inorganic P from monoester soil organic phosphates (Dinkelaker and Marschner 1992; Duff et al. 1994), which are estimated to account for about 30–80 % of total P in agricultural soils (Gilbert et al. 1999; Fig. 11.1).

11.5 Role of Organic Acids in P Transformations

OAs are low molecular weight compounds that perform a number of pivotal metabolic roles, including the provision of C for respiration and biomass production. They are intermediates of the tricarboxylic acid (TCA) cycle (e.g., citrate, malate), in which acetate derived from carbohydrates, fats, and proteins is oxidized to acetyl coenzyme A (acetyl-CoA). The TCA cycle occurs in the mitochondrial matrix of eukaryotes and cytosol of prokaryotes. In prokaryotes, the proton gradient for ATP production is generated across the cell surface (plasma membrane), while in eukaryotes, the gradient is generated across the inner membrane of the mitochondrion (Lodish et al. 2000). It needs to be clarified here that OAs in the rhizosphere are of both plant and microbial origins, and both organisms play a vital role in extracting the poorly available P reserves from the soil.

(a) *Plants*

OAs of plant origin enter the soil mainly as root exudates and lysates of plant cells. Such acids include lactic acid, acetic acid, oxalic acid, succinic acid, fumaric acid, malic acid, citric acid, isocitric acid, and aconitic acids. The intermediates of Krebs cycle such as citric, malic, succinic, fumaric, and aconitic acids accumulate in root exudates of many plant species suffering from nutrient starvation (Gardner et al. 1983; Hoffland et al. 1992; Jones 1998; Lipton et al. 1987; Ohwaki and Hirata 1992). But other OAs such as acetic, glycolic, malonic, oxalic, formic, and piscidic acids have also been identified in root exudates of a number of plants (Ae et al. 1990; Fox and Comerford 1990; Smith 1969, 1976; Vancura 1964). These acids play a crucial role in nutrient (P, Fe, and Mn) acquisition, for plants growing in low nutrient soils and their release in response to nutrient starvation differ between plant species.

(b) *Microorganisms*

Gluconic acid (GA) and 2-ketogluconic acid are known to play an important role in mineral phosphate solubilization (MPS). GA is reported as the principal OA produced by *Pseudomonas* sp. (Illmer and Schinner 1992; Gulati et al. 2009), *Erwinia herbicola* (Liu et al. 1992), *Pseudomonas cepacia* (Goldstein et al. 1993), and *Burkholderia cepacia* CC-A174 (Lin et al. 2006), while 2-ketogluconic acid production has been reported from *Rhizobium leguminosarum* (Halder et al. 1990), *R. meliloti* (Halder and Chakrabartty 1993), and *Bacillus firmus* (Banik and Dey 1982). Strains of *B. licheniformis* and *B. amyloliquefaciens* produced mixtures of lactic, isovaleric, isobutyric, and acetic acids (Rodriguez and Fraga 1999).

11.5.1 Functional Role of Organic Acids of Microbial Origin in the Soil

Microorganisms with specific attributes for mobilizing RPs and legacy soil P are termed phosphorus mobilizing microorganisms (PMM). These may include bacteria/actinobacteria, root-associated fungi, and mycorrhizae. The evidence for the natural occurrence of PSMs in the rhizo-

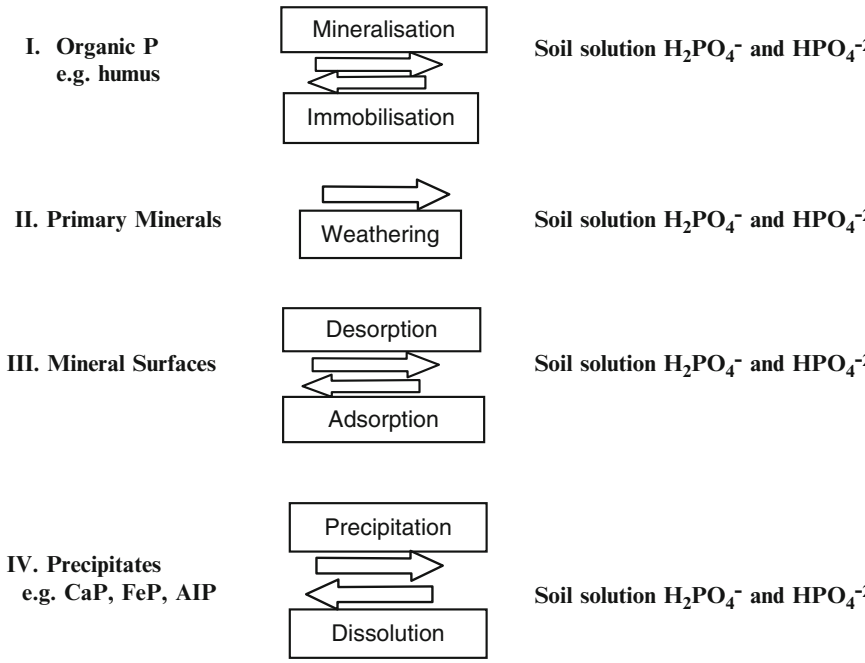

Fig. 11.1 Transformation of P in soil

sphere dates back to 1903 (Khan et al. 2007). Soil bacteria are generally considered to be more effective in phosphorus solubilization than soil fungi (Alam et al. 2002). Phosphate-solubilizing bacteria (PSB) constitute around 1–50 % of the soil microbial community, while phosphorus-solubilizing fungi (PSF) constitute just 0.1–0.5 % of the soil microbial community (Chen et al. 2006). Strains from bacterial genera, namely *Pseudomonas, Bacillus, Rhizobium and Enterobacter*, and the fungal genera, *Penicillium* and *Aspergillus*, are the most powerful P solubilizers (Whitelaw 2000). Insoluble P compounds are mobilized mainly by the production of OAs, which results in acidification of the surrounding medium. The organic and inorganic acids convert tricalcium phosphate to di- and mono-basic phosphates with the net result of enhanced P availability to the plant. The nature of OAs produced and their concentrations differ with different organisms. TCA and dicarboxylic acid are more effective as compared to mono, basic, and aromatic acids. Aliphatic acids are also found to be more effective in P solubilization compared to phenolic, citric, and fumaric acids. OAs may also compete for fixation sites of Al and Fe insoluble oxides and, on reacting with them, result in stable forms called "chelates." This phenomenon commonly occurs in acidic soils where the pH levels are considerably low. Therefore, chelate formation with Al and Fe oxides is the major means of P release from fixed forms of P (Hinsinger 2001). The extent of P solubilization as a result of OA production also depends on the accessory minerals present in the RP. The phosphates solubilized through biological means can react with Ca and Mg ions present in RP and reprecipitate and thereby become unavailable once again. This reaction is possible with an increase in the pH. The presence of free carbonates in RPs also reduces the solubilization efficiency because a portion of the OAs is directed toward neutralization of the free carbonates (Mahdi et al. 2011). The population of PSBs depends on different soil properties (physical and chemical properties, organic matter, and P content) and cultural activities (Kim et al. 1998). The populations of PSBs are usually higher in agricultural and rangeland soils (Yahya and Azawi 1998).

The direct oxidation of glucose to GA is the major mechanism for MPS in Gram-negative bacteria. GA biosynthesis is mediated by the glucose dehydrogenase (GDH) enzyme, while pyrroloquinoline quinone (PQQ) serves as the cofactor (Goldstein 1996). It provides the biochemical basis for highly efficacious phosphate solubilization in Gram-negative bacteria via diffusion of the strong OAs produced in the periplasm in the adjacent environment. Therefore, the quinoprotein glucose dehydrogenase (PQQ–GDH) may play a key role in the nutritional ecophysiology of soil bacteria. Consequently, the acidification of microbial cells and their surrounding leads to the release of P-ions from the P-mineral complex by H^+ substitution for Ca^{2+} (Goldstein 1995; Mullen 2005; Trivedi and Sa 2008). The efficiency of solubilization, however, depends on the kind of OAs released into the medium and their concentration. Furthermore, the quality of the acid is more important for P solubilization than the total quantity of acids produced by phosphate-solubilizing organisms (Scervino et al. 2010). In addition, the simultaneous production of different OAs by the phosphate-solubilizing strains may contribute to the greater potential for solubilization of insoluble inorganic phosphates (Marra et al. 2012). This evidence comes from in vitro experiments, but it is difficult to elucidate the origin of OAs in rhizosphere because of the difficulties encountered in the extraction process of OAs from soils. OA-induced P release in soils depends on many factors, including pH and soil mineralogy (Bolan et al. 1994; Jones and Darrah 1994; Lan et al. 1995). Apart from solubilizing different insoluble phosphates, OAs play multifarious roles in the rhizosphere. Table 11.1 briefly explains the nature and functions of different OAs in the soil.

Table 11.1 Nature and functions of organic acids in the rhizosphere

Organic acids	Functional role in the rhizosphere
Aliphatic organic acids	
Acetic, citric, isocitric, fumaric, tartaric, oxalic, formic, malic, malonic, adipic, and glycolic acids	Serve as a nutrient source, chemoattractant signals for microbes, chelators of poorly soluble mineral nutrients, acidifiers of soil, detoxifiers of Al, mobilizers of P and Zn, besides play a role in allelochemical interactions
	Also play a role in pedogenesis, food web interactions, decontamination of sites polluted by heavy metals and organic pollutants, regulation of soil pH, control of enzymatic activities, and desorption of heavy metals in soil
Cyclic and aromatic acids	
Benzoic, phenylacetic, shikimic, phthalic, ferulic, syringic, *p*-hydroxybenzoic, *m*-hydroxybenzoic, benzoic, caffeic, protocatechuic, gallic, gentisic, sinapic, rosmarinic, and *trans*-cinnamic acids	Allelopathic interactions, inhibition of microbial growth, and weathering of minerals

Note: Cited from Asao et al. (2003), Medvedeva and Yakovlev (2011), Nambu et al. (2005), Bais et al. (2002), Mandal (2001), Grayston et al. (1997), Lima et al. (2009), Halvorson et al. (2009), Liao et al. (2006), Bergelin et al. (2000), Baziramakenga et al. (1995), Sandnes et al. (2005), Van Hees et al. (2002), and Shen et al. (2006)

11.5.2 Behavior of Organic Acid in Different Soils

The pH (hydrogen ion concentration) of the soil affects the crop growth; therefore, soils may be acidic (<7.0), neutral (=7.0), or saline/alkaline (>7.0). The interaction among a number of factors, including parent material, climate, vegetation, and management, determine whether a soil has a neutral, acidic, or alkaline reaction. The pH specifically affects plant nutrient availability by controlling the chemical forms of nutrients. Soils with low pH are injurious to plants because of high toxicity of Fe and Al ions. Low pH also interferes with the availability of other plant nutrients. Information on OA concentrations in the rhizosphere and their nature and origin is still lacking as experimentation in the rhizosphere is

extremely difficult. In addition, the complex nature of the OAs increases the difficulties of such studies. Finer understanding of the behavior of OAs in the soil requires the basic understanding of the properties of the major soil types with respect to their pH.

(a) *Calcareous soils*

Calcareous soils characterized by a high base status and a pH between 7.5 and 8.5 depending on the calcium concentration account for more than 30 % of the earth's land surface (Chen and Barak 1982; Marschner 1995). Availability of nutrients in these soils is limited as most nutrients are poorly soluble at high pH. The excess uptake of cations by plants causes the secretion of HCO^{3-} by roots, in order to maintain the electrical neutrality of the process, which leads to an increase in the rhizospheric pH. As a consequence of this, many vascular plant species are unable to colonize calcareous soils. Under such conditions, root exudates play a significant role in nutrient acquisition (Ström 1997; Jones 1998). Calcicole plants (which thrive in calcium-rich conditions) have enhanced rates of exudation of di- and tricarboxylic OAs, or the anions of these acids (Ström et al. 1994; Tyler and Ström 1995; Ström 1997). The solubility of various calcium phosphate compounds present in alkaline soils determines the phosphorus availability. In alkaline soils, soluble $H_2PO_4^-$ quickly reacts with calcium to form a sequence of products of decreasing solubility. The highly soluble monocalcium phosphate which is commonly applied in the form of superphosphate fertilizer, rapidly reacts first with calcium carbonate ($CaCO_3$) to form dicalcium phosphate, which reacts again with $CaCO_3$ to form tricalcium phosphate with decreasing levels of solubility. The tricalcium phosphate undergoes further reactions to form even more insoluble compounds, such as hydroxy carbonates and hydroxyapatites (Mahdi et al. 2011).

(b) *Acidic soils*

Acid soils are distinguished by a lack of easily soluble salts and an acidic reaction. The low salt content results in low ionic strengths in the solutions (electrical conductivity (EC) values commonly <1.0). In acid soils, the pH-dependent exchange sites become increasingly occupied with hydrogen (non-exchangeable) and the cation exchange capacity (CEC) decreases; that is, the uptake of cations in excess of anions can cause roots to exude H^+ and lower the rhizospheric pH (Breemen et al. 1984). Common causes of acidification of soils include leaching with acid rainfall (the result of industrial pollution) and nitrification following applications of nitrogenous fertilizers (Wild 1988). Two fundamental factors that limit the fertility of acid soils are nutrient deficiencies (e.g., P, Ca, and Mg) and the presence of phytotoxic substances (e.g., soluble Al and Mn). The low P status of highly weathered acid soils is a particular problem because large quantities of P need to be applied in order to raise concentrations of available soil P to adequate levels (Sanchez and Uehara 1980). This is because such soils contain large quantities of Al and Fe hydrous oxides which have the ability to absorb P onto their surfaces and thus interlocks the P (Mahdi et al. 2011). Lime application in acid soils to achieve increases in pH levels above pH 5.5 results in concentrations of soluble and exchangeable Al being lowered to negligible levels, and Al toxicity no longer limits the crop growth. Low molecular weight OAs that are commonly identified in such soils include formic, acetic, propionic, butyric, crotonic, lactic, oxalic, succinic, fumaric, tartaric, and citric acids (Stevenson 1967). The leaves of plants often contain high concentrations of OAs such as malic and citric acids, and to a lesser extent, succinic, fumaric, and oxalic acids, which are added to the soils through leaf litter (Stevenson and Vance 1989). In addition, a wide range of OAs are produced by the soil microbial biomass (Rovira and McDougall 1967). In acidic soils, OAs form stable chelate complexes with Al^{3+} and other polyvalent cations. Hydroxy acids, such as citric acid, form stronger complexes than those containing a single COOH group (Stevenson and Vance 1989). The concentration of OAs in soil solution is normally low (about 1–5 mM), but substantially higher concentrations can often be found in the rhizosphere. Their concentrations are also significantly higher in soils amended with organic manures (Iyamuremye and Dick 1996).

(c) *Buffering capacity of soils*

There are a number of processes in soils and sediments that generate or consume protons and therefore affect the pH. Water flow through the system is an important variable as it removes weathering products and supplies protons for exchange reactions (Bohn et al. 2002). A system is known as "well buffered" if it would accept either some strong acid or strong base without changing the pH very much. A fundamental concept is that only free, unbound H^+ ions affect the pH. It is paradoxical that though PSMs are abundant in soils and in the rhizosphere of most plants (Kucey et al. 1989), phosphorus is still one of the major limiting nutrients for the plant growth. Though inoculation of PSMs into soils has been shown to increase the population of PSMs in the rhizosphere, only a few studies have shown consistent enhancement of phosphorus uptake by plants (Selvakumar et al. 2011, 2013). This inconsistency in growth enhancement and P uptake of inoculated plants arises due to the inability of some PSMs to release P from soils. As the P solubilization ability of microorganisms in soils is quite different from laboratory conditions, most PSMs isolated under unbuffered conditions (Kucey et al. 1989) fail to meet their potential in soils that have a very strong buffering capacity (Ae et al. 1990). This buffering nature of soils limits the solubilization of soil phosphates by microorganisms, mainly under alkaline conditions, as it is well established that solubilization of Ca–P complexes occurs by lowering the pH of the medium (Sperber 1957; Kucey et al. 1989; Halder and Chakrabartty 1993). To overcome this, it has been recommended to carry out screening for efficient PSMs in buffered media conditions in order to mimic calcareous soil conditions (Gyaneshwar et al. 1998).

11.5.3 Fate of Organic Acids in the Rhizosphere

After their release into the rhizosphere, the OAs can undergo a number of reactions, including complexation with metals that induces mineral dissolution and enhances the solubility and diffusion of nutrients toward the root (Ryan et al. 2001). This response is typically enhanced by co-acidification of the soil via the roots H^+-ATPase or via release of OA anions in a protonated form (Jones 1998). However, the behavior of OAs in soil is complex, and a range of processes that can reduce the magnitude of the nutrient mobilization response, including consumption of the OAs by the soil microbial community, immobilization on anion exchange sites, precipitation (e.g., calcium oxalate), abiotic mineralization, and leaching down the soil profile, can occur (Jones 1998).

A number of studies have shown that the rate of OA turnover in soil is extremely rapid with half-lives ranging from 1 to 5 h in organic topsoil and from 5 to 12 h in subsoil (Jones 1998). Across a broad range of ecosystems, the concentration of OAs in soil solution has been shown to be low, typically ranging from 1 to 50 μM (Baziramakenga et al. 1995; Krzyszowska et al. 1996; Strobel 2001). The microbial population that excretes OAs can also utilize the acids as a source of C and hence acts as both a source and a sink for OAs in the rhizosphere (Archana et al. 2012). Since present-day methods used for quantification of OAs measure the content of OA in sum of all soil pools but not the internal microbial pool, it is very difficult to accurately quantify the extent of OA production by microbial populations in the rhizosphere.

11.6 Conclusion

The rhizosphere is characterized by a significant increase in the numbers and activities of microorganisms that are stimulated by the release of soluble sugars, photosynthetic carbon exudates, and organic anion exudates from plant roots. The rhizosphere therefore becomes a rich source of OAs, of both plant and microbial origins, which play a vital role in the P nutrition of plants. Though OAs entering the rhizosphere represent a minor fraction of the primary carbon entering into the soil system and have shorter half-lives in comparison with sugars and amino acids, their role in the dissolution of soil P reserves is crucial under both

alkaline and acidic conditions. But unfortunately, because of the complexities involved in deciphering the nature and properties of these OAs in the rhizosphere, little information has been generated on the distribution and role of OAs of microbial origin in the rhizosphere. Therefore, it is highly desirous to elucidate the OA profile of efficient PSMs under in vitro and soil conditions (both acidic and alkaline), in order to gain a meaningful insight into microbe-mediated P dissolution mechanisms in the rhizosphere. This would not only help us understand the P solubilizing mechanisms better but also help in the selection of efficient PSMs for use as inoculants in different soil types.

Acknowledgments Chandandeep Kaur, was supported by a grant from the Department of Science and Technology, Ministry of Science and Technology, Government of India, under the WOS-A scheme.

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Formulations of Biofertilizers – Approaches and Advances

12

P.K. Sahu and G.P. Brahmaprakash

Abstract

The use of microorganisms in agriculture as bioinoculant is a very important practice and a growing need too. In spite of countless research, the rate of success is remarkably low. To get success, there is a need to examine this aspect from various differ angles apart from conventional approaches. This chapter focuses on a few of these aspects of biofertilizer formulations, along with current approaches, and discusses the ideal bioformulation; the present scenario of solid-carrier-based bioformulations; liquid inoculants and their benefits; polymer entrapped formulation and its slow releasing quality; advances in formulations: fluid bed dried bioformulation technique and its scope; forms of mycorrhizal inoculants; bottlenecks which prevent from realization of inoculant potential; major factor for the failure of bioinoculant: rhizocompetence; different forms and their role in the success of bioinoculant, and an outlook for furtherance of biofertilizer formulation. The chapter set sights on the present scenario of biofertilizer formulation, pros and cons of on-hand techniques, and latitude of advancement.

Keywords

Microorganisms • Biofertilizers • Bioinoculants • Mycorrhiza • Rhizocompetence

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12.1 Introduction

Biofertilizers are emerging as a panacea for organic and sustainable agriculture. Scientifically, it is augmenting the soil with agriculturally beneficial microorganisms for attaining sustainability in agriculture. Nutrients are entailed for the growth of all living beings, counting plants as

D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_12

well (Brahmaprakash and Sahu 2012), a continuous withdrawal from soil, exhaust it. There are seventeen essential plant nutrients, and hardly three to four are added back to the soil, in much lesser quantity than withdrawn. Soil is the greatest buffer of nutrients although, but till when? It also needs restocking of nutrients, essentially by the organic means as we can't add all the nutrients harvested by the crops in inorganic forms.

Now, there is a growing trend of using various bioinoculants for improving soil health and crop productivity. The concept of inoculation is more like a mothers' recipe, transferring one generation to the next from time immemorial. Centuries before, farmers knew that the soil taken from previous legume-sown field to nonlegume field often improves the yield, and a similar thing was followed till the end of the nineteenth century for legume seed inoculation (Smith 1992). In fact, one can say it was the equally scientific bioformulation containing inoculant (*Rhizobium*), a carrier (soil particles), cell protectant (soil colloids), and moisture to sustain live cells.

12.2 Biofertilizer Formulations

Biofertilizers are the preparations containing live or latent microorganisms, and a formulation enables easy handling, long-term storage, and effectiveness of biofertilizers. It is a delivery vehicle of live microorganisms from the factory to the field. A bioformulation is a state of carrier in which live or latent microorganisms (bioinoculants) are being supplied to the target, either plant or soil.

The success of bioinoculant technology depends on two factors, microbial strain and inoculant formulation. Practically, formulation determines potential success of inoculants (Fages 1992). Technological advancement in formulation is independent of strain of microbes used, as they share many physiological properties. In such a situation, the technology for one strain can be used for other related strains with minor modifications (Bashan 1998).

Many kinds of bioinoculants are being used in agriculture like N fixers, P solubilizers, P mobilizers, biocontrol agents, PGPR, etc. For application, these bioinoculants have to be put in carrier, either liquid or solid based, along with osmoprotectant, sticking agents, nutrients, etc.; the complete assembly thus prepared is called a bioformulation. A formulation may differ according to the bioinoculant's use, type of soil, type of plant, nature of application, availability of resources, etc. Thus, the understanding of bioformulation is very much necessary as it affects the abundance and performance of bioinoculants. Hitbold et al. (1980) and Lupwayi et al. (2000) showed that the quality of microbial inoculants depends primarily on the number of viable cells present in the inocula. Thus, the formulation step is very crucial to developing a successful biofertilizer.

Research and development in the formulation advancement is gaining momentum in recent years but is concentrated mostly towards product development. The exclusive approach for basic research in formulation advancement is still ignored in spite of its central role in a successful inoculant technology.

The scientific literature exclusive on the formulation development is limited and largely fragmented (Xavier et al. 2004). Most of them are just comparative studies of different carriers, and approaches were more agronomic than the bioengineering based.

As the available bibliographic database reveals, more extensive work has been done over the development of improved strains through different approaches. Indeed, many improved strains have been constructed and granted patent in many developed countries but failed to appear on the commercial market, perhaps because of inappropriate formulation technology used for them (Brahmaprakash and Sahu 2012).

One major challenge in bioformulation technology is harsh and unpredictable environmental conditions. This situation is more critical for a researcher in semiarid and developing countries like India, as it enhances the unpredictability of performance. In low-input agriculture, the challenges are more; a farmer can't afford to take a second chance if he had not experienced appropriate output from biofertilizers because it

takes additional cost and technical knowledge. Semiarid conditions make survival difficult for introduced inoculum; harsh environmental conditions, including droughts, lack of sufficient irrigation, high salinity and soil erosion, may quickly diminish the introduced bacteria (Bashan 1998). These challenges are the opportunities; it will be a huge achievement if a practically feasible bioformulation can be developed for such conditions.

12.3 Qualities of Model Bioformulation

The desirable qualities of a formulation are discussed by many workers. According to Xavier et al. (2004), the formulation comprises viable bacteria in a suitable carrier, together with additives that provide:

- Stabilization of cells
- Protection of microbial cell during storage and transport and at the target

The formulation should:

- Be easy to handle
- Also be easy to apply so that it is delivered to the target in the most appropriate manner and form
- Be able to protect bacteria from harmful environmental factors
- Also maintain or enhance the activity of the organisms in the field; therefore, several critical factors, including user preference, have to kept in the mind

Besides these, the following are a few more qualities of a model bioformulation that can be considered at present scenario:

- It should support higher number of viable cells of microorganisms, because there is an array of literature (Hitbold et al. 1980; Smith 1992; Lupwayi et al. 2000) proving the relationship between number of cells in bioformulation and its effectiveness.
- The inoculant formulation should have a sufficient shelf life at room temperature (Bashan 1998).
- The inoculant should be easily manufactured and mixed by existing industrial processes and should allow for the addition of nutrients (Bashan 1998).
- It should be cost-effective to be commercially viable (Sivasakthivelan and Saranraj 2013).
- It should not have adverse effects on the environment (Bashan 1998).
- It should help in improving soil properties (Wu et al 2005; Pandya and Saraf 2010).
- It should be able to resist pH changes during storage (Bashan 1998).
- The inoculant has to compete with native soil microorganisms for the nutrients and habitable niches and has to survive against grazing protozoa (Bonkowski 2004).
- It should be able to provide protection from external extremes and should also be able to give some ecological competence upon application to soil (Bashan 1998).
- The release of bioinoculants in entrapped formulation should not be too fast or too slow (Bashan and Carrillo 1996).
- It can be applied by using standard agrochemical machinery (Bashan 1998).
- It should be suitable for as many bacteria and strains as possible (Bashan 1998).
- Polymer entrapped formulations should be nontoxic and free from preservatives that may harm the inoculant microorganisms (Deaker et al. 2004).
- It should not increase contamination during storage (Bashan 1998); nutrient-poor condition will not develop contamination, but the enhancement in cell number will not occur and at the same time nutrient-rich formulation will increase the cell number during storage and also increase contamination if proper protection is not taken.
- It should complete the BIS standards for biofertilizers (Yadav 2009).

12.4 The Qualities of Model Carrier

A suitable carrier is imperative in bioformulation. A carrier is a delivery vehicle employed for transferring live microorganism from industrial fermentor to rhizosphere. A good-quality inoculant should be made from an outstanding carrier material. The most important characteristic feature required for a carrier is the capacity to deliver the right number of viable cells in good physiological condition at right time (Fages 1990). The other characters of a superior-quality carrier material for microbial inoculants include:

- High water-holding and water-retention capacity and suitable for as many bacteria as possible (Mishra and Dahich 2010)
- Free from lump-forming material (Keyser et al. 1993)
- Cost-effective (Mishra and Dahich 2010)
- Available in adequate amounts (Bazilah et al. 2011)
- For carriers used for seed treatment, should assure the survival of the inoculants on the seed since normally seeds are not immediately sown after seed coating (Muresu et al. 2003)
- For carriers that shall be used for seed coating, should have a good adhesion to seeds (Hegde and Brahmaprakash 1992)
- No heat of wetting (Smith 1992)
- Chemically and physically uniform (Bashan 1998)
- Near sterile or easy to sterilize by autoclaving or by other methods like gamma irradiation (Keyser et al. 1993)
- Nontoxic in nature (Bazilah et al. 2011)
- Easily biodegradable and nonpolluting (Smith 1992)
- Nearly neutral pH or easily adjustable and good pH buffering capacity (Keyser et al. 1993)
- Supports growth and survival of bacteria (Smith 1992)
- Amenable to nutrient supplement (Smith 1992)
- Manageable in mixing, curing, and packaging operations (Smith 1992)

12.5 Solid-Carrier-Based Bioformulation

Solid formulation is a preparation in which inoculum is mixed to a solid carrier in appropriate proportion. A carrier is an inert material, used for transporting microbes from laboratory to land (Brahmaprakash and Sahu 2012). Solid carrier materials can be more advantageous because they are proven better in increasing the supply of phosphorus to plant, resistance to soil-borne plant pathogens, and biological degradation of organic pollutants (Warren et al. 2009).

Most of the studies on bioinoculants have given emphasis on the selection of bacterial strains for biofertilizer preparation, whereas very little work has been carried out on the selection of carrier material and its effect on growth and yield of crop. Carrier as being the vehicle of inoculum transfer, it does affect the performance of bioinoculant.

Initially, the biofertilizers were formulated in a solid-based carrier only. Since natural soil provides hostile environment to inoculant cells (Ho and Ko 1985), the soil was used initially as a carrier for rhizobia (Madhok 1934).

Bashan (1998) has divided carriers into four basic categories:

1. Soils: peat, coal, clays, and inorganic soil
2. Plant waste materials: composts, farmyard manure, soybean meal, soybean and peanut oil, wheat bran, press mud (a by-product from the sugar industry, agricultural waste material, spent mushroom compost, and plant debris)
3. Inert materials: vermiculite, perlite, ground rock phosphate, calcium sulfate, polyacrylamide gels, and alginate beads
4. Plain lyophilized microbial cultures and oil-dried bacteria: these preparations can later be incorporated into a solid carrier or can be used as such

These four classes are very broadly defined and perhaps left no space for adding any more category, but if we consider the strict meaning of "categories of carriers" in present scenario then two more can be added:

5. Liquid carriers: broth, broth + polyvinylpyrrolidone (PVP)
6. Capsule-based carriers: pelleted spores and cells in capsules

12.5.1 Traditional Peat Formulations

Peat was the carrier of choice and admired worldwide for decades. It was popular due to successful field results obtained under commercial cultivation, but had shortcomings too.

12.5.1.1 Advantages of Peat

- Since adopted decades ago, farmers have been quite comfortable with peat.
- Governmental agencies usually know how to monitor its quality.
- It was successful under commercial cultivation.
- The bacteria are metabolically active, and in some inoculants, bacterial multiplication continues during the storage period.
- It has high water-holding capacity.
- It has a high surface area that assists growth and survival of inoculants.

12.5.1.2 Principle Drawbacks

- Quality is variable and dependent on source (Van Elsas and Heijnen 1990), which affects inoculant effectiveness between different manufacturers and between different batches from the same manufacturer (Bashan 1998).
- It sometimes releases toxic components to bacteria upon heat sterilization (Chao and Alexander 1984).
- Bacteria have a lower tolerance for physical stress during storage in peat carrier, in particular for temperature variations.
- Peat formulations are prone to contamination that can reduce the shelf life of the inoculant (Fages 1992; Olsen et al. 1994; Van Elsas and Heijnen 1990).
- Some types of peat can even reduce plant growth (Huber et al. 1989).
- Peat powder is blown away from the seeds by the commonly used seed air delivery system used by the planter (Smith 1995).
- Addition of adhesives to the inoculant during its application to the seeds or slurry application will improve its adhesion, but that requires additional time and labor for a process that is already labor intensive (Smith 1995).
- It interferes with the seed-monitoring mechanism of the planters (Smith 1995).
- Availability is restricted to a very few countries (Bashan 1998).

All this downside had made researchers look towards other alternatives. Many solid materials have been evaluated apart from soil talc, fly ash, etc.

Some of the alternative carriers evaluated for bacterial inoculants include lignite and soybean meal (Kandaswamy and Prasad 1971), farm yard manure and tank silt (Bajpai et al. 1978), low-grade coal (Dube et al. 1980), clays and inorganic soils (Chao and Alexander 1984), charcoal and filter mud (El Shafie and El Hussein 1991), talc (Sahu et al. 2013), compost (Akhtar et al. 2009), vermicompost (Gandhi and Saravanakumar 2009; Shariati et al. 2013), biochar (Saranya et al. 2011).

Peat and lignite, though good carriers, are not easily available and are expensive. The low cost and easy availability of carrier material are the major requirements for bioformulation in developing countries (Saha et al. 2001).

As time went, people tried many waste materials as a carrier with a dual aim of cleaning the premises and getting a good base for inoculant in solid formulation at low cost. Coir dust was used by Iswaran (1972) as carrier material for *Rhizobium*. It was found suitable when mixed with an equal proportion of soil and was superior to soybean meal. Kumar Rao et al. (1983) observed that either sugarcane press mud or coffee waste could be used as a substitute for peat in many developing countries. Spent agricultural waste material (Sadasivam et al. 1986), spent

mushroom compost (Bahl and Jauhri 1986), dried sugarcane vinasse and urban compost (Figuiredo et al. 1995), wheat bran and sugarcane bagasse (Alla and Omar 2001), peanut shell, corn cobs, and paddy husk (Aparna et al. 2012) were also used.

Apart from these many other synthetic and inert materials, vermiculite (Sparrow and Ham 1983; Sharma et al. 2009), perlite, ground rock phosphate, calcium sulfate, polyacrylamide gel (Dommergues et al. 1979), alginate (Jung et al. 1982), diatomite (Figuiredo et al. 1995) have also been evaluated.

12.5.2 Talc as a Carrier

Talc is a mineral composed of hydrated magnesium silicate. Its most common use is as talcum powder as it is the softest known mineral. Talc is a common metamorphic mineral in metamorphic belts. Talc is a commonly used carrier of biocontrol agents such as *Trichoderma viride*. Nandakumar et al. (2001) investigated a bioformulation containing PGPR mixture of PF1, FP7, and PB2 for management of sheath blight of rice and higher grain yield. These PGPR had been applied individually and in combination with liquid and talc-based formulation. Both the individual and strain mixtures significantly reduced the incidence of rice sheath blight and enhanced grain yield. Talc-based formulations of PGPR *Bacillus atrophaeus* and *Burkholderia cepacia* inhibitory to the growth of *Fusarium oxysporum* f. sp. *gladioli* (FOG) were developed for the corm dressing and soil application in gladiolus. Corm production increased to 150 percent with less vascular wilt and corm rot incidence in green house (Shanmugam et al. 2011). Sahu et al. (2013) used talc for developing an innovative formulation consisting of a consortium of agriculturally important microorganisms.

12.5.3 Biochar as a Carrier

Biochar is a class of charcoal produced by pyrolysis of biomass under limited oxygen availability. Soil functions can be improved by biochar addition, and it also has appreciable carbon sequestration value. The large-scale production of biochar for carbon sequestration provides an opportunity for using these materials as inoculum carriers to deliver plant-growth-promoting rhizobacteria (PGPR) into agricultural soils (Hale et al. 2014).

Glaser (2007) showed that the application of biochar with bacterial inoculant enhances plant performance. Saranya et al. (2011) formulated carrier-based preparations of *Azospirillum lipoferum* (AZ 204) inoculant, using two different sources of biochar (acacia wood and coconut shell) as a carrier and evaluated in comparison with lignite. Among the different carriers, coconut-shell-based biochar recorded a maximum population of log 10.79 CFU g^{-1} of carrier in 180 days after inoculation. It was also found that seedling vigor index of green gram (CO 3) was utmost in response to coconut-shell-based biochar. The coconut-shell-based biochar was found to increase the survival of *Azospirillum lipoferum* up to 180 days (6 months) of storage period at a required population compared to acacia-wood-based biochar and lignite.

Hale et al. (2014) evaluated the suitability of a biochar produced from pinewood pyrolyzed at 300 °C as a carrier for *Enterobacter cloacae* strain UW5 genetically modified to produce a green fluorescent protein marker which enabled tracking of the inoculum after application. Selective plate count assays and quantitative PCR (qPCR) revealed that cell survival was slightly improved by addition of bacteria to soil using biochar as a carrier for the inoculant, as compared to soil directly inoculated. Here total bacterial abundance was not influenced by biochar. All treatments resulted in same bacterial colonization of roots at population densities of approximately 10^5 CFU g^{-1} root mass. It is clear from this study that there is an effect of biochar in improving plant growth but there was very little effect of inoculum as such.

12.5.4 Sterilization of Carrier

Target microorganism can be introduced into a sterile or nonsterile carrier to produce inoculants.

A sterile carrier has distinct advantages from a purely microbiological point of view. Disadvantages with sterilized carriers include a higher cost of production, increased labor, the necessity for a sterilizing unit, and the necessity for aseptic procedures during packaging. The type of carrier used in inoculant production usually depends on the mode of application. There are two types of inoculants commonly produced: those for seed treatment and those for direct application to the soil. Owing to differing methods of delivery, these formulations can either be powder for seed treatment or granulated for soil application (Walter and Paau 1993).

The work performed on solid carriers has many success stories. The usual solid formulations do have some pitfalls like:

- Involving a significant amount of cost, labor, and energy-intensive processing such as mining, drying, milling, and neutralization
- Short shelf life
- Poor quality
- High contamination
- Unpredictable field performance
- Clump formation upon drying, which leads to significant loss of viability

Today, advancement in inoculant technology is concerned with improving quality, extending useful shelf life and developing new formulations for use under less favorable conditions. Liquid inoculants and alginate-based granular formulations are two key inoculant formulations which are an alternative to peat/lignite-based ones. Solid carriers perhaps need a lot of innovativeness because the users are now interested more on other new formulations.

12.5.5 Additives and Amendments in Solid Formulation

The carriers were also tried with the additives and amendments to achieve greater success. Kandaswamy and Prasad (1971) reported that the viability of *Rhizobium* cells could be enhanced when lignite was mixed with soybean powder. Sharma and Verma (1979) found threefold more survival of *Rhizobium* when cultured in lignite with 10 % lucern hey meal than that of *Rhizobium* cultured on lignite alone. Vermicast was used with lignite in different combinations (0:1, 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, and 1:0) as carrier substrate for biofertilizers (*Azotobacter chroococcum*, *Bacillus megaterium*, and *Rhizobium leguminosarum*). The increase of vermicast proportion in carrier materials showed an increase in the survival rate. The results of the present study suggest that the vermicasts can be used as an alternate carrier material for *A. chroococcum, B. megaterium*, and *R. leguminosarum* (Sekar and Karmegam 2010).

Other materials as amendment might be involved to add to its effectiveness. Evidence shows that the addition of nutrients to seed pellets may be beneficial for enhancing inoculant survival (Moënne-Loccoz et al. 1998). Antifungal metabolite production by *Pseudomonas* BCAs improved by adding carbon source and thus improve biocontrol efficacy (Duffy and Défago 1999).

An increase in chitinolytic microbial populations and a significant reduction in the incidence of fungal diseases were recorded by amending soil with chitin (Bell et al. 1998). Chitin supplementation also found to support the survival of *Bacillus cereus* and *B. circulans* in the groundnut phylloplane and resulted in better control of early and late leaf spot disease (Kishore et al. 2005). The improved disease control results are related to increase in the population of the introduced biocontrol agent in presence of chitin.

Different organic amendments, i.e., sawdust, straw powder, paddy wood, charcoal, poultry manure, farmyard manure, and lignite as carrier material, were used for enhancing the shelf life of *Azospirillum* bioinoculant. It was observed that sawdust sustained high population of log 9.80 CFU g-1 of carrier (Stella and Sivasakthivelan 2009).

12.5.6 Liquid Inoculants

Liquid formulations typically are aqueous, oil, or polymer-based products. They are a formulation containing not only the desired microorganisms

and their nutrients but also special cell protectant and additives that promote cell survival in storage and after application to seed or soil (Brahmaprakash and Sahu 2012).

Peat is the most preferred carrier for many years, but the availability is limited and it is also depleting fast, so researchers are now looking for possibility of liquid inoculants for all kinds of biofertilizers. Rapidly liquid inoculants are being adapted for advanced seeding equipment, as it can be sprayed onto the seed as it passes through the seed auger and dries before it travels into the seed bin on the planter (Smith 1995).

It can be produced by a simple fermentation process, packed directly from the fermentor aseptically, and stored for a long time without loss of viability. It is cost-effective as it avoids processing and sterilization of solid carrier material. No contamination during the storage can be detected as complete sterilization could be achieved with liquid formulations. The quantity of inoculum required is also less compared to carrier-based formulations, hence easier for farmers to handle.

Liquid inoculants are not the usual broth culture from a fermentor or water suspension of the carrier-based biofertilizers, as often considered to be. Liquid inoculants consist of medium containing carbon, nitrogen, and vitamin sources for the growth of microorganisms and certain compounds which serve as cell protectant. These cell protectant and additives are added to the broth for improving inoculant quality like:

- Prevention from osmolysis
- Better adhesion to seed
- Stabilizing the product
- Binding or inactivating of soluble seed coat toxins
- Enhancing of rhizobial survival during storage
- Protection of inoculum after exposure to extreme environmental conditions upon inoculation to seed and planting

Legumes are sometimes sown into soil with temperatures reaching 40 °C. As high temperature affects rhizobia survival and nitrogen fixation, these additives protect rhizobial cells on seed at such high temperature and during desiccation. Liquid cultures containing cell protectants not only maintain high microbial numbers but also promote the formation of resting cells such as cysts and spores, which offer higher resistance to abiotic stresses, thus increasing the survivability of bacteria.

Selection of additives is based on their ability to protect bacterial cells in storage and on seeds at extreme conditions such as high temperature, desiccation and toxic condition of seeds, and seed chemicals. High molecular weight polymers with good water solubility, nontoxicity, and complex chemical nature are good additives (Deaker et al 2004) and are able to limit heat transfer and possess good rheological properties and high water activities (Mugnier and Jung 1985). Commonly used polymers are polyvinylpyrrolidone (PVP), methyl cellulose, polyvinyl alcohol, polyethylene glycol, gum Arabica, trehalose, glycerol, Fe-EDTA, sodium alginate, tapioca flour, etc. (Singleton et al. 2002).

The nature and concentration of additives affect the performance of inoculum. Dayamani (2010) has tried different osmolytes in different concentrations to optimize it for liquid inoculants of *Azotobacter* sp., *Azospirillum* sp., *Acinetobacter* sp., *Bacillus* sp., and *Pseudomonas* sp. This study has shown that each organism responds variably to different osmolytes and its concentration. *Pseudomonas* sp. and *Bacillus* sp. perform best with PVP K-15 at 2 % concentration. Polyethylene glycol (PEG) 4000 at 2 % concentration found best for *Acinetobacter* sp. Glycerol at 2 % level supports higher population density of *Azotobacter* sp. The population of *Azospirillum* sp. was higher in PVP and PEG both at 1 % and 2 % levels.

Supplementing growth medium with 3 % molasses and 0.1 % (w/v) NH_4Cl improves the inoculant quality. Addition of L-ascorbic acid (0.02 % w/v) was found improving the effectiveness of protective substances (Patil et al. 2012).

Some concentrations of various additives to yeast extract mannitol (YEM) media promoted higher cell density compared to cells cultured in YEM media alone. Six different polymeric addi-

tives (polyvinylpyrrolidone (PVP), polyethylene glycol (PEG), polyvinyl alcohol (PVA), gum arabic, cassava starch, and sodium alginate) were evaluated for their ability to support growth and promote survival of several strains of bradyrhizobia and rhizobia during storage. Shelf life of the liquid inoculants found depended on the strain of rhizobia and additives. It was found that liquid inoculant performance was as good as that of peat-based inoculant (Tittabutr et al. 2007).

A liquid inoculant for pyrene biodegradation has been developed by Nopcharoenkul et al. (2011) using *Pseudoxanthomonas* sp. Liquid formulation of RN402 was developed by suspending RN402 in phosphate buffer containing 1 % glycerol. This formulation could be stored at 30 °C for at least 6 months and maintain high efficacy in the treatment of pyrene-contaminated soil.

Albareda et al. (2008) assayed different liquid culture media employing mannitol or glycerol as C sources on *Sinorhizobium* (*Ensifer*) *fredii* SMH12 or *Bradyrhizobium japonicum* USDA110. Inoculants which were cured for 15 days led to a higher survival in comparison with recently made inoculants. These liquid formulations on soybean produced seed yields that were not significantly different from those produced by peat-based inoculants.

Polyvinylpyrrolidone is a water-soluble polymer made from the monomer *N*-vinylpyrrolidone. It was known to bind toxic compounds exudes from seeds during inoculation and seed germination. It has a high water-binding capacity and causes slow drying of an inoculant after application. Polyvinylpyrrolidone solution tends to come into ridges on their seed coat as it dries, perhaps providing a thicker layer of protection than some other compounds. Its sticky consistency may also enhance adherence to seeds (Tittabutr et al. 2007). Liquid *Rhizobium* inoculants prepared with PVP as an osmoprotectant had improved shelf life, nodulation, and nitrogen fixation on par with lignite-based inoculants in cowpea (Girisha et al. 2006).

The first yardstick to measure the quality of biofertilizers is the viable cell density of desired microorganisms which essentially provides adequate number of microorganisms on each seed. The liquid inoculants developed were known to have population of *Rhizobium* sp., *Azotobacter* sp., *Azospirillum* sp., and PSB up to the level of 10^8 cells per ml (Sridhar et al. 2004; Vithal Navi 2004; Dayamani 2010; Velineni and Brahmaprakash 2011). A strong correlation existed between the number of surviving cells on seeds and nodulation in legumes; hence, it is important to have more number of cells per seed, which are sufficient to compete with native *Rhizobium* and to offset death of cells due to biotic and abiotic stresses. Since the liquid biofertilizer has high cell count, each seed receives more than thousands of cells. Additives in liquid biofertilizer protect the cells on the inoculated seeds against toxicity, desiccation, and osmotic shock (Vithal Navi 2004).

Liquid inoculants can be used for stress alleviation. Imposition of little stress to bacteria results in an adaptive response. This causes changes in regular metabolic processes in cells, which then alters protein profiles (Saxena et al. 1996). Synthesis of additional 19 salt stress proteins (SSPs) in *Rhizobium* (40–52 kDa), 10 SSPs (ranging from 19 to 82 kDa) in *Anabaena* sp. L-31 under salt stress (Apte and Bhagwat 1989), and synthesis of 19 heat shock proteins (ranging from 8 to 60 kDa) in *Bradyrhizobium japonicum* at 43 °C have been reported (Munchbach et al. 1999). *Bradyrhizobium* sp. (*Arachis*) on exposure to heat stress showed the presence of bands of proteins of 60 and 47 kDa in liquid inoculant. Similarly, under salt stress (0.05 M NaCl), *Bradyrhizobium* sp. (*Arachis*) grown in liquid inoculant synthesized the extra proteins of 66 kDa but not in YEMB (Brahmaprakash et al. 2007). This kind of mechanism provides the potential to grow and perform at different types of soil as we know that performance of inoculants depends largely on soil conditions.

12.6 Advantages of Liquid Inoculants

- Less amount of inoculant needed
- No need of any sticker material unlike carrier-based inoculants
- Supports higher number of cells for longer time
- Easy to produce
- Easy to sterilize completely, thus prevents contamination
- Compatible with modern agriculture machineries for its application
- Easy transport of large number of inoculum in small bottles
- Easy to apply also as fertigation
- Can be used for stress alleviation

12.6.1 Polymer Entrapped formulation

The progress in the inoculant technology brought out polymer entrapment as a method of inoculant formulation. In this technique, the cells after mass multiplication are mixed with polymer and subjected to chemical solidification. It forms the uniform beads entrapping live cells inside. These beads are fermented for further growth in polymer matrix and dried. These beads upon application are degraded by soil microorganisms and release the entrapped cells in soil.

During the last decade, several experimental formulations based on polymers have been evaluated. These polymers have demonstrated potential as bacterial carriers (Jung et al. 1982) that offered substantial advantages over peat. These formulations encapsulate the living cells, protect the microorganisms against many environmental stresses, and release them to the soil gradually. Different inert materials were also evaluated as carriers like polyacrylamide gel (Dommergues et al. 1979). Jung et al. (1982) used *Rhizobium* entrapped in sodium alginate and a mixture of xanthan and carob gum as legume inoculants and successfully stored them for over 90 days.

The dry beads give an excellent survival rate over a long period. An experiment started in 1983 with two plant-growth-promoting bacteria (*Azospirillum brasilense* Cd and *Pseudomonas fluorescens* 313) immobilized in two types of alginate-bead inoculant (with and without skim milk supplement) and later dried and stored at ambient temperature for 14 years. These beads recovered in 1996 and found that the population in each type of bead had decreased, yet significant numbers 10^5–10^6 CFU g^{-1} beads survived (Bashan and Gonzalez 1999). The morphology as well as plant-growth-promotion activity were similar to their 1983 cultures.

12.6.2 Advantages of Polymer Entrapped Formulation

- It can be stored at ambient temperatures for prolonged periods (Bashan 1998).
- It is easy to produce and handle (Bashan 1998).
- It is nontoxic in nature (Fages 1992).
- It offers a consistent batch quality (Bashan and Gonzalez 1999).
- It provides a better defined environment for the bacteria (Bashan 1998).
- It can be manipulated easily according to the needs of specific bacteria (Bashan and Gonzalez 1999).
- These inoculants can be amended with nutrients to improve the short-term survival of the bacteria upon inoculation, which is essential to the success of the inoculation process, especially with associative PGPB (Bashan 1998).
- It temporarily protects the encapsulated microorganisms from the soil environment and microbial competition (Bashan and Gonzalez 1999).
- It releases microbes gradually for the colonization of plant roots (Digat 1991).

12.6.3 Major Constraints

- Polymers are expensive compared to peat-based inoculants (Bashan 1998).
- Requires more handling by the industry (Fages 1992).

- More labor intensive (Bashan and Gonzalez 1999).
- The low oxygen transfer limits the survival of inoculum.

The major benefit from polymer entrapped inoculant can be addressed in comparison with peat in tropical agriculture. In such areas, there is always a chance of prolonged dryness after sowing and microbial inoculation in rainfed areas. Microorganisms in the encapsulated formulations are at low metabolic activities as they are already desiccated due to lower water activity. These beads are degraded by soil microorganisms when they get sufficient moisture; by this time, seeds also germinate by available moisture. This coincidence of release of microorganism from beads with germination of seeds makes it very effective.

The survival of microorganisms in formulation depends on water availability in the product, since water activity (a_w) is a better representative of moisture available for living organisms. Mugnier and Jung (1985) had studied the effect of water activity on the survival of fungi, bacteria, and yeast in polymer gels. They found that survival remains constant for more than 3 years when the water activity is kept below 0.069. Survival decreases when the water activity rises from 0.069, proving that the less moisture in the polymer gels gives more protective effects to the inoculum. The survival rates differ with the type of solute used for the culturing of the organism. Low molecular weight compounds had a negative effect on the survival of microorganisms, whereas the high molecular weight gave protective effects. The high molecular weight compound such as polysaccharides does not affect the osmolarity of the cell solute; thus, it gives protective effects.

12.6.4 Alginate-Based Formulations

Alginate is a naturally occurring polymer commonly used for encapsulation of microorganisms. It is composed of β-1,4-linked D-mannuronic acid and L-guluronic acid. It is derived from different brown algae and bacteria. The main advantages of alginate preparations are their nontoxic nature, biodegradability, and slow release of microorganisms into a soil (Kitamikado et al. 1990). Fages (1992) had used alginate for encapsulating the plant-beneficial bacteria *Azospirillum brasilense* and *Pseudomonas fluorescens* with a successful inoculation on wheat plants under field conditions.

Field inoculation of polymer entrapped bacteria shows that they survived in the field long enough and their populations were comparable with other carrier-based inoculants (Bashan et al. 1987). Root colonization by beneficial cells released from the beads was superior to that achieved by direct soil inoculation in wheat. The results from these studies provide evidence of slow-releasing efficiency of alginate beads and also protection from harsh environment.

A wide array of polymer entrapped preparations had been tried for the encapsulation of vesicular-arbuscular mycorrhiza (VAM) fungi (Ganry et al. 1982), ectomycorrhizal fungi (Le Tacon et al. 1985), *Frankia* inoculation (Sougoufara et al. 1989), bacterial biocontrol agents (Aino et al. 1997), and fungi (Fravel et al. 1985).

12.6.5 The Process of Alginate Bead Formation

Microorganisms are cultured in respective nutrient broths in a rotary shaker till they attain maximum growth phase under standardized conditions. Entrapment of bacteria within beads is carried out under sterile conditions in a laminar flow hood with sterilized alginate. The bacterial culture is aseptically mixed with 2 % sodium alginate powder and stirred gently for 1 h to ensure complete dissolution of all ingredients. The mixture is added dropwise with the aid of a sterile syringe into gently stirred, sterilized 0.1 M $CaCl_2$ at room temperature. The beads immediately form in the $CaCl_2$ solution. The resulting alginate beads entrapped the bacterial cells whose mean diameter depends on the pore size of syringe. The beads are maintained in the solution at room temperature for an additional 1 to 3 h to

obtain regular solid beads. The $CaCl_2$ solution is then taken out, and the beads are washed twice with sterilized tap water. After washings, the beads are incubated in fresh nutrient broth medium for an additional time of 24–48 h in the rotary shaker to allow the bacteria to multiply inside the beads. Then the beads are washed, collected, and dried.

12.6.6 Attempts for Reducing Cost of Alginate-Based Bioformulations

Alginate is costly as far as economic viability of biofertilizer industry is concerned, but its massive production in the Far East countries in the past few years has given potential opportunity for its use in inoculant industry (Fages 1992). However, attempts were made to amend alginate with other cheap materials to reduce the total formulation cost. Materials like rock phosphate, cement, bentonite clays, granite powder, gypsum, lignite, talc, etc. have been tried with alginate by which cost of production can be minimized (Lewis and Papavizas 1985). Few of the amendments have been added to increase the performance of alginate-based formulation. The addition of clay and skim milk to the beads significantly increased bacterial survival over alginate beads alone. Alginate mixed with perlite was used to entrap *Rhizobium* (Hegde and Brahmaprakash 1992).

Further, a few materials like pero-dextrin, which is a by-product of the starch industry, was used as a carrier. It has shown to improve cell survival and maintain prolonged survival rates and nitrogenase activity. It had been prepared using natural polymers, i.e., arabic gum (5 %), pero-dextrin (20 %), starch granules (10 %), or gelatine (20 %) impregnated with cells of tested diazotrophs. The effluent supported good growth of *Azotobacter chroococcum*, *Enterobacter agglomerans* and *Klebsiella pneuomoniae*, *Azospirillum brasilense*, *Bacillus polymyxa*, and *Pseudomonas putida*. With storage, entrapped cells of *B. polymyxa* were viable up to 160 days, while gradual decreases in *Azospirillum* numbers were recorded (Ali et al. 2005).

12.7 Novel Approach – Fluid Bed Dried Bioformulation

Fluid bed dryer (FBD) is a dryer in which material is maintained suspended against gravity in an upward flowing air stream creating a fluidized condition. Electrical heaters are employed to generate heat for drying the material. This hot air expands the bed of material at its terminal velocity, and creating turbulence in the product (terminal velocity is the minimum velocity of the air sufficient to keep the given particle hanging in the air). This phenomenon is known as fluidization and offers more surface area for drying as the complete particle then comes into contact with heated air. As it produces full agitation of solid particles, it results in high rate of heat transfer and uniform drying. In quest of an appropriate drying technology for bioinoculants, the idea of fluid bed drying was borrowed from the food-processing industry. Fluid bed dryer is commonly used in food industries for making instant coffee powder and other drying operations (Brahmaprakash and Sahu 2012).

The fluid-bed method of wet granulation is very common in the pharmaceutical and other industries as a one-step, enclosed operation. It is very useful as several ingredients can be mixed, granulated, and dried in the same vessel; the technique reduces material handling and shortens process times compared with other wet granulation processes (Srivastava and Mishra 2010). With all these features, the FBD can be a better candidate for use in the inoculant industry.

12.7.1 Need of Drying in Inoculant Industry

Short shelf life of biofertilizers and contamination in it are two major drawbacks in their production and use. Research was needed to reduce the moisture content of carrier-based inoculants so as to reduce contaminants in it. Improved production processes for dried inoculants are required as they have the potentiality of maintaining higher shelf life and performance at field level. Initial studies have been done on the preparation of dried granular inoculants of *Rhizobium* by air drying.

The benefit of FBD for bioinoculant drying is low temperature drying. The product can be dried at 37–38 °C (Sahu et al. 2013). The temperature of the drying chamber is adjustable, and even less temperature can be tried for more sensitive organisms. After drying, the moisture content of inoculants reduces to a level that does not allow the contaminants to grow and outcompete the target microorganisms. The FBD formulation does not allow constituent microorganisms to interact because of very low water activity and thus maintain a nearly constant number of cells till delivered to plant rhizosphere. It also supports the findings of Mugnier and Jung (1985) that low water activity in bioformulation results in good survival.

12.7.2 Research Gap in FBD Formulation

Fluid bed drying has the potentiality to be used in biofertilizers, but still no investigation has been carried out on use of fluid bed dryer for production of bioinoculants. An investigation was done by Sahu (2012) to study its suitability for the production of biofertilizers consisting of nitrogen fixing, phosphorus solubilizing, and plant-growth-promoting rhizobacteria inoculants with a special focus on microbial consortium. This investigation had been carried out as an initiative in investigating and revealing a novel biofertilizer formulation which would be an excellent substitute for rolling formulations in future. Fluid bed drying is basically designed for food processing, thus the researches needed to work out its protocols in biofertilizer production process. This study also had significance in determining the drawbacks associated with present design of machine in accordance with live microorganisms. The research and its feedback to manufacturers can bring out more suitable and effective drying procedures and thus a revolution in high scale and efficient biofertilizer production, storage, and application.

Sahu et al. (2013) had prepared an FBD inoculant formulation of microbial consortium. The survival of all three microorganisms in consortium was observed till 180 days of storage. FBD formulated consortium maintained a more or less constant number of cells till the end of 180 days. There was no contamination observed in any dilution. This was a very specific result achieved from FBD to maintain a contamination-free inoculant as it was speculated.

12.7.3 Advantages of FBD Inoculant Formulation

- The decline in number of cells is very limited (Sahu et al. 2013).
- Absolutely no contamination builds up (Brahmaprakash and Sahu 2012).
- Several ingredients can be mixed and dried (Srivastava and Mishra 2010).
- Ambient temperature is used for drying (Sahu 2012).
- Possibility to change the drying temperature according to need (Sahu 2012).

12.8 Mycorrhizal Inoculant Formulation

The arbuscular mycorrhizal (AM) fungus is a proven potential biofertilizer. It mobilizes phosphorus from soil into plant roots and provides many benefits to the host plant. It has a wide host range which adds to its utility in inoculant industry. Being biotrophic, the large-scale commercial production of AM fungi is still difficult. As it cannot be grown in artificial media with appropriate success, the only method of production is in association with host plant by pot culture.

Different types of AM inocula are used for different purposes. The spores of AM fungi are used as inocula generally for in vitro experiments but large-scale production of spores is difficult (Bagyaraj et al. 2002).

The potential of using AM fungi on a large scale depends upon a few key points (Hua 1990):

- Axenic AM fungi growth technique
- Economic production of a large volume and high quality of inoculant

- Formulation of AM inoculant preparations with higher shelf-life and easy-handling characteristics
- Development of strains superior to indigenous soil AM fungi

12.8.1 Infected Root Inoculum

Infected roots are one of the important inoculant techniques. It is made possible by various methods like aeroponics (Hung and Sylvia 1988), hydroponics (Dehne and Baekhaus 1986), and on Ri t-DNA transformed roots (Diop and Piche 1990) etc. Infected roots contain mycelium and spores. Infected roots colonize the host after 1 or 2 days of inoculation. The root inocula without spores should be used within a week. In vitro reproduction of few AM fungi on tissue-cultured roots has been demonstrated (Napamornbodi et al. 1988). The production process is difficult and expensive.

12.8.2 Culture of AM Fungi on *Agrobacterium rhizogenes* Transformed Roots

Agrobacterium rhizogenes is now becoming popular for genetically transforming roots by its Ri (root inducing) plasmid. This transformation causes higher root proliferation and gives "hairy roots." The mycorrhiza is then cultured on these transformed roots. This in turn provides more number of units of host root to AM fungi for infection. This gives high infection per plant by increasing the roots or inflectional units. A new thrust came in the mycorrhizal production by this method as it is obligate symbiont. The growth of these roots is faster and doubles within hours (Johnson et al. 1997). For the transformation, tissue culture plants of 25–30 days old are chosen and infected with *A. rhizogenes*. This technique is now being used for mass multiplication of AM fungi.

12.8.3 Soil-Based Inoculum

This is the most commonly used inoculant technique. Soil inocula are produced using traditional pot-culture techniques by multiplying AM inocula in the soil sand mixture. The success of good soil inoculum production depends on the selection of host plant, efficient AM strain, and a suitable substrate in which AM fungus can be mass multiplied (Bagyaraj et al. 2002).

Various host plants with different substrates have been tried. Rhodes grass (*Chloris gayana*) was found to be the best host for *Glomus fasciculatum* to support highest percentage of mycorrhizal colonization (Sreenivasa and Bagyaraj 1987).

12.8.4 On-Farm AM Inoculum Production

On-farm mass production of AM fungus can be done by enriching the site by growing the mycorrhizal with its host. An experiment was conducted to enrich AM fungi in the farm itself for 3 years. Starter cultures for the study were produced in pots. The hosts used for three cycles were sudan grass (*Sorghum sudanese*), maize, and carrot in first year; maize, sudan grass, and onion (*Allium cepa*) in second year; and sudan grass, maize, and oats (*Avena sativa*) in third year. An increase of 15–47-folds in inocula of AM fungi in the farm soil was observed after 3 years (Douds et al. 2000). This method can give large benefits for producing large-scale inoculum production and also saves transportation cost.

12.8.5 Peat-Based Inoculants

It is also known as nutrient film technique (NFT). In NFT, host plants preinfected with AM fungi are placed in an inclined tray over which a layer of nutrient solution flows. The pH of the nutrient solution can be adjusted as per the requirement. In legumes, 0.05 to 0.10 strength Hoagland's solution with nitrogen can be provided (Douds et al. 2000).

Arbuscular mycorrhizal inocula obtained from pot cultures are incorporated into peat and then compressed into blocks. Lettuce plants are allowed to grow in the peat block for 2–5 weeks then the blocks are transferred to nutrient film technique (NFT) channels (Cooper 1985). The nutrient solution flows in NFT channels. Plants are allowed to grow in NFT channels for 8–10 weeks. During this time, mass reproduction of the AM fungus takes place. The peat blocks are then allowed to dry, chopped, and used as VAM inoculant. Shelf life of such inoculants is around 6 months (Bagyaraj et al. 2002).

12.8.6 Mixed Bacterial Inoculants

Arbuscular mycorrhizal fungi are also tried in consortium with other microbes. Mixed inoculants that interact synergistically are beneficial in inoculant technology. Plant studies have shown the beneficial effects of coinoculating other beneficial microbe with AM fungi. Synergistic interaction between *Rhizobium* and AM fungi in legume plants is well established (Bagyaraj 1984). Mixed inoculation with nitrogen-fixing bacteria and AM fungi creates synergistic interactions that may result in significant increase in growth and phosphorus content, enhanced mycorrhizal infection, and an enhancement in uptake of mineral nutrients such as phosphorus, nitrogen, zinc, copper, and iron (Li and Huang 1987; Garbaye 1994).

12.8.7 Bottlenecks in Bioformulation Technology

Bioformulation technology is very critical as the plant where it has to work and the soil where it has to apply both are dynamic systems. Merely mixing the isolates, fermenting, and putting into a carrier do not make a successful inoculant. The inoculants grown in the lab with ambient supply of nutrients find it difficult to compete with native microflora in harsh and competitive soil conditions, more so in rhizosphere.

The most important constraints for the adoption of biofertilizers in India have been listed by Wani and Lee (1991):

- Poor quality of inoculants produced
- Lack of knowledge about inoculation technology for extension personnel and farmers
- Lack of effective inoculants delivery/supply systems
- Lack of committed policy to exploit biofertilizers successfully

Bashan (1998) listed a few parameters for the low performance of biofertilizers in the field:

- Erratic root colonization with some PGPB on peat.
- Release of bacteria in most of the inoculants cannot be controlled.
- Difficult quality control.
- During seed treatment, the adhesion of inocula to the seeds is poor and much of the inoculants are lost during mixing and application.
- Formulation does not shield the inocula from the soil environment and predation by soil microflora.

Although these constraints have been realized during the late nineties, but still in 2015 the situation is not very different now. A lot of efforts have been made for improvement and different quality control measures were standardized (Yadav 2009), but its conversion to successful output is very slow. The following bottlenecks are a few factors that affect the performance of biofertilizers in the field:

- A lot is yet to be understood about soil ecology.
- The endophyte profile is dependent on plant species; not much is known about its interaction with applied inoculant.
- The third major problem in inoculants is that they are "live"; it is difficult to store and transport without affecting its viability and efficiency at a reasonable cost, as it is more prone to stress.

- The behavior of the organisms in the formulation and impact of variation of formulation in the organisms is largely unknown.
- Short shelf life and contamination, which render it inefficient.
- Fear of fake products in market.
- No stringent check for the release of bioinoculants.
- Fear of crop loss, small and marginal farmers of tropical countries do not have the risk-bearing ability and capability to try new product.
- Unpredictable weather conditions every year.
- Reproducibility of in vitro and green house trial in field conditions is limited. There is a need to device more natural type of in vitro assays which can mimic the original soil condition.

12.9 Rhizocompetence

The performance of the microorganisms in the rhizosphere depends not only on the capacity of an organism to promote plant growth or protect it from pathogens but also on its ability to survive in nutrient-poor soil environment and compete with other microflora for the niche. The microbial inoculant, grow lush in the laboratory medium, it is not an easy go for it to work in the soil environment. The reasons behind are very poor nutrients, many-fold more competition to colonize, harmful biochemical secreted by different organisms, etc., in the soil. The ability of a microorganism to withstand all these harsh heterogeneous soil conditions and make a successful colonization is called rhizocompetence.

The major causes of the failure of bioinoculants are largely undefined "ecological interaction" and "microbial food web." The lab and pot experiments do not replicate in the field because the competition for the nutrients and niche is limited in the lab and pot experiments. Bacteria can produce several beneficial effects for plant in isolation, but realization of its potential is much more complicated than speculated in soil. One can't decipher the effects of bioinoculants without considering rhizocompetence, and the need for discussing rhizocompetence here is to focus the key pillars of the performance of bioinoculants. The major factors that can be discussed under rhizocompetence are:

Availability of niche: the entire part root is not colonized by bacteria (Rovira 1956). There is only a small window of successful niche is available for colonization which generates great competition for the niche. Though an organism is a very potential bioinoculant, it is ineffective if it fails to colonize the plant roots. So the microbes should also be screened for ability to colonize successfully, apart from its actual beneficial effects (Lugtenberg and Kamilova 2009).

Availability of nutrients: plants are an important source of nutrient by secreting root exudates. Nutrients available in the rhizosphere are many-folds lesser than in the mass multiplication medium. As a result, microbe does not attain a minimum population for effective performance (Validov 2007).

Availability of conducive physical environment of soil: soil is a heterogeneous and highly dynamic medium to which lab-grown organism is not exposed. A microbe should be able to thrive the stresses in soil.

Predation by the soil protozoa: the effect of bioinoculant can be realized only if it can protect itself from vanishing by predation (Jousset et al 2006).

Realization of a sufficient microbial population is more crucial when the bacteria express beneficial features only at a high bacterial cell density. This is sensed by the level of quorum-sensing molecules such as acylated homoserine lactones (AHLs) that accumulate in the surroundings (Berg et al 2005). A bioinoculant is absolutely not effective until it attains a minimum population. Development of this effective population is highly dependent on the rhizocompetence of the given organism, and it can be considered as a key element in the success of inoculant technology.

12.10 Conclusion

Biofertilizers are an integral part of sustainable organic agriculture and as such accepted by many, if not all. There is a need for attitudinal change among the agricultural scientists and technocrats in that they have to follow the dictum "as you sow, so shall you reap." These biofertilizers are no longer a low-cost input; good-quality inoculants cost money. Keeping the cost of biofertilizers very less encourages cutting corners and marketing spurious and ineffective ones.

"Think globally, act locally" needs to be adopted for biofertilizers too. The ecological survival of introduced strains in the new environment is improbable. Merely delivering large numbers may not ensure successful survival or competitive ability over local efficient strains. We have to intensify the search for efficient biofertilizer strains of local importance.

Nutrients for inoculant strains during survival in the formulations need to be addressed also. Feast or starve situations as they exist during fermentation to formulation will affect the performance of these living entities. Time lag taken to acclimatize to such changing conditions may vary for different prokaryotes, which are to be investigated.

Ways and means have to be explored for perfect delivery of agriculturally important microorganisms into the rhizosphere. Seed treatment, although popular, has its own disadvantages and may not be suitable for crops and situations. The realization of agricultural prosperity lies in mechanization of agriculture. Time is ripe for microbiologists to discover appropriate methodologies of delivery suitable for mechanization.

Efficient plant-growth-promoting rhizobacteria for different cropping systems have been identified, but the constraint may be in application for plantation and horticultural crops. Education of farmers and extension workers in proper storage and treatment of different biofertilizers is crucial for their success in the field.

Indian agriculture, mechanized or not, cannot afford to do away with biofertilizers. The future is bright, and biofertilizers have to perform in the field.

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Delivery Systems for Introduction of Microbial Inoculants in the Field

13

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Abstract

Undoubtedly choosing correct microbial inoculants is the foremost factor governing the success of a biocontrol program. But making it reach to the field with a suitable delivery method maintaining consistent performance is the next most important challenge. Microbial inoculants are delivered through several means based on the survival nature and mode of infection of the pathogens. These bioagents cannot be applied as spore suspension in field but are applied as powdered or liquid formulation primarily through seed treatment, soil application, root dip, or foliar application. Application of microbial inoculants can influence, at least temporarily, the resident microbial communities and offer protection against a wide range of pathogens. The biocontrol agent applied through different delivery methods multiplies in the soil and remains near the root zone of plants and offers protection even at later stages of crop growth. In this chapter, we have discussed about various microbial bioformulations commercially available and their mode of application in the field. Along with conventional methods of delivery system, other methods such as microbigation, seed biopriming, seed encapsulation, fluid drilling, and consortia method of application are discussed with recent research updates.

Keywords

Microbes • Inoculants • Seed treatment • Biopriming • Microbigation

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13.1 Introduction

Plant diseases are caused by various biotic and abiotic factors viz. fungi, bacteria, viruses, viroids, phanerogamic parasites, protozoans, and nematodes are taking heavy toll of crops. These pathogens are causing substantial losses in differ-

D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_13

ent crops and therefore need to be managed. Several chemicals have been used in the past to manage the diseases caused by various pathogens. No doubt, some degree of control was achieved, but it posed new problems of residual toxicity and development of resistant strains of the pathogens. Delivery of microbial inoculants is being a very attractive option since it would substantially reduce the use of agrochemicals (Berg 2009). Microorganisms play a vital role in cropping systems, particularly plant-growth-promoting microorganisms (PGPMs). Soil or seed inoculation with microbial inoculants may lead to changes in the structure of the indigenous microbial population, which is important with regard to the safety of introduction of microbes in plant microenvironment (Trabelsi and Mhamdi 2013). Many reports indicate that the application of microbial inoculants can influence, at least temporarily, the resident microbial communities; therefore, screening of biocontrol agents (BCAs) with broad-spectrum activity and capacity to elicit systemic resistance in plants and offer protection against a wide range of pathogens needs to be done. Success in the identification of new microbial inoculants that exhibit significant control of various root and foliar diseases in the past few decades has contributed to the rising interest in the biological control of various phytopathogens. Further, an in-depth study of the action of BCAs is needed before using them on a large scale. End number of formulations approved by regulatory authorities around the globe are available for use in disease management. Therefore, biocontrol agents or antagonists as a means of plant disease control has gained importance in recent years. The biocontrol agent multiplies in the soil and remains near the root zone of plants and offer protection even at later stages of crop growth. The antagonistic activity of biocontrol agents against plant pathogens is highly specific against a particular pathogen and/or different races of the pathogen. Delivery system of BCAs mainly depends on the type of pathogen to be managed, the stage of the crop to be protected, the nature and severity of the disease, and the climatic conditions of the region (Desai et al. 2000). For application of a good formulation, a proper delivery method of microbial inoculants is essential. Since bioformulation incorporated in the soil have high densities of viable and efficient microbes for a rapid colonization of host rhizosphere, it may induce at least a transient perturbation of the equilibrium of soil microbial community. However, a modification in the microbial community structure caused by inoculation could be buffered by ecosystem resilience, which is driven by the level of diversity and interactions of the plant–soil biota (Kennedy 1999).

Seed treatment is a practical method of delivery system for both fungal and bacterial biocontrol agents. Biological control agents applied to seed have been shown to protect the seed against many seed-borne pathogens of crops, as well as increase plant growth and vigor (Jambhulkar and Sharma 2013). The use of biological agents as seed treatment is a valuable and an equally effective protection as chemical seed treatment. Physiological seed treatment such as seed priming has been used to quicken seed germination and improve the survival of seedlings (Burelle 2000). Bioformulation may directly be applied to plant roots in the form of root dip, spray, drip, or flood application for the management of soil-borne pathogens (Gasic and Tanovic 2013). Commercialization of good biocontrol agents becomes difficult due to impractical dosage recommendations, limited or inconsistent control efficacy, and improper delivery system. A better understanding of the ecological and epidemiological relationship between microorganisms and suitable delivery systems that will carry fungal strains with enhanced fungicide resistance will help to reduce the gap between experimental results and commercial use of biopesticides. Developing accurate delivery system is foremost important to assure effectiveness of bioformulation under field conditions. Unlike agrochemicals, in which chemical is dissolved in a solvent, most microbial inoculants are particulate suspensions. Problems with suspensions include settling of the microbial pesticides, nozzle blockages, stress affecting the viability of spores, inappropriate droplet size, large number of infective spores packed to a droplet, etc. (Bateman et al. 2007). Researches are in progress to optimize the delivery system for each group of biopesticides. The application of

fluorescent pseudomonads by seed treatment (Niranjana et al. 2009), seedling root dip (Verma 2009), and soil drenching (Jeyalakshmi et al. 2010) has been attempted my many workers to control phytopathogens in various crops. Jambhulkar and Sharma 2013 reported the efficacy of various carrier formulations of *Pseudomonas fluorescens* through seed treatment, seedling root dip and soil drenching in unity and in combination. Work on droplet size revealed that smaller droplets would enhance effectiveness of microbial inoculants (Alves and Bateman 2013), whereas larger droplets are suitable for entomopathogenic nematodes (Bateman et al. 2007). Stable, effective formulations and appropriate delivery system are needed to convince farmers to adopt bioformulations.

13.2 Microbial Inoculants as Biocontrol Agents

Indiscriminate use of chemical compounds as pesticides damages the entire agroecosystem, which encourages the use of biopesticides. In fact, there is great potential of biopesticides in organic and conventional agriculture. Biological control by antagonistic microbial inoculants is a potential nonchemical means for crop protection, which is seen as a very attractive plant protection measure as it would substantially reduce the use of chemical pesticides and fungicides, and there are now an increasing number of inoculants being commercialized for various crops (Berg 2009). There is immense role of microorganisms in agricultural ecosystem, particularly plant-growth-promoting microorganisms (PGPMs). Plant growth benefits are mainly attributed to three major mechanisms: (1) PGPMs acting as biofertilizers (such as nitrogen-fixing bacteria and phosphate-solubilizing bacteria) assist uptake of plant nutrients by providing fixed nitrogen and other nutrients; (2) phytostimulators (microbes expressing phytohormones such as *Azospirillum*) can directly promote the growth of plants usually by producing phytohormones, and (3) biological control agents (such as *Trichoderma, Pseudomonas*, *Bacillus*, etc.) protect plants against phytopathogenic organisms (Mohiddin et al. 2010; Dawar et al. 2010). Microbial inoculants have various benefits over chemical pesticides: they (a) are more safe, (b) show reduced environmental damage, (c) show more targeted activity, (d) are effective in smaller quantities, (e) are able to multiply but are also controlled by the plant and indigenous microbes, (f) have quicker decomposition procedures, (g) are less likely to induce resistance by the pathogens and pests, and finally (g) can be used either in organic and conventional agriculture (Berg 2009).

Bacillus is a genus of bacteria known to elicit induced systemic resistance (ISR) in plants. In addition, *Bacillus* spp. have reduced incidence of viral diseases, for example, cucumber mosaic virus on tomato. On plants that are not challenged with pathogens, it has been reported that *Bacillus* can increase fresh weight and number of fruits and flowers (Kloepper et al. 2004). *Pseudomonas*, a genus of bacteria that can colonize plant roots and suppress pathogens through the production of antibiotics, is a genus that can elicit ISR as well (Kloepper et al. 2004). Bacteria in this genus have a strong potential as biocontrol and growth-promoting agents due to the following characteristics: a) rapid growth in vitro; b) rapid utilization of seed and root exudates; c) ability to colonize and multiply in the rhizosphere and the spermosphere, as well as inside the plants; d) production of metabolites like antibiotics, siderophores, and growth promoters; e) competition with other microorganisms; and, finally, f) ability to adapt to environmental stress (Weller 2007). In early attempts, products made of this *Bacillus* spp. failed due to the instability of the culture and lack of long-term viability (Kloepper et al. 2004). It is known that the majority of bacteria that promote plant growth are rhizosphere inhabitants; they have been designated as plant-growth-promoting rhizobacteria (PGPR). The most promising group of PGPR for biocontrol of plant diseases is fluorescent pseudomonads. Fluorescent pseudomonads associated with plants include *Pseudomonas fluorescens*, *P. putida*, *P. aeruginosa*, and *P. aureofaciens*. Plant-growth-promoting rhizobacteria may stimulate the production of biochemical compounds associated with host defense; mas-

sive accumulation of phytoalexins and phenolic compounds; increase in the activities of PR proteins, defense enzymes, and transcripts; and enhanced lignification. The induction of SAR using various ISR inducers has been of recent interest with quite reasonable success (Meena 2014).

Another group of microbes corresponds to some types of algae that have biotechnological potential as soil fertilizers for plant production and some macroscopic marine algae (*Eklonia maxima*) that improve the growth and yield of plants (Reisser 2010; Crouch and van Staden 1992). Finally, several genera and species of PGPR and different microbes are used as inoculants; the diversity represents an opportunity to start research in this area and provide new solutions for the current necessities of agriculture.

Soil microorganisms form a very complex and dynamic community between different compartments and levels. Microorganisms can survive in the spermosphere, a zone influenced by the seeds, which is full of nutrients that support their growth. In addition, they can inhabit the phyllosphere, a zone that comprises the above-ground parts of the plants, whose most relevant characteristic is the fact that it is in constant fluctuation related to external facts as temperature, radiation, and water availability. Other zones correspond to the vascular tissue, the rhizosphere, and the endophytic sites. Several strains of *Trichoderma* have been described as antagonistic fungi that are able to attack a wide range of phytopathogenic fungi. The production and secretion of fungal-cell-wall-degrading enzymes and compounds affecting the integrity of fungal membrane and cell walls are considered as the key steps in the antagonistic process by *Trichoderma* (Chet et al. 1998). Antagonism may be accomplished by competition, parasitism, and antibiotics or by a combination of these. Parasitism involves the production of several hydrolytic enzymes that degrades cell wall of pathogenic fungi. β-1,3 glucanase and chitinase are the key enzymes responsible for fungal cell and sclerotial cell wall lysis. These enzymes are produced by several fungi and bacteria and may be an important factor in biological control. Moreover, in the rhizosphere, region that includes plant roots and surrounding soil, intensive interactions between plants, soil, and microfauna take place due to its high energy and carbon content. This accumulation in the rhizosphere corresponds to all the compounds produced by plant roots, most of which are organic derived from photosynthesis and other plant processes (Pinton et al. 2001). Different and varied biochemical signal exchanges take place between these communities and their host plants; indeed, a wide diversity of bacteria and plant-associated microbes can interact with plants in a beneficial way, either by enhancing their growth and/or controlling phytopathogens (Beattie 2006; Nihorimbere et al. 2011).

13.3 Formulations Used for Inoculation of Microbial Inoculants

Development of a bioformulation is necessary to commercialize biocontrol technologies by industries. The commercial use of plant-growth-promoting rhizobacteria requires inoculum that retains high cell viability and can easily be transported and applied to seed. It needs extensive studies for large-scale multiplication of a biocontrol agent (BCA), which include suitable and inexpensive medium, method of fermentation (solid or liquid), type of formulation (wettable powder, liquid, granular), nature of filler material, delivery systems, optimum shelf life, and storage conditions. Application guidelines are set by considering all these aspects of a bioformulation. Delivery of free cell form is usually impractical to achieve satisfactory bioremediative effect because microbes are encumbered by biotic and abiotic stresses from the environment (Ting et al. 2010). The aims of formulating viable cells are to ensure that adequate cell viability is sustained to increase the efficacy of the cells and to facilitate the delivery and handling processes (Filho et al. 2001). A bioformulation can improve product stability and shelf life and also protect microbial inoculants against different environmental conditions and provide initial food source (Jambhulkar and Sharma 2014). Application of microbial

inoculants either to increase crop health or to manage plant diseases depends on the development of commercial formulations with suitable carriers that support the survival of microorganism for considerable length of time (Aeron et al. 2011). A formulated microbial product is a product composed of one or more biological control agents mixed with ingredients that will improve its survival and effectiveness (Schisler et al. 2004). Those microbial inoculants formulated for delivery to soil are of especial importance due to their specific court of action, which is the rhizosphere. Microbial inoculants can be applied to the soil as fluid suspensions, as powder formulations, and as granules for soil and spray application. Fluid suspensions are prepared based on culture concentrates diluted in water or a buffer solution prior to application. They can also be prepared as dormant aqueous suspensions, obtained after harvesting the bacteria from a liquid culture, washed free of the spent medium and stored at a specific concentration in sterile water at room temperature (Miranda 2012). Microbial inoculants are formulated as dry formulation for direct application dusts (DP), seed dressing formulations-powders for seed dressing (DS), granules (GR), microgranules (MG), dry formulations for dilution in water-water dispersal granules (WG), and wettable powders (WP); liquid formulations for dilution in water emulsions, suspension concentrates (SC), oil dispersion (OD), suspoemulsion (SE), capsule suspension (CS), ultra low volume formulations (Knowles 2005, 2006). Powdered formulations are more commonly used. They consist of organisms concentrated into dry or wet powders. Depending on the composition of the powders, they can be applied directly to the soil, suspended in water, or dusted onto seeds. The commonest method to formulate granular products is to mix the organism and the ingredients with the granules (Burges 1998). In general, product formed from solid or semi solid-state fermentation does not require sophisticated formulation procedures prior to use. For example, grain or other types of organic matter upon which antagonists are grown are simply dried ground and added to the area to be treated. There are several problems with solid-state fermentation, which may make the system inappropriate for commercial product development. The preparations are bulky, they may be subject to a greater risk of contamination, and they may require extensive space for processing, incubation, and storage. The liquid-state fermentation is devoid of such problems, and large quantities of biomass can be produced within a few days. Either the biomass can be separated from medium and concentrated or the entire biomass with medium can be incorporated into dusts, granules, pellets, wettable powders, or emulsifiable liquids. The carrier material may be inert or a food base or a combination of both. Inoculant formulations depend directly on the carrier used for the delivery of the products because target microorganisms are mixed with it before being applied. Carriers are the inert ingredients that hold or dilute the microorganism to the desired concentration and improve coverage and distribution (Burges 1998). Commercial application of PGPR either to increase crop health or to manage plant diseases depends on the development of commercial formulations with suitable carriers that support the survival of bacteria for a considerable length of time. Carriers constitute the key for the effective release of the different products; they need to be effectively chosen due to their diversity (e.g., water, vermiculite, calcium sulphate, mineral soil and sand, vegetable oil, corn cob) that starts from classic ones to new and unconventional ones (Bashan 1998; Burges 1998). Certain specific conditions might increase the efficacy of a formulation. Addition of organic acids to *T. koningii* formulations and polysaccharides and polyhydroxyl alcohols to *T. harzianum* increases the activity of BCAs (Connick et al. 1991). The carrier represents the principal portion of inoculants. The materials from which they are made define their effectiveness. Moreover, they have to fill certain requirements in order to be efficient. First, they need to have the capacity to deliver the correct concentration of viable cells at the time they are needed. The reason is because there are certain ranges of concentrations that can be inoculated in certain crops. In addition, as inoculants should be sterile; carriers should be chemically consistent and able to provide enough

water-holding capacity for microbial growth (Bashan 1998). Moreover, carriers need to be easily available and able to be mixed with other compounds like nutrients in order to provide a good environment for the live cells. In fact, they need to be easy to mix and easy to fabricate as they are intended to be used massively. Furthermore, they need to be easy to handle and have longer shelf life because they will be used by farmers who will use them periodically and will need to have reservoirs of the products for rapid use (Bashan 1998). Foliar application of fluorescent pseudomonads was attempted by few workers (Gnanamanickam and Mew 1992; Bahadur et al. 2007; Prathuangwong et al. 2013), all of whom used bacterial cell suspension for seed treatment, soil application, or foliar sprays. Use of bacterial suspensions is impractical for large-scale application to control foliar diseases in the field. A powder formulation with longer shelf life would be beneficial (Tables 13.1 and 13.2).

13.4 Delivery Systems

Plant-growth-promoting rhizobacteria are delivered through several means based on the survival nature and mode of infection of the pathogen. It is generally delivered through seed treatment, root dip, soil application, and irrigation water. An ideal formulation is expected to facilitate the delivery of the living biocontrol agents in its active state, at the right place, at the right time. While the formulated microbial products must be effective at the site of action and compatible with agronomic practices, they should be easy to apply to and adhere to plant parts such as seeds, tubers, cuttings, seedlings, transplants, and mature plants or be available in the soil medium.

13.4.1 Seed Treatment

Biological formulations applied to seeds greatly help to deliver the agents to the spermosphere of plants, where, in general, extremely conducive environments prevail. The BCAs are therefore provided an excellent opportunity to survive, multiply, persist, and exercise control of soil-borne phytopathogens (Cook and Baker 1983). Seed treatment has the potential to deliver microbial agents "in the right amount, at the right place and at the right time." With increasing public awareness of the potential environmental and health hazards of both agrochemicals and the advances in biotechnology to improve the performance in microbial products, the application of microbial inoculants to seeds (Chandra and Greep 2010; Chandra et al. 2006) is likely to increase in the future. With an aim to deliver the active ingredients as close to the target as possible, this approach continues to receive considerable attention from end users. Significant advances in seed treatment technology has been achieved due to consistent work done around the globe, and this approach is an attractive means for introducing biological control agents into the soil–plant environment, as these introduced organisms are offered the selective advantage to be the first colonizers of plant roots. At the time of planting seedlings, the formulated products can be used directly (powders, liquids) without stickers. Powders for seed treatment are formulated by mixing an active ingredient, inert carrier to facilitate product adherence to seeds by mixing seeds with formulated product (Woods 2003). Additives such as gum arabic and xanthan gum are used to prolong the survival of microbial agents applied to seeds. Alginate hydrogel, used as a seed encapsulation material, maintains the entity in a viable state and protects it from other stresses. Seed priming, in which seeds are mixed with an organic carrier and then moisture content, is brought to a level just below that required for seed treatment which has been used to deliver *T. harzianum* to control *Pythium*-induced damping-off on cucumber (Callan et al. 1990). In another process of seed treatment, an industrial film-coating process which was developed for the application of chemicals and biological crop protection agents is being utilized for the application of *Trichoderma* spp. on radish and cucumber seeds through a film coating and was shown to be effective against damping-off (Cliquet and Scheffer 1997). Prathuangwong et al. (2013)

Table 13.1 Commercial formulations of biocontrol agents available in India

Product	Bioagent(s)	Target organism	Delivery system	Developing agency
Antagon-TV	*T. viride*	*R. solani, Macrophomina phaseolina*	Seed treatment, soil application	Green Tech Agro Products, Coimbatore
Biocon	*P. fluorescens*	Bacterial wilt and rot diseases	Spray	Tockalai Experimental Station, Tea Research Association, Jorhat, ASSAM
Bioguard	*T. viride*	*Fusarium*	Spray	Krishi Rasayan Export Pvt. Ltd. Solan (HP)
Bioshield	*Pseudomonas fluorescens*	*Fusarium, Rhizoctonia, Pythium, Colletotrichum, Phytophthora*	Seed treatment, spray	POABS Biotech, Kuttoor, Kerala
Biotik	*Metarhizium anisopliae*	Termites, red ants, root grubs, grasshoppers	Seed treatment, spray, soil application	SS Biotech Guwahati Assam
Ecoderma	*T. viride*	*Fusarium, Rhizoctonia, Pythium, Phytophthora*	Seed treatment, drenching, soil application, seedling dip	Margo Biocontrol Pvt. Ltd., Bangalore
Bioderma	*T. viride*+ *T. harzianum*	*Pythium, Rhizoctonia, Phytophthora, Fusarium*	Seed treatment and spray	Biotech International Ltd., New Delhi, India
Ecofit	*Trichoderma viride*	*R. solani, Macrophomina phaseolina*	Seed treatment	Hoechast and Schering AgrEvo Ltd., Mumbai
Funginil	*T. harzianum*	*Botrytis, Pythium, Fusarium, Macrophomina, Rhizoctonia, Sclerotinia*	Seed treatment, soil application	Crop Health Bioproduct Research Centre, Gaziabad
Kalisena SD Kalisena SL	*Aspergillus niger* AN-27	*Pythium, Fusarium, Macrophomina, Rhizoctonia, Sclerotinia*	Seed treatment, foliar spray, soil application	Cadila Pharmaceuticals Limited, Ahmedabad
Pant Biocontrol Agent-1 (Biowilt-X)	*T. harzianum*	*Pythium, Fusarium, Macrophomina, Rhizoctonia, Sclerotinia*	Seed treatment, soil application	G. B. Pant University of Agriculture Technology, Pantnagar
Pant Biocontrol Agent-2	*Pseudomonas fluorescens*	*Fusarium*	Seed treatment, soil application	G. B. Pant University of Agriculture Technology, Pantnagar
Pusa Th3	*Trichoderma harzianum*	*Fusarium, Rhizoctonia, Sclerotium*	Seed treatment, soil application	Div. of Plant Pathology, IARI, Pusa, New Delhi
Sun Agro Derma Sun Agro Derma H	*T. viride* *T. harzianum*	*Fusarium, R. solani, Macrophomina phaseolina, Colletotrichum*	Seed treatment, seedling root dip, soil application	Sun Agro Chemicals, Chennai
Tricho-X	*T. viride*	*Fusarium, Rhizoctonia, Sclerotium, Pythium*	Seed treatment, foliar spray, soil application	Excel Industries Limited, Mumbai
Trichostar	*T. harzianum*	*Fusarium, Rhizoctonia, Sclerotium, Pythium*	Seed treatment	GBPUAT, Pantnagar
Gliostar	*Gliocladium.* spp.	*Fusarium, Rhizoctonia, Sclerotium, Pythium*	Seed treatment, drenching	GBPUAT, Pantnagar

(continued)

Table 13.1 (continued)

Product	Bioagent(s)	Target organism	Delivery system	Developing agency
Monitor	*Trichoderma* sp.	*Fusarium, Rhizoctonia, Sclerotium, Pythium*	Seed treatment and spray	Agricultural and Biotech Pvt. Ltd. Gujrat
Trichoderma	*Trichoderma* sp.	*Fusarium, Rhizoctonia, Sclerotium, Pythium*	Seed treatment	Innovative Pest control Lab, Bangalore
Phule Trichokill	*Trichoderma* sp.	*Fusarium, Rhizoctonia, Sclerotium*	Seed treatment	Department of Plant Pathology, MPKV, Rahuri
Biowilt-X Bionem-X Biocomp-X	*T. harzianum* *Pochonia chlamydosporia* *P. fluorescens*	*Fusarium oxysporum* f.sp. *ciceris* and *F. udum*, *Meloidogyne incognita*, and wilt disease complex (*Fusarium* + *Melodiogyne*)	Seed treatment	Dept. of Plant Pathology, AMU, Aligarh
Soil Guard	*T. viride*	*Fusarium, Rhizoctonia, Pythium, Colletotrichum, Phytophthora*	Seed treatment, soil application	POABS Biotech, Kuttoor, Kerala
Myco-Jaal	*Beauveria bassiana*	Diamond black moth	Spray	Pest Control of India, Bangalore

applied kaolin-based formulation of *Pseudomonas fluorescens* SP007s as seed treatment and spray to reduce fungal population in rice plants.

13.4.2 Seed Biopriming

Biopriming, a seed treatment system that integrates the biological and physiological aspects of disease control, involves coating the seed with fungal or bacterial biocontrol agents (El-Mougy and Abdel Kader 2008). It is a method of treating seeds with microbial inoculants and incubating under warm and moist conditions until just prior to radical emergence. Priming is one of the simple techniques which improve the vigor, seedling establishment, and plant efficiency in the field. There are three main large-scale priming approaches using different methods to regulate water potential, which are quite popular in European countries: (1) Osmoconditioning: seeds are incubated in an aerated solution of an osmoticum such as polyethylene glycol, or an inorganic salt such as potassium nitrate or phosphate, using high liquid–seed ratio (e.g., 10:1) in stirred bioreactors of various designs. At the end of the process, seeds are rinsed before further processing. (2) Solid-matrix priming technique: seeds are mixed with equivalent quantity of friable, nonclumping, inert material, e.g., a carbonaceous, preferably ligneous shale or coal, with osmotic component at least 90 % of the equilibrium water potential, moistened sufficiently to equilibrate seeds to the correct water content. Extraneous solid material is sieved off after incubation. (3) Basic priming method: incubate damp seeds and bring the seed directly to predetermined moisture content by various means, without using external matrix or osmotic agent to regulate seed water potential (McQuilken et al. 1998). Priming allows the early DNA transcription and RNA and protein synthase which repair the damaged parts of the seeds and reduce metabolic exudation (Entesari et al. 2013). These agents thus improve the seed germination charac-

Table 13.2 Commercial formulations of biocontrol agents available worldwide

Biocontrol agent	Product	Target disease/organism	Manufacturer	Delivery system
Agrobacterium radiobacter strain 84	Galtrol	*Agrobacterium tumefaciens*	AgroBioChem, USA	Spray
A. radiobacter strain 1026	Nagol	*Agrobacterium tumefaciens*	Bio-Care	Spray
Bacillus subtillus strain GB34	GB34	*Rhizoctonia, Pythium, Phytophthora, Fusarium*	Gustafon, USA	Drenching during sowing and transplanting
B. subtillus strain GB 03	Kodiac, companion	*Rhizoctonia, Aspergillus*	Growth Products, USA	Drenching during sowing and transplanting
Pseudomonas aureofaciens strain TX-1	Bio-jet, spot less	*Pythium, Rhizoctonia solani*	Eco Soil Systems	Overhead irrigation, can only be used with BioJet automatic fermentation system
Pseudomonas fluorescens A506	Frostban, Blightban A506	Fire blight, frost damage, bunch rot	Plant Health Technologies	Spray at blooming flower and fruiting
Streptomycine griseoviridis K61	Mycostop	Soil-borne pathogens	Kemira Agro Oy, Finland	Drenching, spraying, or through irrigation
Trichoderma harzianum T-22	Root shield or BioTrek T-22G	Soil-borne pathogens	BioWorks, Inc., USA	Granules mixed with soil or potting medium, powder mixed with water and added as soil drench
T. harzianum T-39	Trichodex	*Botrytis cinerea*\	BioWorks, Inc., USA	Spray
T. asperellum T34	T34 Biocontrol	*Fusarium oxysporum* f.sp. *dianthi*	Fargro Ltd., Littlehamptom, West Sussex, UK	Drenching during sowing and transplanting, root dip of cuttings
Ampelomyces quisqualis M-10	AQ10	Powdery mildew	Ecogen, USA	Spray
Aspergillus flavus AF 36	Alfa guard	*Aspergillus flavus*	Circle One Global, USA	Seed treatment, foliar spray, soil application
Gliocladium catenulatum strain JI446	Prima stop soil guard	Soil-borne pathogens	Kemira Agro Oy, Finland	Seed treatment, foliar spray, soil application
Trichoderma sp.	Bio-Fungus	*Sclerotinia, Phytophthora*, *R. solani, Pythium* spp., *Fusarium, Verticillium*	De Cuester, Belgium	Seed treatment, foliar spray, soil application
Candida oleophila	Aspire	*Botrytis* spp., *Penicillium* spp.	Ecogen, Inc., Langhorne, PA	Postharvest to fruit as drench, drip, or spray

(continued)

Table 13.2 (continued)

Biocontrol agent	Product	Target disease/organism	Manufacturer	Delivery system
T. harzianum (ATCC20476) and *T. polysporum* (ATCC20475)	Binab T	Wilt, tale-all, root rot	Bio-Innovation AB, Sweden Henry Doubleday Research Association, UK	Spray, mixing with potting substrate, as paste painting on tree wounds
Fusarium oxysporum (nonpathogenic)	Biofox C	*F. oxysporum, F. moniliforme*	SIAPA, Bologna, Italy	Seed treatment or soil incorporation
Pseudomonas syringae ESC-10	Bio-save 100 Bio-save 1000	*Botrytis cinerea, Penicillium* spp., *Mucor pyriformis, Geotrichum candidum*	EcoScience Corp, Orlando, Florida	Pellets, postharvest to fruit as drench dip or spray
P. syringae ESC-11	Bio-save 110	*Botrytis cinerea, Penicillium* spp., *Mucor pyriformis, Geotrichum candidum*	EcoScience Corp, Orlando, Florida	Pellets, postharvest to fruit as drench dip or spray
P. chlororaphis	Cedomon	Net blotch, stripe disease, *Fusarium* spp., spot blotch, leaf spots	BioAgri AB, Sweden	Seed dressing
P. fluorescens	Conquer	*Pseudomonas tolaasii*	Mauri Foods, Kittanning, PA	Spray
Coniothyrium minitans	Contans	*Sclerotinia sclerotiorum* and *S. minor*	Prophyta Biologischer Pflanzenschutz, Germany	Spray
Burkholderia cepacia	Deny	*Rhizoctonia, Pythium, Fusarium*	Stine Microbial Products	Seed treatment, aqueous suspension for drip irrigation
Bacillus subtilis	Epic	*R. solani, Fusarium* spp., *Alternaria, Aspergillus* spp.	Gustafson Inc., Dallas, TX	Added to slurry, mix with chemical fungicides for seed treatment
F. oxysporum (nonpathogenic)	Fusaclean	*F. oxysporum*	Natural Plant Protection, France	In drip to rock wool, incorporate in potting mix; in rows
Pseudomonas cepacia	Intercept	*R. solani, Fusarium spp., Pythium spp.*	Soil Technologies, Fairfield, IA	Seed treatment, foliar spray, soil application
Trichoderma spp.	Monitor SD	Soil-borne plant pathogens	M/s Agriland Biotech Pvt Ltd., Baroda, India	Seed dressing
Trichoderma spp.	Monitor WP	Soil-borne plant pathogens	M/s Agriland Biotech Pvt Ltd., Baroda, India	Soil application
Agrobacterium radiobacter	Nogall, Diegall	*Agrobacterium tumifaciens*	Bio-Care Technology Pvt. Ltd, Australia	Root dips
A. radiobacter K84	Norbac 84C	*Agrobacterium tumifaciens*	New BioProducts, Corvalis, OR	Root, stem, cutting dip, or slurry

(continued)

Table 13.2 (continued)

Biocontrol agent	Product	Target disease/organism	Manufacturer	Delivery system
Paecilomyces lilacinus	Bioact or Paecil	Various nematodes	Technological Innovation Corporation Pvt Ltd	Drenching
Pythium oligandrum	Polygandron	*Pythium ultimum*	Plant Production Institute, Slovak Republic	Seed treatment and soil incorporation
Gliocladium catenulatum	Primastop	*Pythium* spp., *R. solani, Botrytis* spp.	Kemira Agro Oy, Finland	Drenching and soil incorporation
Bacillus subtilis FZB24	Rhizo-Plus	*R. solani, Fusarium* spp., *Alternaria* spp., *Sclerotinia, Streptomyces scabies*	KFZB Biotechnik GmBH, Germany	Seed treatment, soil drenching, root dip application
T. harzianum	Root Pro	*R. solani, Fusarium* spp., *Alternaria* spp., *Sclerotium rolfsii*	Mycontrol Ltd., Israel	Mix with growing media at time of seeding or transplanting
B. subtilis	Serenade	*Pythium, Cercospora, Alternaria, Helmithosporium,* fire blight	AgraQuest Inc., Davis, CA	Spray
Gliocladium virens GL-21	SoilGard	Damping-off, root rot pathogens, *R. solani, Pythium* spp.	Thermo Trilogy, Columbia, MD	Granules incorporated in soil
B. subtilis GB03	System 3	Seedling pathogens	Helena Chemicals Co., Memphis, TN	Seed treatment
T. viride	Trieco	Soil-borne pathogens	Ecosense Labs Pvt. Ltd., Mumbai, India	Seed treatment, tuber or seed dressing, soil drenching
Trichoderma sp.	Trichoderma 2000	Soil-borne pathogens	Mycontrol Ltd., Israel	Seed treatment, tuber or seed dressing, soil drenching

teristics and the seedling emergence under unfavorable conditions and priming results in a stronger plant. Seed priming can improve the physiological responses and increase seed tolerance to environmental stress (Khan et al. 2008).

This technique is more useful over simple coating of seeds as it results in rapid and uniform seedling emergence. *Trichoderma* conidia germinate on seed surface and form a layer around bioprimed seeds. These bioprimed seeds tolerate adverse soil conditions better. Biopriming may also reduce the amount of biocontrol agents that is applied to seeds (Ramanujam et al. 2010). Enhancements of seed inoculation with biological agents in combination with priming which will stabilize the efficiency of biological agents have been reported by previous workers (Callan et al. 1990; Warren and Bennett 1999). Nayaka et al. (2008) bioprimed maize seeds with conidial suspension of *T. harzianum* for the control of *F. verticillioides* and fumonisins in maize. It was found that the pure culture of *T. harzianum* was more effective in reducing the *F. verticillioides* and fumonisin incidence, followed by talc formulation. Biopriming with microbial inoculants is potentially able to promote rapid and uniform seed germination and plant growth. Moeinzadeh et al. (2010) reported the application of UTPf76 and UTPf86 strains of *Pseudomonas fluorescens* on improving sunflower seed germination and promotion of seedling growth. These bioprimed strains enhanced seed factors such as germina-

tion index, germination percentage, germination rate and vigor index, and also seedling growth indices, including root length, shoot height, dry and wet weight of seedlings, and numbers of lateral roots. In biopriming, the selected strains were applied to the seed during osmopriming with NaCl.

13.4.3 Seed Encapsulation

The reproducible results following introduction of microbial inoculants into soil relies on the survival rate of the inoculated microbes in heterogenous soil environment, and it can be achieved by improved encapsulation technique (Young et al. 2006). It is a specialized seed-coating process which involves enveloping the seed, microbes, and possibly few other components such as pesticides or micronutrients, in a gelatinous or polymer gel matrix, thereby prolonging the survival of microbial inoculants on seed. A gel-encapsulation system developed with hard alginate prill to coat or pellet seeds for making formulation of biocontrol fungi (Lumsden and Lewis 1989). The gel-like matrix allows the cell to remain viable with its catalytic ability for longer duration. Alginate forms microbeads immediately in the presence of polyvalent cations by binding the cation to guluronic acid units (Witter 1996) in a single step with a sufficient mechanical strength. Digat (1991) has patented a process to produce granules of up to about 8-mm diameter, with a core containing liquid microbial inoculants and an outer protective coating layer. GEL COAT is an example of seed encapsulation which is an alginate hydrogel product patented as a delivery system for entomopathogenic nematodes (Boyetchko et al. 1999). This method of delivery system has a distinct advantage of being user friendly and environmentally safe, since the active ingredients are effectively sealed until they are released during germination. Major factors that need to be taken care of while adopting this technique are seed inoculum density, coating stability, both for microbes viability and coat integrity, in association with user feasibility and cost of production. *Pseudomonas putida* CC-FR2-4 and *Bacillus subtilis* CC-pg104 encapsulated in alginate supplemented with humic acid and inoculated to *Lactuca sativa* L. seedlings and observed significant plant growth by Rekha et al. (2007). Encapsulation of *Bacillus megaterium* was attempted by Sivakumar et al. (2014), with bacterial alginate by enriching the bead microenvironment with humic acid, and high viability of encapsulated bacteria with minimum cell loss after 5 months of storage was observed, thereby achieving successful plant-growth promotion of rice seedlings. This novel technique clearly demonstrates that inoculation of encapsulated microbial inoculants promotes plant growth and is feasible for application in agricultural industry.

13.5 Soil Application

Soil treatment is preferred when biocontrol agents are too sensitive to desiccation (Warrior et al. 2002). The biocontrol agent (BCA) establishes a high population in the soil, making them suppressive to the disease. Niche exclusion also becomes operative in such cases, as the increase in number of the introduced microbes renders essential nutrients unavailable to soil pathogens and other less beneficial microflora (Lumsden et al. 1995). Soil acts as repertoire of both beneficial and pathogenic microbes; delivering of microbial inoculants to soil will increase the population dynamics of augmented bacterial antagonists and thereby suppress the establishment of pathogenic microbes on to the infection court. Many species of *Trichoderma* have also been formulated extensively, using cellulosic carriers and binders and modern thin-film coating techniques, in an attempt to introduce them into the rhizosphere regions of seedlings to protect them from diseases such as *Rhizoctonia solani* and *Pythium ultimum*. However, the major limitation of fungi as seed coatings remains; so they do not colonize the rhizosphere as readily as the bacterial agents (Warrior et al. 2002). Numerous attempts have been made to control several soil-borne pathogens by incor-

porating natural substrates colonized by antagonists of pathogen into soil (Sesan and Csep 1992). Though drenching of soil with aqueous suspensions of bioagent propagules was carried out, there will not be any even distribution of bioagents in the soil. Bankole and Adebanjo (1998) reported that soil drenching with suspension of *T. viride* was very effective in reducing infection from cow pea seeds infected with *Colletotrichum truncatum* (brown blotch). Soil drenching with *T. harzianum* has given good control of stem rot of groundnut caused by *S. rolfsii* (Kulkarni and Anahosur 1994). An aqueous drench containing conidia of *T. harzianum* controlled wilt of chrysanthemum by preventing reinvasion by *F. oxysporum*.

Weststeijn (1990) found that root rot in tulip caused by *P. ultimum* was reduced by mixing *Pseudomonas* suspensions thoroughly through the soil to a concentration of 10^8 cells per gram dry soil before planting the bulbs. Wilt disease of sunflower was found to be suppressed when *P. cepacia* strain N24 was applied to the seedbeds at the rate of 500 ml per m^2 under greenhouse conditions (Hebber et al. 1991).

A technique of enrichment of farmyard manure (FYM) with *Trichoderma* culture for soil and nursery bed application is widely accepted and appreciated by farmers for soil treatment against soil-borne pathogens. This technique involves less labor and time to multiply *Trichoderma* culture to manifold for soil application. Vidhyasekaran and Muthamilan (1995) stated that soil application of peat-based formulation of *P. fluorescens* (Pf1) at the rate of 2.5 Kg of formulation mixed with 25 Kg of well-decomposed farm yard manure, in combination with seed treatment, increased rhizosphere colonization of Pf1 and suppressed chickpea wilt caused by *Fusarium oxysporum* f.sp. *ciceris*. Application of *Trichoderma harzianum* Th3-enriched farm yard manure in soil, along with seed treatment, before sowing of chickpea to ward off against root rot caused by *Rhizoctonia solani* exceptionally reduce the disease and increased yield (Jambhulkar et al. 2015).

13.6 Foliar Application

Liquid formulations are being commonly applied on foliar parts of the plants for control of foliar pathogens. The efficacy of the foliar application mainly governs by the microclimate of the crop canopy. The crop canopy has varied concentration of nutrients like amino acids, organic acids, and sugars exuded through stomata, lenticels, hydathodes, and wounds. It affects the efficacy and survival of antagonists in phylloplane.

Kelly Cartwright (1995) reported that three spray applications of *Pseudomonas cepacia* to cuttings during a 2-week period were more effective than either one or two bacterial sprays in the control of *Rhizoctonia* stem rot of poinsettia. Rice blast (*P. oryzae*) can be effectively controlled by foliar spray of talc-based powder formulation of *P. fluorescens* strain Pf1 (1 kg ha^{-1}). The effectiveness of spraying persisted up to 2 weeks. When the bacterial product was sprayed on plants grown from treated seed, the effectiveness was higher than when spraying was carried out without any prior seed treatment (Vidhyasekaran et al. 1997). Foliar application of *Pseudomonas chlororaphis* (PA-23), *Bacillus amyloliquifaciens* (BS6), *Pseudomonas* sp (DF41), and *B. amyloliquifaciens* (E16) was found very effective against causal agent of stem rot of canola, *Sclerotinia sclerotiorum* in field (Fernando et al. 2007). *Trichoderma* species can be applied as foliar spray to control diseases affecting above-ground parts. Biological control of foliar diseases is not so developed as biocontrol of soil-borne diseases. The reasons for the paucity of examples of biocontrol of foliar diseases may be the availability of cheap and effective chemical fungicides and the ease of application to the foliage, and results obtained with biocontrol agents were not so good as those obtained with common fungicides (Elad and Kirshner 1992).

The dosage and frequency of application have to be standardized based on the crop value, which could be a reliable and practical approach. Selected strains from many genera

of bacteria isolated from these suppressive soils have the potential to reduce plant diseases when applied to the plant root environment (Weller et al. 2002). Today liquid bioformulations with high potency, cost-effective with good suspension properties, and good stability are available and being successfully adopted globally. Additives are important for application in monocots which facilitates adhesion of microorganisms on plant tissues. Additives such as stickers, spreaders, adjuvants, and emulsifiers in foliar sprays facilitate adhesion of microorganisms on plant tissues (Harvey 1991).

13.7 Root Dip

The nature of pathogen may be seed borne or soil borne; it may establish host parasite relationships by entering through the root. Hence, protection of the rhizosphere region by prior colonization with PGPR will prevent the establishment of host–parasite relationship. Seedling roots can be treated with spore or cell suspension of antagonists either by drenching the bioagents in nursery bed or by dipping roots in microbial inoculant suspension before transplanting. This method is suitable for the vegetable crops and rice where transplanting is practiced (Singh and Zaidi 2002). In an experiment, Jambhulkar and Sharma (2013) dipped paddy seedlings in suspension of talc-based bioformulation of *Pseudomonas fluorescens* for 2 h before transplanting, and it showed reduction in bacterial leaf blight of rice. Similarly, dipping of rice seedlings in talc-based formulation of *P. fluorescens* (PfALR1) prior to transplanting reduced sheath blight severity and increased yield in Tamil Nadu, India (Rabindran and Vidhyasekaran 1996). Nandakumar et al. (2001) reported that *P. fluorescens* strain mixtures by dipping the rice seedlings in bundles in water containing talc-based formulation of strain mixtures (20 g/l) for 2 h and later transplanting it to the main field suppressed sheath blight incidence.

13.8 Fluid Drilling Technology

Fluid drilling, also referred to as fluid sowing or gel seeding, is the technology of sowing seeds that have been germinated, using a gel to suspend and transfer them to the seedbed. This delivery system involves the incorporation of biocontrol agents into fluid drill gels. The major advantage of sowing germinated seed compared to dry seed is earlier and more uniform emergence. The gel protects the exposed radicle from mechanical damage and also provides the growing seedling with an initial water source. Unfortunately, the gel tends to attract microorganisms, including soilborne pathogens, which may result in an increased incidence of disease. Conway 1986 has used fungicides as adjuvant to the gel matrix to decrease damping-off disease caused by *R. solani* in chili peppers. Fluid drilling offers an ideal system for the delivery of a biocontrol agent such as *Trichoderma* for the control of soil-borne disease problems (Fisher et al. 1983). In one study, vegetable or fruit tree seedlings were dipped into gels incorporated with antagonists so that the root area was surrounded by a thin layer of gel before the seedlings were planted. Fluid-drilling gels have been used to deliver *T. harzianum* for the control of *R. solani* and *S. rolfsii* on apple (Conway 1986). This innovative approach, utilizing the benefits derived from fluid drill technology, offers considerable promise for the formulation and application of biocontrol microorganisms. But in future, the technique of fluid drilling will be successful only if sowing of primed seeds rather than germinated seeds are used in carrier gel (Pill 1991). The positional advantage due to additive incorporation in the fluid-drilling gel shows an efficient, cost-effective, and environmentally sound application method for bioagents.

13.9 Microbigation

Applying microbial biocontrol agents to control weeds, soil pathogens, and soil insect through drip irrigation system is called “microbigation” (Boari et al. 2008). The uniform and precise

application of microbial particles close to the target organism and to the plant to be protected can increase the success of a biological control treatment. To make acceptability of biocontrol application among farmers, use of systems or technologies that are usually available in agriculture can be modified and enlarge the market. An exploratory drip irrigation system was carried out by Boari et al. 2013, using dripper lines, drippers, filters, and other tools commonly used in irrigation and precision agriculture in the greenhouse to evaluate their suitability for applying microbial biocontrol agents. Conidial suspensions of marketed or marketable agents were used, i.e., *Fusarium oxysporum, F. solani*, *T. harzianum*, and *Paecilomyces lilacinus*. They demonstrated that conidial suspensions (10^6 conidia ml^{-1}) can pass through the drippers without causing clogging, regardless of their size, and remained viable. A further advantage could be the limitation of the applied doses to the crop root zone and not the whole field, and therefore a reduction of the costs for treatment. Several biocontrol agents could be applied at the soil level through this system, such as mycoherbicides (Charudattan 2001), antagonists (Whipps and Lumsden 2001), and biopesticides (Copping 1999). They can be applied at plant transplanting or through soil drenching or root dip (Alabouvette et al. 1993).

13.10 Coaggregation Assay

Microbial inoculant formulation has a very important effect in the inoculation process as it determines the potential of the bioagents (Bashan et al. 1984). Poor performance of bioformulations in agriculture was reviewed by Van Veen et al. (1997), and suggested to use multiple microbial consortia for multipronged attack against phytopathogens and to thrive together in unique ecological niches in ideal proportions, instead of using a single strain, for a single trait. Coaggregation is a bacteria–bacteria, fungus–fungus, or fungus–bacteria interaction, and the interactions are highly specific that only few or certain species are consortial partners.

Coaggregation was first reported by Gibbons and Nygard (1970), who referred to it as interbacterial aggregation, and it was readily observed with naked eyes (Cisar et al. 1979). Coaggregation is effective only when equal numbers of partners are present and genetic stability of coaggregation is mediated by surface components that recognize a carbohydrate on the cell of the partner (Kolenbrander and Phucus 1984). Coaggregation has been reported earlier among certain bacterial species. Bougeu and Mc Bride (1976) and Kolenbrander and Phucus (1984) reported that *Actinomyces viscosus* T14V and *Streptococcus sanguis* 34 co-aggregated by a mechanism which is not inhibited by 1 M NaCl and is independent of dextran, requires calcium and pH in the range of 8.0 to 8.5. Recently, Sivakumar and Joe (2008) attempted coaggregation of *Azorhizobium caulinodans* with *A. brasilense*, *A. chroococcum*, *Bacillus megatherium*, and *Pseudomonas fluorescens* to develop coaggregates with multiple benefits using seed powders of many plants, viz., *Moringa oleifera, Strychnos potatorum*, and *Sappindus emaignatus*. There is wide scope of using coaggregates to deliver microbial inoculants for obtaining multiple benefits in different crops against various soil-borne pathogens. Studies on coaggregates open up the possibilities for further investigation of the genetic basis of effective coaggregation and also the nature of cellular mechanism.

13.11 Consortia Application

Judicious use of microbial inoculants as biocontrol agent (BCA) is a potentially important component of sustainable agriculture. The principal biocontrol mechanism involved includes mycoparasitism, antibiosis, competition, and induced resistance; additional mechanisms are hypovirulence mediated through fungal viruses and inhibition of enzymes involved in plant pathogenicity (Kapat et al. 1998). Individual biocontrol mechanism could be predominant for some BCAs, but there are also many instances where more than one mechanism may operate in a given BCA iso-

late. The ecological processes determining the fate of such biological control are complex. Thus, it is not surprising that, in addition to variable control efficacies, biocontrol success in field crops has been limited despite much research effort. Most biocontrol success has been achieved in greenhouse cultivation (Paulitz and Belanger 2001), where ecological parameters are less variable. Because of the inconsistent or limited biocontrol achieved in the field, BCAs have also been used in combination with fungicides or cultural practices (Shtienberg and Elad 1997).

Use of mixtures of cultivars (Mundt 2002) or fungicides (Brent and Hollomon 2007) has been successfully adopted in many crops to increase and maintain disease control efficacy when individual cultivars or fungicides may not be able to control disease effectively. To improve biocontrol efficacies achieved through the use of a single BCA, there has been increasing interest recently among researchers in using mixtures of BCAs to exploit potential synergistic effect among them (Xu et al. 2011). Number of biocontrol mechanism may operate in mixed BCA populations, and we need to consider both direct and indirect interactions between different BCA populations. Compared with the more efficacious BCA, combined use of two or more BCAs may lead to increased, reduced, or similar biocontrol efficacy (Xu et al. 2011).

Biocontrol agents applied individually are not likely to perform consistently against all pathogens of the crop or under diverse crop conditions. A combination of biocontrol agents is more likely to have a greater variety of traits responsible for the suppression of one or more pathogens, and also it is likely to have these traits expressed over a wide range of environmental conditions (Crump 1998). Numerous studies (Meyer and Roberts 2002; Roberts et al. 2005) have reported increased performance in the suppression of pathogens or disease by combinations of biocontrol agents. Incompatibility among microbes combined in biocontrol preparations is possible since biocontrol agents are typically selected based on their antagonistic behavior towards other microbes (Leeman et al. 1996; Meyer and Roberts 2002). Several researchers have indicated that strains combined in biocontrol preparations must be compatible for increased disease suppression to occur (Raupach and Kloepper 1998; Roberts et al. 2005). Accumulating evidence from literature has shown that compatible multiple strains appear to be an important prerequisite for the desired effectiveness of strains and more consistent disease suppression (Ganeshmoorthi et al. 2008). Compatible strains of *P. fluorescens* (Pf1, Py15 and Fp7) and *Bacillus subtilis* strains (EPCO 16 and EPC 5) were found to effectively inhibit the growth of *Alternaria solani* in tomato crop (Sundaramoorthy and Balabaskar 2012). Similarly, experiments for the biological control of the bacterial blight pathogen revealed that different species of *Bacillus* applied to rice plants as a seed treatment before sowing, a root dip prior to transplantation, and two foliar sprays prior to inoculation could afford up to 59 % suppression of the disease. These treatments could also bring about a twofold increase in plant height and grain yield (Vasudevan and Gnanamanickam 2000). Efforts are in progress, including formulation of synergy hypothesis in relation to biocontrol mechanism to exploit microbial mixture for uses in biocontrol of plant diseases.

13.12 Conclusion

Today in the light of the growing concern towards environmental safety, suppression of plant diseases through biocontrol agents is gaining ground as an alternative to traditional disease management strategies. Now it is necessary to focus on the challenges involved in testing, formulating, and delivering newer potential biocontrol agents within the context of integrated disease management. Undoubtedly choosing correct microbial inoculants is the foremost factor governing the success of biocontrol program. But making it reach to the field with a suitable delivery method maintaining consistent performance is the next most important challenge. Thus, the major challenge for plant pathologists is to develop a specific delivery system for a particular bioagent against a specific pathogen. There is a trend prevalent among farmers to use or apply a

single biocontrol agent, but research in the area of consortia application with multiple mechanism of disease control against more than one pathogen is the need of the hour. Also, future research strategies should emphasize on achieving viable and stable biological product. In addition, future research should be attempted to develop a systematic approach to select a suitable method of delivery system based on the characteristics of the microbial inoculants. Another research area in the wake of less labor and increasing mechanization in agriculture, a model of pilot irrigation system such as microbigation, needs to be invented for applying microbial inoculants in field level to the diseased plant in the form of viable spores or fungal conidia through drippers. Overall, a multifaceted management program will require helping the end user to grow a disease-free crop.

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Advances in Formulation Development Technologies

14

Mona S. Zayed

Abstract

The increasing need for environmentally friendly agricultural applications is motivating the use of fertilizers based on beneficial microorganisms called biofertilizers. These biofertilizers could be defined as formulations containing one or more beneficial and efficient microbial strains (or species) loaded on economically safe and easy-to-use carrier material. Productions of biofertilizers require integration of physical, chemical, and biological parameters to increase the populations and survival of these microorganisms. The most common biofertilizers are nitrogen fixers; phosphate solubilizers; potassium mobilizers; sulfur oxidizers; *Pseudomonas fluorescens*, which is known as the most common plant-growth-promoting rhizobacteria (PGPR); and mycorrhizae. Productions of efficient biofertilizers require selection of good microbial strain(s), selection of good carrier, and using a suitable formulation process. Selected strains must be effective and competitive against soil indigenous populations. Good carriers must be characterized by their ability to deliver the right number of viable cells in good physiological condition and at the right time. The formulation process refers to the laboratory or industrial process for unifying the carrier with the bacterial strain. There are different formulation technologies that were utilized during the last decades at which four basic dispersal types from microbial inoculant were produced (powder, granule, slurry, and liquid). High-quality microbial inoculants should meet farmers' and manufacturers' requirements, which include the following: contains large population of one or several strains; has

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_14

consistent and reproducible efficacy under different field conditions; free from significant contamination and opportunistic pathogens for humans, animals, and plants; has an extended shelf life and resistance to mishandling by the farmers.

Keywords
Pseudomonas fluorescens • Rhizobacteria • Carriers • Formulation • Inoculant

14.1 Introduction

In recent years, biofertilizers have emerged as an important component containing living cells of efficient strains, which have an ability to convert nutritionally important elements from unavailable to available form through biological processes (Vessey 2003). Such integrated nutrient supply system has a great promise to improve crop yields through environmentally better nutrient supplies as most of the bacteria included in biofertilizers have close relationship with plant roots (Mishra and Dadhich 2010).

The term "biofertilizer," which is also called "microbial inoculant," can be generally defined as a formulation containing one or more beneficial and efficient microbial strains (or species) loaded on economical, safe, and easy-to-use carrier material (Board 2004).

Plant-growth-promoting rhizobacteria (PGPR), also called plant-growth-promoting bacteria (PGPB) or plant-growth-promoting microorganisms (PGPM), have been defined as bacterial strains that can fulfill at least two of three important criteria such as aggressive colonization, plant growth stimulation, and biological control. They may colonize the rhizosphere, the surface of the root, or even superficial intercellular spaces of plant cells (Vessey 2003; Weller et al. 2002). Cited literature stated that PGPR inoculants are currently commercialized as plant growth promoter through one or more from the following mechanisms: (1) improvement of soil structure; (2) suppression of plant diseases either by antagonizing plant pathogens or through induction of the plant systemic resistance; (3) production of antibiotics; (4) improvement of nutrient acquisition by inducing specific ion flux in plant cell; (5) fixation of atmospheric nitrogen that is transferred to the plant; (6) solubilization of phosphorus and potassium; (7) production of siderophores that chelate iron and make it available to the plant root; (8) production of phytohormones like IAA, GA3, and cytokinin; (9) increasing of yield, germination rate, tolerance to drought, shoot and root weights (Fattah et al. 2014; Gholami et al. 2009; Kloepper et al. 2004; Lucy et al. 2004; Mishra et al. 2010; Zahir et al. 2003; Zehnder et al. 2001).

14.2 Microbial Formulations

Microbial formulations, also called microbial inoculants or formulated biofertilizers, are carrier-based preparations containing beneficial microorganism(s) in a viable state intended for seed or soil application (Bashan 1998).

14.2.1 Most Common Microbial Biofertilizers

14.2.1.1 Nitrogen-Fixing Biofertilizers

14.2.1.1.1 *Rhizobium* spp.

Rhizobium spp. are symbiotic nitrogen-fixing motile prokaryotes defined by their ability to nodulate legumes. Growth-promoting diazotrophs can enhance the growth and development of associated legumes by transferring fixed N_2 or by improving nutrient uptake through modulation

of hormone-linked phenomena in inoculated plants (Biswas et al. 2000).

14.2.1.1.2 *Azotobacter* spp.

Azotobacter spp. are a common soil bacteria belonging to nitrogen-fixing biofertilizers. They have the potential to increase plant yield due to biological nitrogen fixation (enhancement of the uptake of nitrate, ammonium orthophosphate, potassium, and iron); stimulation of root development and branching; production of plant growth hormones like indole acetic acid (IAA), gibberellic acid, and vitamins; and improving of the water status of the plant (Guleri et al. 2005).

14.2.1.1.3 *Azospirillum* spp.

Azospirillum spp. are isolated from various geographical regions of the world and characterized as the best genus of plant-growth-promoting rhizobacteria (Burdman et al. 1996). *Azospirillum* bears great promise as a growth-promoting and N_2-fixing biofertilizer under microaerobic conditions. Several studies indicated that *Azospirillum* spp. can increase the growth of various crops (Bashan and Holguin 1997).

14.2.1.1.4 *Peanibacillus polymyxa*

Peanibacillus polymyxa (nitrogen-fixing spore-forming bacteria) is a common soil bacterium belonging to plant-growth-promoting rhizobacteria (PGPR). The activities associated with *Peanibacillus polymyxa* include increase of soil porosity (Gouzou et al. 1993), nitrogen fixation (Heulin et al. 1994), soil phosphorus solubilization (Jisha and Alagawadi 1996), as well as producing great variety of auxins and indolic and phenolic compounds (Lebuhn et al. 1997). Also, they suppress several plant pathogens (Kharbanda et al. 1999).

14.2.1.2 Phosphate-Solubilizing Bacteria (PSB)

Phosphorus is the most abundant nutrient in soils in both organic and inorganic forms. However, due to its unavailability, it is frequently a major or even the prime limiting factor for plant growth. Phosphorus status may be improved by applying certain bacteria that release its fixed form. A number of PGPRs solubilize unavailable P in the rhizosphere by secreting organic acids and phosphatase (Kim et al. 1997) and make it available to host plants, which results in better plant growth and higher crop yield (Gaur et al. 2004). Strains from the genera *Pseudomonas* spp., *Bacillus* spp., and *Rhizobium* spp. are among the most powerful phosphate-solubilizing bacteria (van Straaten 2002).

14.2.1.3 Potassium-Mobilizing Bacteria (KMB)

Potassium is a major essential macronutrient for plant growth and development; hence, it is commonly added as fertilizer to optimize yield. Potassium-solubilizing bacteria (KSB) enhance mineral uptake by plants through solubilizing insoluble K and releasing it from silicate in soil (Friedrich et al. 1991). The strains *Bacillus circulans* and *Peanibacillus polymyxa* are considered the most important potassium-solubilizing bacteria (Sheng et al. 2002).

14.2.1.4 Sulfur-Oxidizing Bacteria

Sulfur is one of the essential plant nutrients that affect the yield and quality of crops (Vidyalakshmi et al. 2009). Plants take up sulfur in sulfate form. The transfer of sulfur between the inorganic and organic pool is entirely caused by the activity of the soil microflora, such as *Thiobacillus thioxidans* (Kuenen et al. 1982). Also, the acidity caused due to the formation of sulfate helps in solubilizing other nutrients and improves alkaline soil (Stamford et al. 2002).

14.2.1.5 *Pseudomonas fluorescens*

Pseudomonas spp. are receiving worldwide attention, under the broad general category known as plant-growth promoting rhizobacteria (PGPR) or plant-health-promoting rhizobacteria (PHPR) (Kloepper et al. 1989). The strains of *Pseudomonas fluorescens* are known to survive both in the rhizosphere and phyllosphere. These bacterial strains are well known for improving plant growth through direct effects on plants by producing plant-growth-promoting substances (Glick 1995; O'sullivan and O'Gara 1992), increasing the availability and uptake of mineral nutrients (Gaskins et al. 1985), excreting siderophores that are Fe-chelating agents (Hemming

1986), degrading pollutant (Brazil et al. 1995); protecting plants against many fungal, bacterial, and viral diseases; and suppressing the soil-borne pathogens or other deleterious microorganisms (Saravanakumar et al. 2009).

14.2.1.6 Mycorrhizae

Mycorrhizae are widespread symbiotic, nonpathogenic permanent associations between plant and fungi, both in natural and cultivation environment, and characterized by a bidirectional transfer of nutrients, where plants provide sugar to the fungi, and these help the plants in the acquisition of mineral nutrients from the soil (Smith and Barker 2002).

Seven types of mycorrhizal fungi can be distinguished according to their structural and functional features (Smith and Read 1996). The most important mycorrhizal types are ectomycorrhizae and endomycorrhizae.

Arbuscular mycorrhizal (AM) fungi are soil microorganisms that establish a mutual obligate symbiosis with the majority of higher plants, providing a direct physical link between soil and plant roots (Strullu and Plenchette 1990).

Ectomycorrhizal (ECM) fungi are also widespread in their distribution and establish a mutual nonobligate symbiosis with 3 % of vascular plant families (Smith and Read 1996). These two groups of mycorrhizal fungi play an important role in soil-water extraction, increasing the drought tolerance of the host (Mathur and Vyas 2000). These associations are also reported to improve the plant's ability to tolerate heavy metal toxicity (Khan 2001), as well as attacks by pathogens (Fusconi et al. 1999). Briefly, both ectomycorrhizae and endomycorrhizae play an important role in sustainable agriculture and forestry (Futai et al. 2008; Siddiqui and Pichtel 2008).

14.3 Production of Microbial Inoculants

Microbial inoculant is best defined as a biologically active product containing one or more beneficial microbial strains loaded on economical carrier materials. The objectives considering the production of efficient inoculant is to find the best bacterial strain(s), selection of good carrier, and the formulation processing (Herridge 2007).

14.3.1 Strain Selection

Selection of an appropriate organism is a critical process in the production of inoculants. Therefore, selected strains must be more competitive against soil indigenous populations and be more efficient (Stephens and Rask 2000). Also, it is important to select different genera or strains that are compatible with each other for the development of an inoculant that can be used for a range of crops since it is preferable than single strain inoculants (Fattah et al. 2014).

14.3.2 Selection of Carrier Materials for the Preparation of Biofertilizer Formulations

Carrier refers to the abiotic substrate (solid, liquid, or gel) that is used in the formulation process. It has been shown to improve the survival and effectiveness of inoculants by physically protecting the microbial culture from biotic and abiotic stresses (van Veen et al. 1997).

14.3.2.1 General Characteristics of a Good Carrier

Good carriers must be characterized by its ability to deliver the right number of viable cells in good physiological condition and at the right time (Stephens and Rask 2000; Trevors et al. 1992). Other desirable properties of a good carrier have been listed before and could be summarized as follows.

14.3.2.1.1 Chemical and Physical Characteristics

The carrier should (1) be nearly sterile or cheaply sterilized (be able to sterilize either by autoclaving, gamma radiation, or other methods); (2) be chemically and physically uniform as possible; (3) have high water-holding capacity; (4) be suitable for many bacterial species and strains as possible; (5) permit gas exchange, particularly oxygen; (6)

have high organic matter content and favorable H+ concentration (Bashan 1998; Stephens and Rask 2000; Ferreira and Castro 2005).

14.3.2.1.2 Manufacturing Qualities

The carrier should (1) be easily manufactured and mixed by existing industry, (2) allow for the addition of nutrients, (3) have an easily adjustable pH, and (4) be made of a reasonably priced raw material in adequate supply (Catroux et al. 2001).

14.3.2.1.3 Farm-Handling Qualities

A good carrier (1) allows for ease of handling (a major concern for the farmers), (2) provides rapid and controlled release of bacteria into the soil, and (3) can be applied with standard agrotechnical machinery (Date 2001).

14.3.2.1.4 Environment Friendly

From an environmental point of view, the carrier should be (1) nontoxic, (2) biodegradable, (3) nonpolluting, and (4) without environmental risks such as the dispersal of cells to the atmosphere or to the ground water and leaving no carbon footprint.

14.3.2.1.5 Storage Quality

The suitable carriers are expected to overcome the loss of viability in living organisms during the storage period and donate their long shelf life and stability over the range of 5 °C to 30 °C (Deaker et al. 2004).

14.3.2.2 Types of Existing Carriers

The carriers are divided by two ways according to their natural sources as well as their chemical composition (Herridge et al. 2008; Bashan 1998).

14.3.2.2.1 The Types of Carriers According to Their Natural Sources

Carriers can be divided into four basic categories:

1. *Soils*: these are coal, clays, and inorganic soil (Smith 1995). Different mixtures from soil materials could be used as carriers, such as commercial mixtures of talc and lyophilized cultures (Burton 1964), charcoal and coal bentonite mixture (Deschodt and Strijdom 1976), bentonite and talc (Date and Roughley 1977), bentonite and corn oil (Kremer and Peterson 1983), lignite (Dube et al. 1980), lignite, coal, clays, and inorganic soils (Smith 1995).
2. *Plant Waste Materials*: examples are corncob (McLeod and Roughley 1961); cellulose (Pugashetti et al. 1971); soybean meal (Iswaran 1972); sawdust, rice husk (Khatri et al. 1973); composts, farmyard manure (Iswaran 1972); manure, powdered coconut shells, ground tea leaves, and combinations of these substances (Tilak and Subba-Rao 1978); soybean and peanut oil (Kremer and Peterson 1983); agricultural waste material, spent mushroom compost (Sadasivam et al. 1986); plant debris from wheat bran, corn meal, wheat, barley, maize, sorghum, rice husk, wheat straw, and peanut hulls (Zayed et al. 2014)
3. *Inert materials*: belonging to this category are ground rock phosphate, calcium sulfate, and polyacrylamide gel (Dommergues et al. 1979); entrapped alginate beads (Bashan 1986); vermiculite (Paau et al. 1991); vermiculite plus additives (Graham-Weiss et al. 1987); and perlite (Daza et al. 2000)
4. *Oil-dried bacteria* (Johnston 1962) *and plain lyophilized microbial cultures* (Mohammadi 1994). These preparations can later be incorporated into a solid carrier or used as they are.

14.3.2.2.2 The Types of Carriers According to Their Chemical Composition

Organic Carriers

Different organic carriers are well known like lignite, charcoal, coir dust, composts of various origins and compositions, sugarcane bagasse, soils mixed with various organic amendments, and vermiculite (Bashan 1998; Singleton et al. 2002).

Inorganic Carriers

Inorganic carriers can be made from natural materials, natural polymers, or synthetic materials. The natural inorganic materials are talc-based formulations (Bharathi et al. 2004; Saravanakumar et al.

2009), perlite, and vermiculite (Albareda et al. 2008). Polymers, either synthetic or natural, have been suggested as alternative carriers, such as polyacrylamide for entrapping fungi and bacteria (Mugnier and Jung 1985), sodium alginate (Fages 1990), agarose, k-carrageenan, and polyurethane. The use of polymeric carrier materials to encapsulate bacteria before soil inoculation has been proven successful in enhancing the survival of bacteria and offering substantial practical advantages over other carriers (Amiet-Charpentier 1999).

14.3.3 The Formulation Process

"Formulation" refers to the laboratory or industrial process of unifying the carrier with the bacterial strain. "Inoculant" refers to the final product of formulation containing a carrier and microbial agent or consortium of microorganisms.

Different formulation technologies were utilized during the last decades. However, immobilization process proved its superiority when compared to other different formulation technologies (Abd El-Fattah et al. 2013), since it encompasses different forms of cell attachment or entrapment into a matrix, which include flocculation, adsorption on surfaces, covalent bonding to carriers, cross-linking of cells, and encapsulation in a polymer gel (Cassidy et al. 1996). As well, encapsulation has proven to be the most promising technique for creating inoculants, with substantial advantages over other types of formulations. Once the living cells are encapsulated, they became protected in a nutritive capsule against mechanical and environmental stresses such as pH, temperature, organic solvent, poison, and predators (Saxena 2011).

14.3.3.1 The General Characteristics of a Proper Inoculant

The good inoculants should have most of the following characteristics: (1) easy to use; (2) compatible with the seeding equipment at the time of seeding; (3) tolerant to abuse during storage; (4) able to work under different field conditions and types of soil; (5) able to facilitate prolonged survival to the inoculated bacteria for the time needed by the plant and growers; (6) maintain microbial shelf life that lasts more than one season; (6) reproducible in the field; (7) human, animal, and plant safe (by eliminating the use of hazardous materials); (8) support the growth of the intended microorganisms; (9) support the necessary number of viable microbial cells in good physiological condition for an acceptable period of time; (10) deliver enough microorganisms at the time of inoculation to reach a threshold number of bacteria that is usually required to obtain a plant response, i.e., the inoculant must contain enough viable bacteria after the formulation process (Abd El-Fattah et al. 2014; Abd El-Fattah et al. 2013).

14.3.3.2 Factors Affecting the Effectiveness of Prepared Formulations

14.3.3.2.1 Growth Phase of Microbial Strain

The growth phase of microbial strain (logarithmic or stationary phase) refers to the time of mixing bacterial cultures with a carrier either active cells, spores, cysts, or flocculated cells of various PGPR species (Bashan 1998), since the incorporation phase influence on the survival and effectiveness of microorganism (s) in the inoculant (Abd El-Fattah et al. 2013).

14.3.3.2.2 Moisture Content of the Carriers

Moisture content of the carriers had a great effect on the survival of inocula. Roughley (1970) noted that in nonsterile peat, rhizobia might be more susceptible to harmful effects of high moisture than rhizobia in sterile peat. He found that 40–50 % moisture content was suitable in unsterilized peat, whereas moisture content of 60 % was recommended for sterilized peat. On the other hand, Van Schreven (1970) found that a final moisture content of 40–55 % appeared to be favorable for most peats used as carriers.

14.3.3.2.3 Packing Materials

The development of the plastic industry has changed the packaging of inoculants as many other goods. Polyethylene has commonly been

used as a packaging material for steam sterilization. Polyethylene of a higher density (higher melting point) can be used, since it is unaffected by γ irradiation and permits high gas exchange, allowing for CO_2 losses and O_2 uptake (Abd El-Fattah et al. 2013).

The majority of the world's inoculant production is marketed in plastic pouches. A choice of plastic material for pouches involves balancing the requirements for gas exchange, moisture retention. Polyethylene bags of low density (0.033–0.051 mm gauge) are used for packing *Rhizobium* spp. inoculants in the USA (Burton 1964) and in Australia (Roughley 1970).

14.3.3.2.4 Sterilization

To produce an inoculant, the target microorganisms are introduced into a sterile or nonsterile carrier. From a purely microbiological point of view, the sterile carrier has significant advantages, while the only disadvantage of the sterilized carriers is the high cost of the production process (Brahmaprakash and Sahu 2012). The purpose of sterilizing carrier materials is to preserve the number of used microorganisms during the storage period and to prevent undesirable dispersion of pathogenic bacteria to agricultural field. In other words, sterilization is essential to reduce the risk of field contamination. Abd El-Fattah et al. (2013) noted that sterile carriers generally support high populations and display much longer shelf lives. Also, Date and Roughley (1977) observed that rhizobia behaved better in sterile than in nonsterile peat. Although it is generally accepted that a sterilized carrier is superior to a nonsterilized one, there are some disagreement on the most suitable sterilization methods. The most common methods used in sterilization are gamma irradiation and autoclaving.

Heat Sterilization

Different methods were used for sterilizing carrier materials to obtain the most suitable one without any effect on their quality. Steam sterilization by autoclaving is the most commonly used and has the superiority among all heat sterilization methods which allows absolutely pure culture of inocula to be prepared (Strijdom and Deschodt 1976). However, Schreven (1970) noted that long sterilizing time of up to 5 h had long-term deleterious effect, while Deschodt and Strijdom (1976) noted that the carriers could successfully steam sterilize intermittently at 121 °C for 2 h for 2 successive days. Actually, the high risk of contaminating sterilized carriers appears when bags are removed from the autoclave before being sealed off, which makes steam sterilization a less attractive method.

Gamma Irradiation

Gamma irradiation is the most suitable way of carrier sterilization because the sterilization process makes almost no change in the physical and chemical properties of the materials, as well as the final product quality, compared to autoclaving (Strijdom and van Rensburg 1981; Abd El-Fattah et al. 2014).

Gamma irradiation even at 10×10^6 rad did not completely sterilize peat; however, the survival of rhizobia was not seriously affected by the contamination level, which does "not exceed 10^7 cells g-1 of peat" (Roughley 1970).

Also, gamma irradiation at a dose of 50 kGr and steam sterilization for 3.5 h were equally effective for the manufacturing of high-quality inoculants with the peat (Strijdom and van Rensburg 1981). Manufacturers preferred gamma irradiation, as it is a more convenient and reliable method.

14.3.3.2.5 Storage Temperature

One of the most critical factors affecting microbial survival during storage is temperature. Pure cultures of rhizobia in sterilized peat may be stored for 6 months at 4 °C, followed by up to 9 months on the shelf (Roughley 1970). However, Somasegaran et al. (1984) reported that the influence of storage temperature on the survival of rhizobia is strain specific and depends on the purity of the culture and moisture loss during storage. However, other studies with previously sterilized peat showed a significant decline in the viability of several strains of rhizobia when the inoculants were stored at 4 °C (Somasegaran 1985).

14.3.3.2.6 Dehydration Rate

The dehydration phase is perhaps the most critical stage of the formulation process, especially for nonspore-forming bacteria. From both commercial and agricultural points of view, the extremely long survival of bacteria in these preparations makes the dry formulations very attractive, since low water content in the final product is responsible for long-term survival during storage. In this way, the bacteria in the formulation remain inactive, resistant to environmental stresses, insensitive to contamination, and more compatible with fertilizer application (Paau 1988). Kosanke et al. (1992) reported that using air-dried and lyophilized preparations are the most common solutions to increase the survival period. By studying the effect of dehydration on encapsulated *Azospirillum* sp. cells, Paul et al. (1993) demonstrated that a large proportion of the cells are destroyed during dehydration. However, when the inoculants are properly dehydrated, the surviving cells are sufficient for inoculation and the bacteria survive for almost a year without a drop in population.

14.3.3.3 The Forms of Microbial Inoculants

Microbial formulations (inoculants) come in four basic dispersal forms. The use of each type depends upon market availability, choice of farmers, cost, and the need of a particular crop under specific environmental conditions (Bashan 1998). These forms could be summarized as follows.

14.3.3.3.1 Powder

This form is used as a seed coating before planting. The smaller the particle size is, the better the inoculant will adhere to the seeds. Standard sizes vary from 0.075 (Strijdom and Deschodt 1976) to 0.25 mm (Burton 1967), at which peat and soil carriers should be ground to pass a 50- to 60-mm mesh sieve. The amount of inoculant used is around 200–300 g/ha depending on the type of biofertilizer.

In powder formulation, the microorganisms that produce heat- and desiccation-resistant spores are preferred since they produce stable dry powder products, as well prolonged shelf life and efficiency (Caesar and Burr 1991). Also, Paul et al. (1993) reported that powder carriers permit retention to living cells, against various stresses during storage and gradual release into the soil. But despite their benefits, these powders are not widely applied as a result of their expensive technology.

14.3.3.3.2 Granule

Granules are made of peat, prill, small marble, calcite, or silica grains that are wetted with an adhesive and mixed with inoculum. The granules are coated or impregnated with the target microorganism(s) (Stephens and Rask 2000). Some studies showed that granular application in rhizobia is superior to peat-based products as well as to liquid inoculants in terms of nodule number and weight, N accumulation, N2 fixation, and total biomass (Herridge 2007). Also, the granular inoculants are especially pronounced under soil stress conditions like soil acidity, moisture stress, cool and wet soils (Lupwayi et al. 2006). These inoculants are applied directly to the furrow, together with the seeds. A particle size between 0.35 and 1.18 mm provides a granule that absorbs the culture rapidly and flows uniformly through a granular applicator. Such granular inoculants are particularly suitable to farming systems in developed countries, where the seeding equipment is commonly multifunctional and includes seed fertilizers and inoculant delivery systems (Hegde and Brahmaprakash 1992).

14.4 Advantages of Granular Inoculants

They are less dusty and easier to handle, store, and apply. The placement and the application rate can be easily controlled, and the limitations of seed applications are overcome, at which the inoculant is placed in a furrow near to the seed to facilitate lateral root interactions (McQuilken et al. 1998; Xavier et al. 2004).

14.5 Disadvantages of Granular Inoculants

The granular inoculants are bulkier; thus, the transport and storage costs are high; consequently, the rates of application also increased (McQuilken et al. 1998; Xavier et al. 2004).

14.5.1 Slurry

This type of inoculant is based on powder-type inoculants suspended in liquid (usually water). The suspension is directly applied to the seeds just prior to sowing until a uniform coverage is achieved or alternatively to the furrow.

14.5.2 Liquid

Liquid inoculants are an upgraded derivative of inoculants. In this type of inoculants, there is no need to prepare and amend a carrier, and usually their application to the seeds or to the field is easier. Essentially, they are microbial cultures or suspensions amended with substances that may improve stickiness, stabilization, surfactant, and dispersal abilities (Singleton et al. 2002).

These inoculants use liquid cultures or liquid formulations either dissolved in water, mineral or organic oils to inoculate seeds by dipping them into the inoculant before sowing or by using a sprayer to uniformly spray the liquid inoculant on the seeds. After drying, the seeds are sown. This method ensures standard coverage of the seeds without intervention with the seed inspection system of the growers or losing the inoculum during the drying (Smith 1995). Also, the liquid inoculants can be sprayed directly into the seeds before sowing in the furrow.

Liquid inoculants gained popularity in developed countries for most crops, especially legumes, because of their easier handling and high cell counts (Schulz and Thelen 2008), as well as because they are compatible with machinery on large farms, such as air seeders and seed augers. Finally, they are preferable in small-scale inoculant manufacturers that lack the capacity to handle peat and other carriers.

14.6 Main Advantages of Liquid Inoculants

The liquid inoculants are distinguished by the following: (1) are easy to handle, (2) allow the manufacturer to add sufficient amounts of nutrients in the inoculants like cell protectants and some additives that are responsible for stimulate cell/spore/cyst formation to improve their performance, (3) offer greater protection against environmental stresses, and (5) have increased field efficacy compared to peat-based inoculants (Singleton et al. 2002).

14.7 Major Disadvantages

Few disadvantages were reported for liquid inoculants: (1) they have limited shelf life in some cases (but not for all); (2) cool or cold conditions are required for long-term storage; (3) they involve increased costs, a fact that limits their use in developed countries and precludes their use in most developing countries (Stephens and Rask 2000); and (4) for bacteria with poor survival in the soil, like *Azospirillum* sp., these formulations are largely useless since they do not provide a protective environment for them (Bashan et al. 1995).

14.8 Common Additive Used in Liquid Inoculants

14.8.1 Sucrose

Sucrose is used to improve the survival of microorganisms in the liquid inoculants, mostly in rhizobia and phosphate-solubilizing bacteria (Taurian et al. 2010).

14.8.2 Glycerol

Glycerol is used because it holds significant amount of liquids as well as protects the cells from dehydration by slowing the desiccation rate (Manikandan et al. 2010). Taurian et al. (2010) mention that the addition of glycerol to liquid formulation containing *Pseudomonas fluores-*

cens maintains the viability of cells during storage for 6 months.

14.8.3 Carboxymethyl Cellulose (CMC)

CMC is a widespread additive as it is readily available and has comparatively a steady batch quality since it is a semi-synthetic polymer. As well, it is used in low concentration "1/5; w/v," which makes it cheap relatively (Jha and Saraf 2012).

14.8.4 Arabic Gum

Arabic gum is a complex mixture of glycoprotein and polysaccharides. It is known as acacia gum as it is extracted from acacia. It is commonly used as adhesive agent for biofertilizers, especially the rhizobia (Rose et al. 2011). It protects the bacteria against dehydration and improves their survival when added to liquid inoculants as an adhesive agent (Wani et al. 2007).

14.8.5 Polyvinylpyrrolidone (PVP)

Polyvinylpyrrolidone is a synthetic water-soluble polymer. It is used as a binder in many microbial inoculants, especially *Bradyrhizobium japonicum*, as it provides protection from dehydration and inhibitory seed coat exudates, which are deleterious to inoculated rhizobia (Singleton et al. 2002).

14.9 Inoculant's Quality in Relation to Its Performance

A high-quality inoculant should meet farmers' and manufacturers' requirements, including the following: (1) contains large population of one or several strains; (2) has consistent and reproducible efficacy under a range of field conditions; (3) free from significant contamination and opportunistic pathogens for humans, animals, and plants; (4) has an extended shelf life; and (5) resistant to mishandling by the farmers (Lupwayi et al. 2000).

In most countries, there are no regulations for the level of contaminants in the most commonly used nonsterile inoculant preparations. However, one should note that the use of nonsterile carrier inoculants has caused no reported health hazards in decades of usage. France, which has the highest standards for inoculant quality and mandated field testing of new formulations, has strict levels of contaminants (0.1 % of the total bacterial population) but at the same time requires high population levels of microorganisms (Thompson 1991).

Different methods were defined to evaluate the inoculants' quality such as the following.

14.10 Count of Viable Cells and the Level of Contaminants

Naturally, any inoculant should contain a level of bacteria sufficient to inoculate plants and produce an economic gain. The required level of bacteria cannot be established as a general standard because it varies from one bacterial species to another (Bashan 1998). High-quality biofertilizers would be expected to have higher population of desired microorganisms with sufficient viability and remain uncontaminated during the storage period (Brahmaprakash and Sahu 2012). Viable cell numbers in broth and inoculants prepared with sterile carriers can be measured using total count procedures. As most inoculant products are prepared using nonsterile carriers and contain so many fast-growing contaminants, plate count procedures are impractical.

The plate count is the most widely used technique to assess the number of viable cells or the number of contaminants in an inoculant, while most probable number (MPN) counts are also widely used to estimate the population of rhizobia (Abd El- Fattah et al. 2014; Lupwayi et al. 2000). The level of rhizobia required in the inoculant varies worldwide (between 10^7 and 4×10^9 cfu/g inoculant) since no set of common international standards exist (Olsen et al. 1995), while in

India the agency for formulating the standard biofertilizers has specified that all the bacterial inoculants should have minimum CFU of 5×10^7 per g of carrier and 10^8 CFU per ml of liquid inoculants and should not have contamination at 10^5 dilution (Brahmaprakash and Sahu 2012).

One of the major drawbacks of these methods is that they only assess the number of viable cells in the inoculants but do not take into account the physiological activity of the cells and do not reflect their ability to survive in the soil after application (Penna et al. 2011).

14.11 Most Probable Number (MPN) Plant Infection Assay

The MPN plant infection assay used with rhizobia is based on the ability of rhizobia to nodulate appropriate host legume plants. A dilution series is prepared, and then aliquots of consecutive dilutions are added onto surface-sterilized seedlings of the appropriate host. This method relies upon the pattern of positive and negative nodulation responses generated, assuming that a single *Rhizobium* sp. cell at a given dilution will cause nodulation. This method is time and space consuming and requires about 30 days for plant growth and adequate greenhouse or growth chamber space; therefore, methods based on serological and physiological properties are preferable.

14.12 Measurement of Cell Metabolic Activity

Evaluation of the biological efficiency of the inoculants by measuring the cell metabolic activities using different physiological and genetical methods was recorded, such as probes (rRNA) and flow cytometry (Catroux et al. 2001), as well as evaluating the important physiological characteristics associated with each species, such as nitrogenase activity with nitrogen fixers, phosphate-solubilizing activity with phosphate-solubilizing bacteria, potassium-mobilizing activity with potassium-mobilizing bacteria; production of plant-growth-promoting substances; and excreting of siderophores with *Ps. fluorescens* (Abd El-Fattah et al. 2014).

14.12.1 Immunological Techniques

When bacteria, including rhizobia, are injected into a mammal, the animal responds to high molecular weight substances (antigens) on the surface of the bacteria by producing antibodies that bind to the bacteria. Since the reaction between antigens and the antibodies produced is quite specific, antibody preparations from animals inoculated with a particular bacterial strain can be used to both detect and identify that strain. Immunological methods are used to confirm culture identity, but they do not determine cell viability as antibodies react with live and dead cells (Lupwayi et al. 2000).

A range of methods based on such antigen/antibody reactions are used to confirm the identity of the bacterial strains in broth culture, peat, soil, etc. (Harlow and Lane 1988): cell agglutination reaction (Somasegaran and Hoben 1994), spot blot (Ayanaba et al. 1986), colony-lift immunoblot tests (Olsen and Rice 1989), a syringe filter enzyme immunoassay (EIA) that can enumerate and identify rhizobial strains in broth within a total testing time of 10 min, using only readily available disposable materials (Olsen et al. 1998).

14.13 Some Important Carriers

14.13.1 Peat

The most common carrier for microbial inoculants used all around the world is peat, which is the most commonly used in the production of legume inoculants due to its bacterial protection properties (Albareda et al. 2008; Stephens and Rask 2000). The choice of peat as preferred carrier for most bacterial species is supported by numerous studies in which it was established that peat remains unchallenged as a carrier (Somasegaran 1985). It is characterized by high water-holding capacity and can usually be used

without additives (Bashan 1998). It usually maintains a high concentration of viable bacteria and is easy to apply. The conventional means to produce the inoculant involves inoculating neutralized, nonsterile peat with a bacterial suspension of 10^7 cfu/g of peat, to reach a high population in the final product. Graham-Weiss et al. (1987) showed that peat inoculants with high numbers of viable bacteria also can be produced by inoculating with 10^4 cfu/g of sterile peat. The bacteria multiplied in the peat to a peak population density of 10^8 to 10^9 cfu/g without serious competition from contaminants.

Several amendments were used to enhance common formulations of peat with various microorganisms. Peat was amended with chitin, vermiculite, heat-killed *Aspergillus niger* mycelium, a spent compost from *Agaricus bisporus* (button mushroom), pyrophyllite (hydrous aluminum silicate), and charcoal. These amendments improved microbial growth, promoted seed germination when inoculums were used as seed treatments, and enhanced plant growth and yield (Meyer et al. 2001).

14.13.2 Disadvantages of Using Peat as a Carrier

Although peat-based carrier material has been widely accepted as superior for use with most microbial inoculants, and showed successful field results, it has some drawbacks as some peats do not meet the requirements of a good carrier such as the following: (1) different batches of peat and peat from various sources differ greatly in composition (which are source dependent), structure, pH, and microbial populations; therefore, the uniformity and quality as well as microbial growth and survival will differ by means of different batches; (2) some peats have been known to contain inhibitors to microbial strains; (3) since peat is organic, complete sterilization by steam or by gamma irradiation is difficult and undesirable because high temperatures and high dosage of irradiation stimulate the peat to produce toxic by-products (Mulligan and Cooper 1985) which are unfavorable for subsequent growth and survival of bacteria (Malusá et al. 2012); (4) peat formulations are prone to contamination that can reduce the shelf life of the inoculants (Olsen et al. 1995); (6) some countries lack natural peat; therefore, any commercial exploitation of peat as a carrier is a remote possibility (Saha and Kapadnis 2001); (7) it increased the complexity in inoculation and abrasiveness to the seeding machinery; (8) processing of peat is a costly investment as it requires several steps (which involves excavation, drying, grinding, neutralization, packing, and sterilization) before it can be used as carrier (Tittabutr et al. 2007).

14.13.3 Inoculant Preparation

Harvested peat must be drained and sieved to remove coarse material before it is slowly dried to about 5 %. The drying step is of major importance since it can lead to the formation of toxic compounds. The drying should be done at the lowest possible temperatures and certainly never exceed 100 °C. Air drying should be used when it is practicable, instead of oven drying. The type of peat and the eventual particle size desired will determine to what extent drying is required, but the moisture content has to be reduced sufficiently to ensure that the subsequent addition of liquid culture brings the final moisture content of the inoculant to the desired level. Once dried, peat is ground, to pass through at least a 250-μm sieve. Most of the peat has a low pH, which must be corrected by liming to reach pH 6.5–7.0 (Tittabutr et al. 2007). The peat is then sterilized, and a sufficient quantity of liquid inoculum is added to the peat. In the case of bacterial inoculant, a final moisture content of 40–55 % is generally acceptable. Inoculated peat is incubated for a certain period of time to allow bacteria multiplication in the carrier. Peat can also be used for AM and ectomycorrhizal inoculants (Zayed et al. 2014).

14.13.4 Wheat Bran

Wheat bran was found to be the best carrier for the mass multiplication of phosphate-solubilizing

fungi (PSF) and ectomycorrhizae (Zayed et al. 2014), which may be attributed to its high water-holding capacity and organic matter content. Although the genera *Pseudomonas* spp. and *Bacillus* spp. have been reported to degrade cellulose, however some (PSF) fail to multiply in this substrate due to the lack of cellulase enzymes which is necessary for the degradation of cellulosic materials.

14.13.5 Alginate

So far, alginate is the material of choice for encapsulations of microorganisms and in application. It is a naturally occurring polymer composed of β-1,4-linked D-mannuronic acid and L-glucuronic acid. It is available mostly from different marine macroalgae in large sustainable quantities (Yabur et al. 2007), as well as several bacteria (Smidsrød 1990). Alginate formulation supported high populations and survival for the microbial inoculants at the elevated storage temperature of 40 °C (Viveganandan and Jauhri 2000). Several alginate-based preparations were evaluated with different microorganisms for agricultural purposes, and were found to be superior over other inoculants, such as encapsulation of *Azospirillum* sp. (Fages 1990), AM (Vassilev et al. 2005), ectomycorrhizal fungi (Marx and Kenney 1982), *Frankia* sp., and phosphate-solubilizing bacteria (Bashan 1998; Bashan et al. 2002). Also, they were found to be superior over liquid inoculants and charcoal-based inoculants for inoculating maize plants under low temperatures (Trivedi et al. 2005).

Plant-growth-promoting rhizobacteria can survive in alginate beads for a long period of time. The superiority of alginate as mentioned by Brahmaprakash and Sahu (2012) is due to lower water activity, as microorganisms will be at a low metabolic activities and are released into soil only after availability of sufficient moisture, which always coincides with the germination of seeds.

Considering the high-price polymers compared to peat-based inoculants, different attempts have been made to amend these formulations with materials like rock phosphate, cement, bentonite, clays, granite powder, gypsum, lignite, and talc by which cost of production can be minimized, besides adding bulkiness to the formulation (Fages 1990). Also, in order to improve the chemical, physical, or nutritional properties of the formulated biomass as a trial to increase its storage period, Schisler et al. (2004) suggested adding amendments that can be grouped as either fillers or extenders. These amendments like water, clay, talc, oil, or others make the formulation safer to handle, easier to apply, and better suited for storage. In some formulations, addition of enrichment materials, comprising nutrient-rich medium such as molasses, trehalose, maltose, and sucrose, enhance the viability of microorganisms (Brar et al. 2006).

14.13.6 Advantages of Using Alginate as a Carrier

The advantages of alginate carrier could be summarized as follows: (1) it is simple to use, (2) uniform, (3) biodegradable by soil microorganisms, (4) nontoxic in nature; (5) it holds large uniform bacterial population, (6) provides slow release of the bacteria for long periods, (7) causes no ecological pollution, (8) is produced on a large scale by the proper industry; (9) its biological characteristics can be effectively controlled by manipulating its chemical features; (10) the beads can be stored for long periods in a relatively small volume without any apparent effect on the size of the bacterial population; and (11) the bacteria released from the beads can inoculate the plants efficiently (Van Elsas and Heijnen 1990).

14.13.7 Inoculant Preparation

Alginate forms a 3D porous gel when mixed with multivalent cations (Ca_2+) (Yabur et al. 2007). To form the beads, microbial cells are dispersed into the polymer and the mixed solution is simply dropped in the cationic solution. Nutrients and other additives can be included to extend shelf life and inoculation efficacy (Malusá et al. 2012).

The beads are then dried for ease packaging and handling. The resulting beads can be produced in two sizes: macrobead (1–4 mm) and microbead (50–200 μm) (John et al. 2011).

14.13.7.1 Macroalginate Beads

Macrobeads are used to encapsulate several plant-growth-promoting bacteria and mycorrhizal fungi. The use of macroalginate beads has two major disadvantages: first, additional treatments during sowing are needed even if the inoculant is planted by the seeding machine. Second, the bacteria released from the inoculant need to move through the soil toward the plants. Under agricultural practices, when beads are loosely mixed with seeds and sown together by planters, the inoculant beads might fall far from the seeds (up to a few centimeters). The bacteria released from the beads must migrate through the soil and during that face competition from the native microflora, which are sometimes more aggressive and more adapted to the soil than the inoculated strain. Sometimes, the absence of a continuous film of water needed for their movement is an additional limiting factor (John et al. 2011).

14.13.7.2 Microalginate Beads

The hypotheses underline that if the beads are small enough, they are still capable of encapsulating a sufficient number of bacteria; therefore, "bead powder-like" formulation has been produced. The seeds are coated with this "bead powder" using seed-handling facilities. Subsequently, the microalginate beads result in a uniform distribution of cells close to the targeted site, even on small seeds which enhance the application efficacy (John et al. 2011), as well as cell movement through soil, and the possibility of off-site drift during application is reduced (Cassidy et al. 1996).

The production of alginate microbeads is simple and involves mixing alginate solution with liquid bacterial culture suspended in a very rich medium; then the mixture is sprayed into a slowly stirred solution of CaCl2 by using low-pressure thin nozzle which extrudes "mist-like" to produce small-diameter alginate beads, then immediately solidify into microbeads at diameters ranging between 50 and 200 μm. These microbeads entrap a large number of bacteria (~10^8 to 10^{10} CFU g–1), which is similar to the population levels entrapped in alginate macrobeads (Bashan et al. 2002).

14.14 Biochar as Inoculant Carrier

Biochar is a product of thermal degradation for organic materials in the absence of air (pyrolysis) and is distinguished from charcoal by its use as a soil amendment (Lehmann and Joseph 2009). Also, it has been described as a possible means to improve soil fertility as well as other ecosystem services and sequester carbon (C) to mitigate climate changes (Lehmann 2007; Laird 2008). However, biochar has also been shown to change soil biological community composition and abundance (Grossman et al. 2010). Therefore, biochar materials have been suggested as inoculant carriers substituting for the increasingly expensive, rare, greenhouse-gas-releasing, and nonrenewable carriers.

Cited literature has focused on the survival of microorganisms during storage, since carrier materials such as peat are rapidly decomposed in the soil and would not improve survival once added to the soil, while biochar will remain in the soil and may positively influence abundance and the efficiency of the inoculated organisms. The rhizobia's carriers should be intended to protect *Rhizobium* spp. against desiccation, adverse pH, toxic substances in the soil; be environmentally safe and nontoxic to the target organisms; release the organisms; and be abundant in supply (Deaker et al. 2004; Stephens and Rask 2000), all of which may theoretically be achieved with appropriately designed biochars. Research is in progress to determine optimal pyrolysis substrates and conditions to optimize biochar properties as an inoculum carrier.

14.14.1 Advantages of Using Biochar as a Carrier

The advantages of biochar as a carrier could be summarized as follows: (1) its large internal

surface area (2–20 uM pore space) provides protected habitat for bacterial and fungal growth in internal spaces, (2) production process makes it a presterilized medium, and (3) it has the ability to adsorb nutrients and growth factors (Lehmann et al. 2011).

14.14.2 Vermiculite as Inoculant Carrier

Vermiculite is a hydrated magnesium aluminum iron silicate which exfoliates at extremely high temperatures (700–1,000 °C). Vermiculite is considered a desirable alternative to peat for the production of bacterial inoculants. Good-quality inoculants can be produced consistently in vermiculite with many bacterial species without the need of expensive fermentation and incubation facilities, which makes vermiculite especially attractive for the production of inoculants (Graham-Weiss et al. 1987).

14.14.3 Advantages of Using Vermiculite as a Carrier

Advantages of vermiculite as a carrier could be summarized as follows: (1) the exfoliation process kills microorganisms (contamination); (2) its inorganic and preexpanded nature allows it to be sterilized easily by common sterilization processes without the risk of producing toxic by-products or causing further structural changes; (3) it is relatively inexpensive and is widely available (Graham-Weiss et al. 1987); (4) the multilamellate structure of vermiculite provides superior aeration and space for microbial proliferation; (5) it is anticrusting (Hemphill Jr 1982); (6) it acts as a plant-growth-promoting substance (Lima et al. 1984); (7) its particle sizes in the range of 45–80 mm meshes, which provide the best moisture-holding capacity and enable the final inoculant product to adhere uniformly to the seed surface; (8) it has good seed-sticking properties; (9) the number of viable microorganisms on the seeds does not change significantly for at least 1 day if stored at room temperature (Graham-Weiss et al. 1987).

14.15 Conclusion

This chapter discusses the progress in formulation development technologies during the last decades and the future of the microbial inoculant industry, and its prospective usefulness for sustainable agriculture depends on improving inoculant quality both numerically and in effectiveness.

The advances made in latest years have shown that it is possible to get inoculants with high microbial counts, free of contaminants and long shelf life. Also, different carriers and technologies of inoculation as well as formulations have been distinguished.

The future challenge is to produce improved microbial inoculants, which should be characterized by higher microbial count in field conditions, extended shelf life, effectiveness, resistant against soil's biotic and abiotic stresses, ease to use, economic, and has the ability to create different impacts on sustainable agriculture.

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Formulations of Plant Growth-Promoting Microbes for Field Applications

15

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Abstract

Development of a plant growth-promoting (PGP) microbe needs several steps starting with isolation of a pure culture, screening of its PGP or antagonistic traits by means of different efficacy bioassays performed in vitro, in vivo or in trials under greenhouse and/or field conditions. In order to maximize the potential of an efficient PGP microbe, it is essential to optimize mass multiplication protocols that promote product quality and quantity and a product formulation that enhances bioactivity, preserves shelf life and aids product delivery. Selection of formulation is very crucial as it can determine the success or failure of a PGP microbe. A good carrier material should be able to deliver the right number of viable cells in good physiological conditions, easy to use and economically affordable by the farmers. Several carrier materials have been used in formulation that include peat, talc, charcoal, cellulose powder, farm yard manure, vermicompost and compost, lignite, bagasse and press mud. Each formulation has its advantages and disadvantages but the peat based carrier material is widely used in different part of the world. This chapter gives a comprehensive analysis of different formulations and the quality of inoculants available in the market, with a case study conducted in five-states of India.

Keywords

Formulation • PGP microbes • Peat • Talc • Lignite • Viability

15.1 Introduction

Public health and safety concerns about the environmental impact of chemical fertilizers and pesticides have led to exploration of PGP microbes for sustainable agriculture. Development of PGP

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_15

microbes is a multi-step starting with isolation of a pure culture, screening of its PGP or antagonistic traits by means of an array of *in vitro* and *in vivo* bioassays followed by demonstration under greenhouse and field conditions. In order to maximize the potential of an efficient PGP microbe, it is essential to optimize carefully crafted microbial screening procedures, mass multiplication protocols that promote product quality and quantity and a product formulation that enhances bioactivity, preserves shelf life and aids product delivery. Depending on the PGP microbial groups (viruses, bacteria, yeast or fungi and nematodes), the methods used for industrial scale-up varies; for instance, bacteria and yeast are usually produced in liquid fermentation while fungi are produced in a solid state fermentation (Montesinos 2003). PGP microbe that cannot be cultured on synthetic media, such as viruses and nematodes, are usually scaled-up using an alternate host or tissue culture, as done for nuclear polyhedrosis virus (NPV).

Formulation typically consists of an active ingredient either as microbe(s) or as a product of microbe(s) in a suitable carrier material (sterile or non-sterile) with additives, which help in the stabilization and protection of the microbial cells during storage, transport and at the target site. Selection of formulation is very crucial as it can determine the success or failure of a PGP microbe. A sterile carrier has advantages over non-sterile carrier for delivering the right microbe at the precise concentration and thus avoids the unpredictable potential of an indigenous microorganism(s) to suppress cell numbers (Bashan et al. 2014). A good carrier material should be able to deliver the right number of viable cells in good physiological conditions. Some of the additional characteristics of a good carrier material include: (1) it should be easily sterilized, chemically and physically uniform as possible, having high water-holding capacity and suitable for many microbes; (2) should be reasonably priced, easily manufactured and mixed by existing industry; (3) should allow addition of nutrients and adjustment of pH; (4) should be easily handled by the farmers; and (5) should be non-toxic, biodegradable, non-polluting and have sufficient shelf life (at least 1–2 years at room temperature) (Bashan et al. 2014). Several carrier materials are used in formulation that includes peat, talc, charcoal, cellulose powder, farm yard manure, vermin-compost and compost, lignite, bagasse and press mud (Kumar 2014).

Formulations are of many types, which include dry products (such as granules, dusts and wettable powders), liquid products (such as emulsions, oil and water; usually contains one but sometimes two strains of active ingredient) and microencapsulation. The efficacy of microbial inoculants largely depends on the type of formulation and the delivery technology that extends the shelf lives for at least few months and in all cases the PGP/antagonistic activity is retained. The production cost also has to be considered and kept to a minimal while developing a microbial formulation. A good formulation should be easy to handle and apply so that it is delivered at the target site and protects the PGP microbes and enhances its activity from harmful environmental factors under field conditions. A detailed review on different types of formulations, additives used and PGP/antagonistic microbes used on various crops was reported by Nakkeeran et al. (2005) and Bashan et al. (2014). It is understood that the major role of a formulation is to provide more suitable micro-environment that prevents the rapid decline of an introduced PGP microbe in the soil.

15.2 Ingredients of the Formulations

In order to combat the loss of bioactivity of PGP microbes in formulation, certain ingredients are added. Any formulation, be it an experimental or commercial, requires an amendment for multiplication of PGP microbes and/or products for improving the physical, chemical or nutritional properties of the formulated biomass. Some of the ingredients include stickers/binders such as corn flour, gum arabic and carboxymethyl cellulose (CMC); surfactants such as Tween 80; dispersants such as microcrystalline cellulose; thickeners such as xanthan gum; desiccants such

as silica gel and anhydrous sodium sulphate; stabilizers such as lactose and sodium benzoate; and UV protectants (da Costa et al. 1998; Schisler et al. 2004). Irrespective of formulation ingredients and storage conditions used, the PGP microbes will inevitably be exposed to environmental stresses; however, most microbes have intrinsic cellular mechanisms to protect themselves against hostile environments. Hence, there is a need to understand these cellular mechanisms against environmental stress factors and utilize these effects at the time of stabilization. Many reports support the competitive colonizing ability of bacteria and its impact on plant productivity (Dekkers et al. 2000; Fuente et al. 2001; Gopalakrishnan et al. 2014).

15.3 Types of Formulations

Among the various types of formulations available for PGP microbes, the following six are widely used by the researchers:

15.3.1 Liquid-Based Formulations

The PGP microbes are typically formulated in a liquid buffer with or without added protectants such as sugars. For instance, addition of 10 % lactose or 5 % trehalose increased the storage survival of yeast *Pichia anomala* to varying degrees depending on storage temperature and duration compared to non-supplemented control (Torres et al. 2003; Melin et al. 2006). Addition of sucrose or glycerol was also demonstrated to improve survival of rhizobia, phosphate solubilizing bacteria and *Pseudomonas fluorescens* (Taurian et al. 2010). Liquid formulation has been extensively used in enhancing agricultural productivity under field conditions. For instance, inoculation with *Azospirillum brasilense* as liquid formulation enhanced not only vegetative growth but also harvested grains in wheat (Diaz-Zorita and Fernandez-Canigia 2009). The main advantage of liquid formulation is that it is a simple preparation and no cells are killed during the formulation; while the drawback is the actual weight of the products and shorter shelf life, especially when stored at elevated temperatures (Melin et al. 2011).

15.3.2 Talc-Based Formulation

Talc is composed of minerals in combination with chloride and carbonate and referred as steatite or soapstone or magnesium silicate (Nakkeeran et al. 2005). It is one of the common means of application of bacterial inoculants to soil and is reported effective against plant diseases (Meena et al. 2002; Hassan-El and Gowen 2006). Talc-based formulation of *Streptomyces griseus*, either as single or with chitin, was demonstrated to have stable shelf life of up to 105 days and control *Fusarium oxysporum* f. sp. *lycopersici*, which causes Fusarium wilt in tomato (*Lycopersicon esculentum*) (Anitha and Rabeeth 2009). *Bacillus subtilis* and *P. fluorescens* in talc-based formulations were found to control early blight of tomato caused by *Alternaria solani* and sheath blight of rice caused by *Rhizoctonia solani* (Nandakumar et al. 2001; Sundaramoorthy and Balabaskar 2012). *Ochrobactrum anthropi* TRS-2, a plant growth-promoting bacteria, was found to survive in talc-based formulation up to 9 months and also suppressed brown root rot disease of tea (*Camellia sinensis*) plants (Chakraborty et al. 2009).

15.3.3 Sawdust-Based Formulation

The use of sawdust as carrier is highly recommended where it is easily available, as it contains inherent ability of high organic matter and water-holding capacity compared to other carrier materials (Arora et al. 2001; Kolet 2014). Sawdust was demonstrated as the best carrier among the five tested carriers, viz., alginate beads, charcoal, sand, sugarcane bagasse and sawdust (from *Shorea robusta*), for *P. fluorescens* and *Rhizobium leguminosarum* as both mono-inoculants as well as co-inoculants on *Trifolium repense* (white clover) (Arora et al. 2008). Further, Arora et al.

(2008) also reported that the co-inoculants containing both rhizobial and pseudomonad population proved much better in enhancing the seedling biomass and the nodule number on *T. repense* in addition to increasing the fertility of rhizosphere soil. Recently, Kolet (2014) demonstrated the use of sawdust as carrier material for five cellulolytic bacteria, viz., *Chaetomium globosum*, *C. crispatum*, *C. olivacerum*, *C. nigricolor* and *C. virginicum*. Ambardar and Sood (2010) reported the usefulness of sawdust as carrier material for *P. fluorescens* and *B. cereus*. Chakraborty et al (2013) demonstrated the usefulness of sawdust, talc powder and rice husk as bio-formulations for *Bacillus amyloliquefaciens*, *Serratia marcescens* and *Bacillus pumilus* and reported survivability of up to 9 months of storage.

15.3.4 Fly Ash–Based Formulation

Fly ash, generated in large quantities in thermal power stations, is generally considered as a waste and an environmental hazard. However, it can be used as carrier material as it contains good mineral contents for bio-formulation development of PGP microbes. Fly ash has been reported to promote crop growth and improve soil structure (Kumar et al. 1999). Kumar (2014) noted encouraging results with fly ash as carrier material for *Bacillus* spp., *Azotobacter* spp. and *Pseudomonas* spp. when compared to other formulations. The advantage of using fly ash as bio-formulation is that it increases soil pH and aids in nutrient availability (Dwivedi and Chauhan 2007). Fly ash alone and in combination with other materials was demonstrated in bio-formulation of *Rhizobium* (Kumar and Gupta 2008) and *Trichoderma viride* and *T. harzianum* (Kumar et al. 2012).

15.3.5 Encapsulation-Based Formulation

Encapsulation of PGP microbial cells in polymeric gel (alginate or gluten) is a well-known and established technology where the gel-like matrix allows the cells to remain viable for longer duration (Fravel et al. 1985; Park and Chang, 2000). The main objectives of encapsulation of PGP microbes is to protect them from harsh environment(s) under field conditions, to reduce natural microbial inhabitant competition in soils and to release them gradually to facilitate colonization on host plant roots (Bashan et al. 2002). Immobilization of PGP microbial cells such as *Bacillus megaterium* and *T. viride* using alginate or gluten as the matrix has proved to be advantageous over other methods (Cassidy et al. 1996; Cho and Lee 1999; Sivakumar et al. 2014). Namasivayam et al. (2014) reported enhancement of seedling emergence and PGP of green gram (*Vigna radiata*) and black gram (*Vigna mungo*) upon using encapsulated formulation of *Rhizobium* spp., *Azotobacter* spp. and *Azospirillum* spp. Encapsulation of PGP bacteria, *B. subtilis*, in alginate beads enriched with humic acid effectively protected the bacteria from adverse conditions of the soil for their successful establishment in the rhizosphere (Young et al. 2006). The advantage of using alginate inoculant over peat inoculant is well described (Bashan 1998). It is understood that the use of encapsulation has several advantages over other free cell formulations such as protection from biotic stress (Smit et al. 1996), abiotic stress (Cassidy et al. 1997), inhibitory effect of toxic compounds, enhanced survival and improved physiological activity (Weir et al. 1995) and supply of encapsulated nutritional additives (Trevors et al. 1993).

15.3.6 Peat-Based Formulation

Peat is a carbonized vegetable tissue formed in wet conditions by the slow decay of aquatic and semiaquatic plants such as sedges, rushes, reeds and mosses (Nakkeeran et al. 2005). Peat-based formulation is the most marketed PGP microbial inoculants in developed countries and is most commonly used in rhizobia inoculation industry. In peat-based formulations, bacteria are metabolically active and multiplication continues during the storage period as long as sufficient nutrients, moisture and the optimum temperatures are maintained (Bashan, 1998). The techni-

cal details of production of the peat-based formulations are well described by Catroux et al. 2001; Deaker et al. 2011). Peat-based formulations are coated on seeds or pelleted for sowing in furrows for rhizobia (Toomsan et al. 1984). Of the four formulations (bentonite, talc, rice bran and peat) tested on two different strains of *P. fluorescens*, peat was found more effective as it enhanced the stability and effectiveness of the biocontrol agents (Ardakani et al. 2010). *P. fluorescens* in peat formulation enhanced soybean plant growth under greenhouse conditions when compared to other formulations such as tapioca flour and coconut water in palm oil (Habazar et al. 2014). The main drawback of the peat formulations is its unavailability in many countries.

15.4 ICRISAT's Experience in Using Peat Formulation

International Crops Research Institute for the Semi-Arid Tropics (ICIRSAT), based at Patancheru, Hyderabad, India, has been using peat-based formulation for rhizobial inoculants for chickpea (*Cicer arietinum* L), pigeon pea (*Cajanus cajan* L) and groundnut (*Arachis hypogea* L) crops. ICRISAT hypothesized that one of the main reasons why farmers are not using rhizobial inoculants is that they are not getting quality inoculants. Quality of inoculants can be enhanced only if good carrier materials are used for multiplying and maintaining a PGP microbe in it. In order to find a suitable carrier material, a total of 16 rhizobia (six specific for chickpea such as IC-53, IC-59, IC-76, IC-2002, IC-2018 and IC-2099 and five each specific for pigeon pea such as IC-3195, IC-4059, IC-4060, IC-4061 and IC-4062 and groundnut such as IC-7001, IC-7017, IC-7029, IC-7100 and IC-7113) were inoculated on sterilized peat-based carrier material and allowed to multiply at room temperature (28 ± 2 °C) for 2 weeks. At the end of 2-week incubation, formulated peat inoculants were evaluated for rhizobial survival and longevity and this was considered as 0 month. The rhizobial colonies were represented as colony forming units (CFU) and the CFU was enumerated at 1-month interval for a period of 10 months. The results showed that all 16 rhizobia survived and maintained (at least 10^8 CFU/ ml) up to 9 months (except IC-59, IC-2099 and IC-3195; where population started declining from 9th month onwards) in peat formulations. It was concluded that peat-based carrier material is found to be suitable for rhizobia of chickpea, pigeon pea and groundnut (Table 15.1).

15.5 Survival of PGP Microbes in Formulation

The PGP microbe, when inoculated under field conditions, often finds it difficult to establish a niche for survival amongst the predators (such as protozoans) and competitors (such as better adopted native microflora) in addition to unpredictable fluctuating environmental factors. There are also several other factors such as soil type, plant species, type of native bacteria, inoculant density and sunlight that play a key role in declining the inoculated bacterial density and thereby fail to elicit the intended plant response. Sunlight probably is one of the most important factor in reducing bioactivity of aerial PGP microbial agent application to field crops (Slininger et al. 2003) and this has been demonstrated in bacteria (Hughes et al. 1997), virus (Shapiro and Argauer 1997) and fungus (Yu and Brown 1997). Viability of PGP microbe in an appropriate formulation for a certain length of time is essential for commercialization of the technology. For example, *Bacillus*, *Pseudomonas* and *Ochrobactrum* formulations are reported to survive up to 1 year or more in several bio-formulations (Trivedi et al. 2005; El-Hassan and Gowen 2006; Chakraborty et al. 2009). Sawdust, talc powder and rice husk were used as bio-formulations for *B. amyloliquefaciens*, *Serratia marcescens* and *B. Pumilus*, which showed good survivability even up to 9 months of storage (Chakraborty et al. 2013). Hence, it is concluded that survival and establishment of PGP microbe under field conditions in the rhizosphere in competition with native microbial flora is absolutely essential in order to avail the maximum benefits out of it.

Table 15.1 Viability and longevity of 16 rhizobia in peat formulations over 10 months

Rhizobial isolates	Colony forming units (CFU/ml) at different months (values are mean of 3 replications)										
	0	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
Chickpea rhizobia											
IC-53	8.5×10^9	8×10^9	6.5×10^9	2.7×10^9	1.2×10^9	2.9×10^8	2.8×10^8	2.8×10^8	2.5×10^8	2×10^8	1.1×10^8
IC-59	3.3×10^9	2.3×10^9	2.2×10^9	1.7×10^9	2.3×10^8	1.5×10^8	1.3×10^8	1.2×10^8	1×10^8	3×10^7	2.0×10^7
IC-76	4.6×10^9	3.5×10^9	2.3×10^9	2×10^9	1.7×10^9	4.2×10^8	4×10^8	3.9×10^8	3.7×10^8	2.65×10^8	1.2×10^8
IC-2002	16×10^9	12×10^9	6.6×10^9	4.3×10^9	1.5×10^9	2.5×10^8	2.2×10^8	2×10^8	1.7×10^8	0.9×10^8	2.3×10^7
IC-2018	7.5×10^9	7.2×10^9	5.6×10^9	4.2×10^9	16×10^8	4.7×10^8	4.2×10^8	3.6×10^8	3.2×10^8	1.8×10^8	1.3×10^8
IC-2099	4.4×10^9	3.8×10^9	2.4×10^9	2.1×10^9	7×10^8	2.1×10^8	2×10^8	1.7×10^8	1.5×10^8	9×10^7	7.0×10^7
Pigeon pea rhizobia											
IC-3195	16×10^9	8.7×10^9	3.4×10^9	2.5×10^9	1.9×10^8	1.1×10^8	9×10^7	8×10^7	4×10^7	1×10^7	4×10^6
IC-4059	8.6×10^9	7.5×10^9	5.8×10^9	3.6×10^9	1×10^9	5.2×10^8	4.9×10^8	3.4×10^8	2.3×10^8	1.1×10^8	4.1×10^7
IC-4060	18×10^9	17×10^9	7.6×10^9	4.1×10^9	1×10^9	4.5×10^8	4.3×10^8	4.1×10^8	3.1×10^8	1.3×10^8	7×10^7
IC-4061	15×10^9	11×10^9	9.7×10^9	4.2×10^9	1.7×10^9	4.4×10^8	4.2×10^8	4.1×10^8	3.6×10^8	2.2×10^8	1.1×10^8
IC-4062	7.7×10^9	6.3×10^9	2.2×10^9	1.9×10^9	2.3×10^8	3.4×10^8	2.6×10^8	2.1×10^8	1.4×10^8	1×10^8	6×10^7
Groundnut rhizobia											
IC-7001	5.2×10^9	4.8×10^9	4×10^9	2×10^9	1.9×10^8	1.2×10^8	2.3×10^8	2.210^8	2×10^8	1×10^8	2.4×10^7
IC-7017	7.6×10^9	6.6×10^9	3×10^9	2.1×10^9	2.2×10^8	1.7×10^8	1.6×10^8	1.3×10^8	1.1×10^8	7×10^8	3.3×10^7
IC-7029	8.2×10^9	6.8×10^9	5.2×10^9	3.6×10^9	2.0×10^9	5.8×10^8	5.5×10^8	5.2×10^8	4.8×10^8	1.3×10^8	1.5×10^7
IC-7100	6.1×10^9	8.2×10^9	6.3×10^9	3.6×10^9	1.7×10^9	3.7×10^8	3.2×10^8	3×10^8	2.7×10^8	1.3×10^8	7×10^7
IC-7113	8.1×10^9	7.5×10^9	5.4×10^9	4.5×10^9	2.1×10^9	5.5×10^8	5.1×10^8	4.5×10^8	3.7×10^8	1.9×10^8	1.2×10^8

15.6 Regulation and Quality of Commercial Inoculants

An inoculant available in the market should contain sufficient PGP microbe to inoculate plants and produce an economic gain. Many developed countries such as The Netherlands, Thailand, Russia, France, Australia, Canada and the United Kingdom have regulations for inoculant quality which lead to improvements in the quality of commercial inoculants (Bashan et al. 2014). Canada and France has set norms that formulated products should have 10^6 viable rhizobia per seed with no detectable contaminants (Catroux et al. 2001). However, that is not the case in developing countries as most of the inoculants produced are of poor or suboptimal quality. Brockwell and Bottomley (1995) observed that most of the inoculants produced in the world are of relatively poor quality and 90 % of all inoculants have no practical effect on the productivity of crops for which it is used. Upon evaluating 18 different commercial soybean rhizobial inoculants marketed in Argentina, Gomez et al. (1997) found only one liquid inoculant was free of contaminants and carried more than 10^6 *Bradyrhizobium japonicum* while the 17 other inoculants contained between 10^5 and 10^9 contaminants per g product. Olsen et al. (1996) found contaminants in all of the 60 tested commercial inoculants; in addition, the number of rhizobia (5.5×10^5 to 8.1×10^9; per g of product) observed was found to be less than the number of contaminating bacteria (1.8×10^8 to 5.5×10^{10}). The presence and nature of contaminants encountered in inoculants may represent a risk for humans, plants and for the environment, which remains to be assessed.

15.7 Quality of Rhizobial Inoculants Available in the Indian Market – A Case Study

Rhizobia contribute increase in nitrogen fixation and yield in legume crops. Rhizobial inoculants are used where there are no indigenous rhizobia in soil or where the level of the indigenous rhizobia is low. A good quality rhizobial inoculant should be free of contaminants, contains high

number of rhizobia (8.0×10^9 per g of product) and has longer shelf life so that inoculation could be more beneficial for farmers. Even though Bureau of Indian Standards had prescribed certain specifications for rhizobial inoculants to maintain the quality of inoculants (to enable the farmers to obtain certified inoculants), many brands of rhizobial inoculants marketed today in India have been found to vary in quality and reliability. Hence, in order to have a thorough investigation on quality of rhizobial inoculants available in the Indian market, a case study was conducted in 2010–11 by ICRISAT, Patancheru. The major objective of this case study was to check the quality of chickpea rhizobial inoculants available in the market in five states of India.

Rhizobial inoculants of chickpea were purchased from the market in five states of India (Hyderabad in Telangana; Rajanandgoun, Kabirdham and Raipur in Chhattisgarh; Jabalpur, Damoh, Rewa and Satna in Madhya Pradesh; Bhubaneswar in Orissa; and Ranchi in Jharkhand) and stored in refrigerator at 4 °C until processed. A total of 28 samples (14 in May 2010 and another 14 in Nov 2010) were procured and used in this study. All the inoculant samples were analysed for pH, moisture content, purity (plated on yeast extract mannitol [YEM] agar to observe *Rhizobium* like colonies; Log $_{10}$ values), total rhizobial count (Log_{10} values), presence of contamination (Log_{10} values) and further evaluated for their nodulation potential (by plant infection test as per the standard protocol of ICRISAT) in chickpea.

Table 15.2 Identity of the chickpea rhizobial inoculants procured from five states of India

Area	State	Culture type
Batch 1 (May 2010)		
Rajanandgoun	Chhattisgarh	Lignite
Rajanandgoun	Chhattisgarh	Lignite
Rajanandgoun	Chhattisgarh	Lignite
Raipur	Chhattisgarh	Talcum
Bhubaneshwar	Orissa	Liquid
Bhubaneshwar	Orissa	Lignite
Jabalpur	Madhya Pradesh	Lignite
Jabalpur	Madhya Pradesh	Lignite
Satna	Madhya Pradesh	Lignite
Satna	Madhya Pradesh	Lignite
Satna	Madhya Pradesh	Lignite
Satna	Madhya Pradesh	Lignite
Ranchi	Jharkhand	Lignite
Hyderabad	Telangana	Talcum
Batch 2 (Nov 2010)		
Satna	Madhya Pradesh	Lignite
Satna	Madhya Pradesh	Lignite
Satna	Madhya Pradesh	Lignite
Damoh	Madhya Pradesh	Lignite
Jabalpur	Madhya Pradesh	Liquid
Rewa	Madhya Pradesh	Lignite
Rewa	Madhya Pradesh	Lignite
Ranchi	Jharkhand	Lignite
Kabirdham	Chhattisgarh	Lignite
Rajanandgoun	Chhattisgarh	Lignite
Raipur	Chhattisgarh	Talcum
Bhubaneshwar	Orissa	Lignite
Bhubaneshwar	Orissa	Lignite
ICRISAT	Telangana	Peat
Hyderabad	Telangana	Lignite

Of the 28 commercial formulated samples, 23 were made of lignite, three of talcum powder and one of liquid inoculation, whereas the ICRISAT sample was made of peat (Table 15.2). The optimum pH for growing rhizobia is 7.0 while the pH of the rhizobial inoculants from the market varied between 2.1 and 9.4. Among the 28 samples analysed, 13 samples were found highly acidic (pH ranged between 2.1 and 5.8), 7 were alkaline (pH ranged between 8.2 and 9.4) and only 8 samples were found fit for growing *Rhizobium* cultures (Fig. 15.1). The optimum moisture percentage for growing rhizobia in any carrier material is 30 %. Among the 28 rhizobial inoculants, five of them contained less than 15 % moisture while six other sources contained more than 40 % moisture (Fig. 15.2). When the samples were plated on YEM agar to observe *Rhizobium* like colonies, only 15 samples contained *Rhizobium*-like colonies (Fig. 15.3). All but six samples contained contamination and these were found more than the *Rhizobium*-like colonies while the remaining six samples were found to be completely sterile, where neither rhizobia nor any contamination was found (Fig. 15.4). When the 28 samples were analysed for nodulation capability by plant

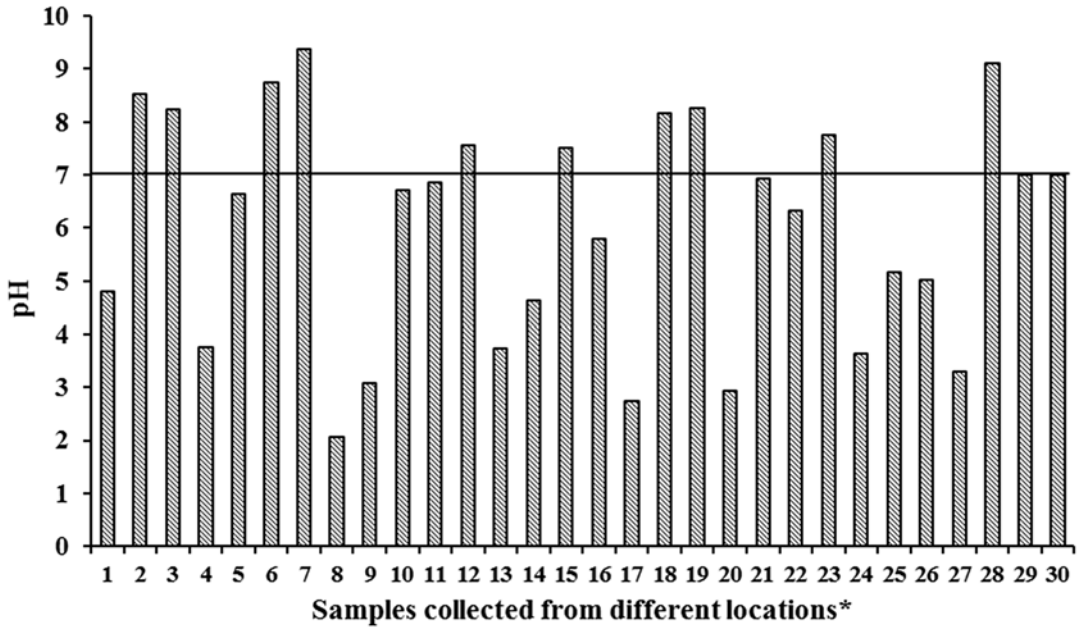

Fig. 15.1 pH of the 28 chickpea rhizobial inoculants procured from five different states of India (Footnote: * = 1–4 from Orissa, 5–11 from Chhattisgarh, 12–13 from Jharkhand, 14–26 from Madhya Pradesh, 27 and 28 from Telangana. Sample numbers 29 and 30 are from ICRISAT)

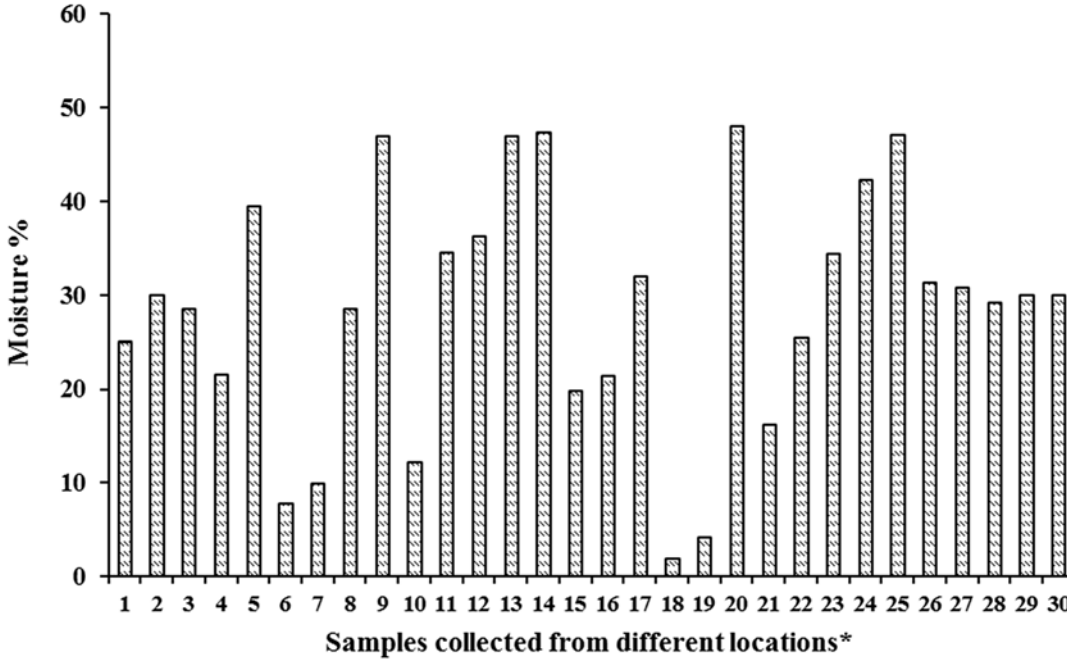

Fig. 15.2 Moisture content of the 28 chickpea rhizobial inoculants procured from five different states of India. (Footnote: * = 1–4 from Orissa, 5–11 from Chhattisgarh, 12–13 from Jharkhand, 14–26 from Madhya Pradesh, 27 and 28 from Telangana. Sample numbers 29 and 30 are from ICRISAT)

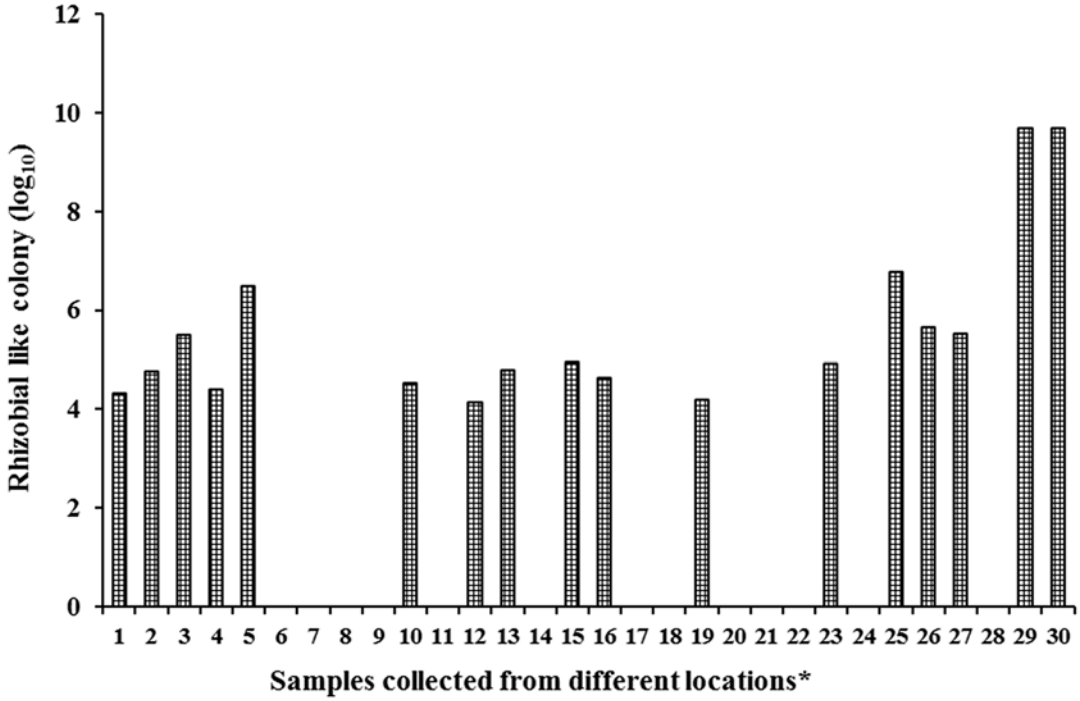

Fig. 15.3 Rhizobia-like colonies present in the 28 chickpea rhizobial inoculants procured from five different states of India. (Footnote: * = 1–4 from Orissa, 5–11 from Chhattisgarh, 12–13 from Jharkhand, 14–26 from Madhya Pradesh, 27 and 28 from Telangana. Sample numbers 29 and 30 are from ICRISAT)

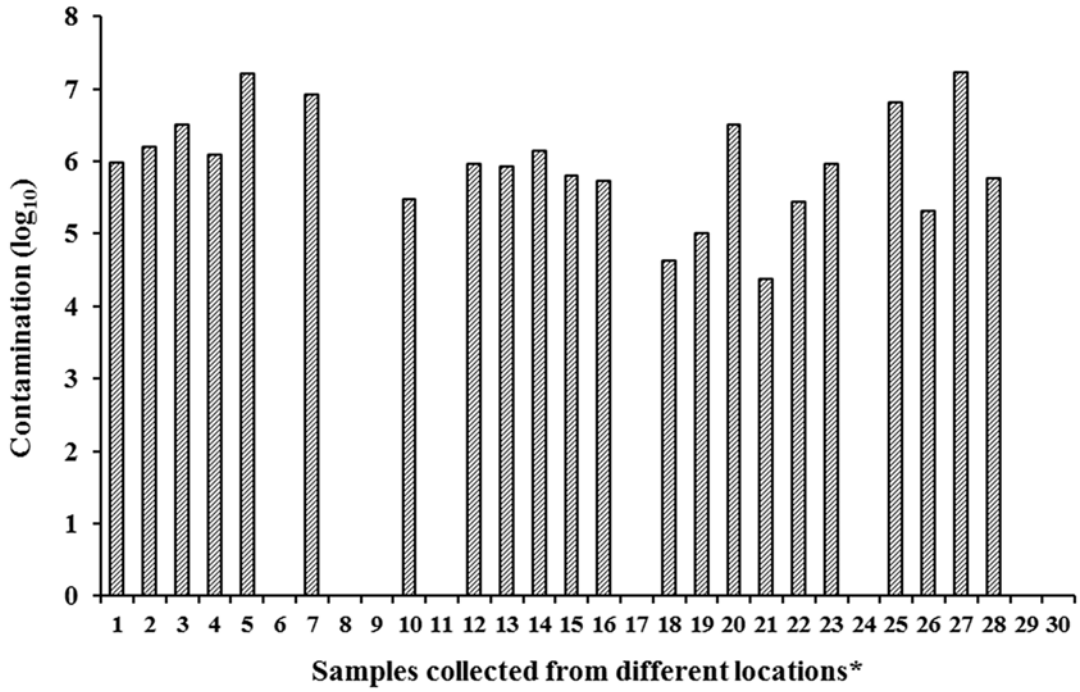

Fig. 15.4 Microbial contaminants present in the 28 chickpea rhizobial inoculants procured from five different states of India. (Footnote: * = 1–4 from Orissa, 5–11 from Chhattisgarh, 12–13 from Jharkhand, 14–26 from Madhya Pradesh, 27 and 28 from Telangana. Sample numbers 29 and 30 are from ICRISAT)

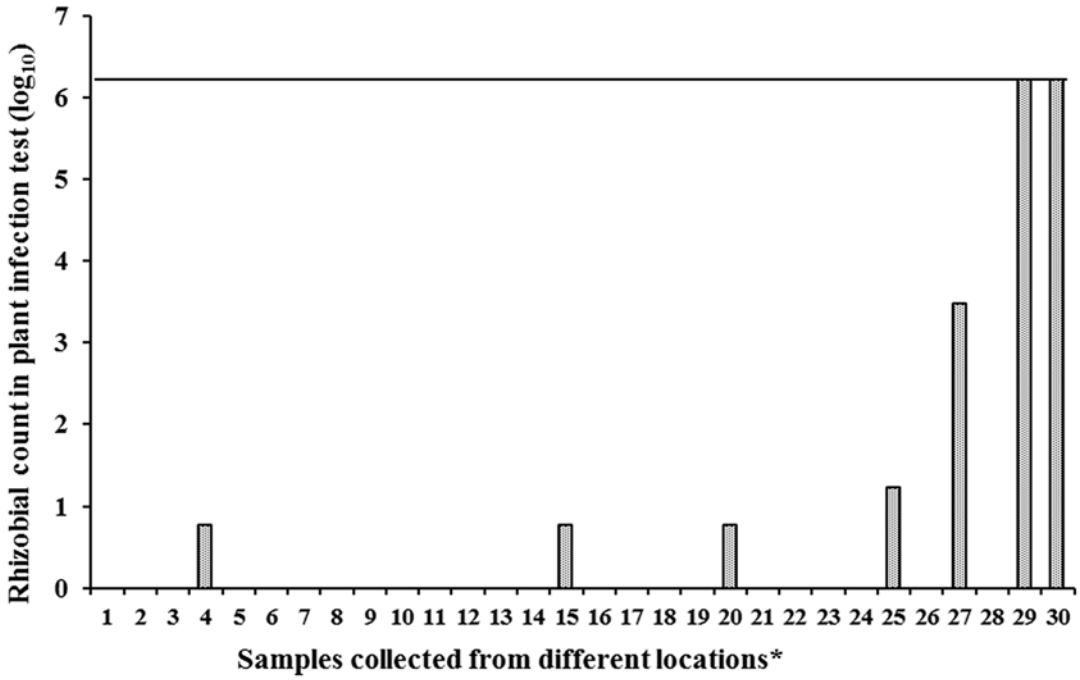

Fig. 15.5 Rhizobial counts in the 28 chickpea rhizobial inoculants procured from five different states of India (Footnote: * = 1–4 from Orissa, 5–11 from Chhattisgarh, 12–13 from Jharkhand, 14–26 from Madhya Pradesh, 27 and 28 from Telangana. Sample numbers 29 and 30 are from ICRISAT)

infection test in chickpea (which tells whether the rhizobia is capable of producing nodules or not), only five rhizobial inoculants were able to produce nodules. Of the five nodulated inoculant samples, rhizobia were found very less (log values 0.78–3.49) compared to positive control (Log values 6.23; where ICRISAT rhizobial inoculants were used (Fig. 15.5). Thus, it was concluded that rhizobial inoculants available in the Indian market contained no or very little rhizobia.

15.8 Conclusion

Application of PGP microbial agents to rhizosphere, phyllosphere and spermosphere particularly under field conditions is less effective or at times totally ineffective. This is mainly due to the type of carrier material used and variation in climatic conditions that suppress growth and survival of PGP microbial agents (Guetsky et al. 2001). Therefore, the efficacy of PGP microbes needs to be improved through the usage of compatible mixed inoculum of PGP microbial agents rather than using a monoculture. Also, for the commercial delivery of a PGP microbe, the beneficial microorganism must be manufactured at industrial scale (in large fermenters), preserved for storage and formulated by means of biocompatible additives in order to increase its survival and stability and to improve the application. The future of PGP microbes depends not only in developing an efficient strain of PGP microbe but also in developing new active ingredients (secondary metabolites from potential PGP microbes). It is not important what formulation is used in developing a PGP microbe but it is important that the formulation has a product shelf life with retained biological activity for up to a year preferably at ambient temperatures. The development of new formulation(s) for PGP microbes is a challenging task as it requires greater effort in terms of funding and research. However, continued research may lead to improvements in for-

mulations for the best PGP microbes/ products. Also, conducting formulation research in the private sector will greatly expedite progress in this critical area for advancing the successful incorporation of PGP microbes and/or their products. Finally, the acceptance of PGP microbes as nutrient/pest management tools is dependent on the development of low-cost bio-agents/products which provide consistent efficacy.

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A Novel Tool of Nanotechnology: Nanoparticle Mediated Control of Nematode Infection in Plants

16

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Abstract

The designing and synthesis of nanoparticle research has been established as an area of intense and dynamic scientific area of research and academia. Due to their unusual physical and chemical properties, nanoparticles have drawn a tremendous amount of attention. Nanobiotechnology holds the promise of controlled release and site-targeted delivery of agrochemicals. A plethora of chemical, physical and biological techniques continues to evolve leading to the production of noble metal nanoparticles. Alongside, biological organisms including plant, fungi and bacteria are an ideal source for green synthesis of nanoparticles with desired shape and size. Some of these nanoparticles also have nematicidal properties, which apply to numerous genera of plant parasitic nematodes and also to plant pathogenic fungi and bacteria. Plant parasitic nematodes are major agricultural pests causing crop losses worth hundreds of billions dollars annually worldwide. Traditional control measures depend upon highly toxic nematicides. In theory, seed treatment for nematode control is optimal, but is largely ineffectual due to poor rhizosphere delivery. Active ingredients of various nanoparticles have also shown evidence of being potentially effective nematicides, which makes these nanoparticles a suitable noble source to control nematode infection in plants. Although very limited reports are available on the use of nanoparticles to control plant nematodes, very encouraging reports are there and research in this area is getting lot of attention. This chapter focuses on the nanoparticles, their synthesis, properties and their use to control nematode infection in plants.

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_16

Keywords
Nanoparticles • Nematode • Fungi • Biocontrol • Inoculants • Nematicides

16.1 Introduction

Nematodes, the most ubiquitous soil organisms found on Earth, are important pests of many cultivated plants. Plant parasitic nematodes cause global losses to crop plants with an estimated loss of 125 billion dollars per year in the tropics (Chitwood 2003). The *Meloidogyne* genus belongs to a group of root-knot nematodes (RKN) and is represented by over 90 species that have been described so far (Moens et al. 2009). Severe infestations of the nematodes cause total crop loss, while yield losses of 5–20 % occur in some crops despite routine use of nematicides. The *Meloidogyne* spp. are sedentary obligate endoparasitic nematodes and are among the most damaging agricultural pests that cause major economical damage globally to a wide range of crops (Sikora and Greco 1993). They are widely distributed in the tropics and subtropics and are common in temperate regions. The disease cycle instigates when second-stage juveniles hatch from eggs, move through the soil and invade root tips forming multinucleate giant cells called galls. Galling restricts root volume and obstructs the normal translocation of water and nutrients within the plant, thereby exhibits above-ground symptoms of stunting, wilting and chlorosis of plants. This damage also inclines plants to attacks by other soil-borne pathogens resulting in a loss in yield and a reduction in the quality and marketability of plant products that are produced underground.

The current major issue concerning nematode damage to plants is the lack of effective chemical treatment methods. The public concern over the chemical nematicides is not only their toxicity and health and environmental risks associated with it but also their loss of efficiency after prolonged use. So several control strategies alternative to chemical control, such as host plant resistance, rotation with non-hosts, destruction of residual crop roots and use of biocontrol agents, have been reported to effectively control root-knot nematodes (Whitehead 1998). Development of resistant cultivars and rootstocks has also been slow because of the genetic diversity of *Meloidogyne* and problem for making interspecific and intergeneric crosses of some plant species and it has proved difficult to prevent the transfer of deleterious genes (Stirling and Stanton 1997). The resistance involves a gene which acts by destroying the giant cells produced by the developing nematode, thereby preventing nematodes from obtaining nutrients from these cells. Crop rotation also has potential for use in managing root-knot nematode but its value is limited by the specificity of resistance genes (Stirling and Stanton 1997). However, it is not always possible to use species identification to determine host range, as populations with different host ranges can occur within one *Meloidogyne* species. Therefore, considerable effort is being committed to the development of alternative control strategies. Biological control using microbial antagonists is one potential alternative to chemical nematicides (Almaghrabi et al. 2013). But this has also disadvantages of possessing a narrow range of treatment effects and a lack of reliability under varying environmental conditions. Moreover, use of plant growth promoting rhizobacteria (PGPR) seems to promote growth through suppression of plant disease-causing organisms, but this approach has also its limitations due to possessing shorter shelf life and a lack of reliability under varying environmental conditions. Therefore, the lack of options for managing nematodes poses a serious problem in plant disease management.

Nanotechnology, a buzzword of present day science, is an immensely developing field, owing to its wide-ranging applications in different areas of science and technology. Any material when attenuated at nanometre scale (<100 nm) exhibits new properties that are entirely different from its bulk counterpart due to small size and high

surface to volume ratio. Nanoparticles have better chemical reactivity, biological activity, catalytic behaviour and high mobility in the body of an organism including cellular entry (Rajan 2004), which may be exploited for the benefit of mankind. Nowadays biosynthesized nanoparticles are more important in agricultural sciences as they are naturally encapsulated by mother protein which makes them stable (>90 days).

Although nanotechnology is the second trend after biotechnology for innovative research in many areas of plant pathology, in plant pathogen, nanotechnological application is still in its infancy. Nanoformulations are viewed to be safer and environment friendly option for plant disease management, but high toxicity of nanoparticles involuntarily released in the environment may pose greater risk to man and other organisms. Therefore, nanotechnological progress is to be viewed with caution and dealt accordingly. The addition of silver nanoparticles (AgNP) for the prevention of bacterial growth to existing products was amongst the first use of nanoparticles in clothing, bandages, disinfectants and food packaging (Seltenrich 2013). AgNP has also shown evidence of being a potentially effective nematicide (Roh et al. 2009), and its toxicity is associated with induction of oxidative stress in the cells of targeted nematodes (Lim et al. 2012). Moreover, it has been reported that chronic exposure of Al_2O_3 nanoparticles shows toxicity against the nematodes with end-points of lethality, growth, reproduction, stress response and intestinal auto fluorescence (Wang et al. 2009; Wu et al. 2011). However, a limited research on nanoparticles application against controlling of nematode has been reported till date. Therefore, approaches are needed to offer new capabilities for preventing or treating plant pathogenic nematodes by using nanoparticles, which would result in the more effective monitoring in the ways not currently possible.

In the present article, we have reviewed processes based on the applications of nanotechnology made in plant pathology for detection and management of plant pathogenic nematodes which offer plant pathologists and nanotechnologists immense possibility of using nanoparticles for plant disease management.

16.2 What Are Nanoparticles?

Nanotechnology is an immensely developing field as a result of its wide-ranging applications in different areas of science and technology. The word nano' is derived from a Greek word meaning dwarf or extremely small (Rai et al. 2009). The term nanotechnology was first coined by Taniguchi (Taniguchi 1974), which largely deals with creation, synthesis and application of nano size particles (1-100 nm) of any material. Nanobiotechnology is a multidisciplinary field and involves research and development of technology in different fields of science like biotechnology, nanotechnology, physics, chemistry and material science (Huang et al. 2007; Rai et al. 2009).

By definition, nanoparticles are atomic or molecular aggregates with at least one dimension between 1 and 100 nm (Ball 2002; Roco 2003), which can drastically modify their physicochemical properties compared to the bulk material (Nel et al. 2006). Nanoparticles are of great scientific interest as they are effectively a bridge between bulk materials and atomic or molecular structures. Because of this very small size scale, they trigger the biochemical activity due to their distinctive crystallographic nature that increases surface to volume ratio, hence the scope of reactivity (Osaka et al. 2006). Nanoparticles can be composed of single constituent material or be a composite of several materials leading to an immense chemical diversity in the form of metals, metal oxides, semiconductors, polymers, carbon materials, organics or biological. Similarly, immense diversity is observed in the morphology of nanoparticles like flatness, different shapes (triangular, spherical, rod, tubes, needles, cubes, cylinders, etc.) and aspect. The large specific surface area of nanoparticles is the origin of a number of their unique applications. The enormous diversity of the nanoparticles arising from their wide chemical origin and nature, various shapes, sizes and morphologies, the medium in which the particles are present, the state of dispersion of the particles and numerous possible surface modifications and functionalization of the nanoparticles can be subjected to make this an important active

field of nanoscience. Nanoparticles serve as the fundamental building blocks for a wide variety of potential applications in biomedical, optical and electronic fields. Moreover, nanoparticles have the potential to improve the environment, both through direct applications of those materials to detect, prevent and remove pollutants, as well as indirectly by using nanotechnology to design cleaner industrial processes and create environmentally responsible products.

16.2.1 Synthesis of Nanoparticles

The method for the synthesis of nanoparticles is the one of the important areas of nanoscience and nanotechnology and therefore mainly three broad categories, namely, physical, chemical and biological procedures have been adopted. However, physical and chemical methods are weighed down with various problems including use of harmful chemical agents, production of hazardous commodities, economically expensive chemicals and technically laborious. Nowadays, there is growing need to develop environment-friendly approach towards nanoparticle synthesis process which have advantages over conventional methods involving toxic chemical agents in the synthesis route (Song and Kim 2009). The formation of nanoparticles mediated by biological route is considered as healthier method than any other approach because catalytic and functional information obtained under close to optimal conditions can help to understand the biochemical and molecular mechanisms of nanoparticles formation. Therefore, plants, algae, fungi, bacteria and viruses have been used to achieve the production of inexpensive, energy-efficient, and eco-friendly metallic nanoparticles.

16.2.1.1 Physical Approach

In physical processes, metal nanoparticles are generally synthesized by evaporation-condensation and laser ablation methods. Various metal nanoparticles such as silver, gold, lead sulphide and fullerene have previously been synthesized using the evaporation-condensation technique, flame pyrolysis, high-temperature evaporation, microwave irradiation and plasma synthesis (Gurav et al. 1994; Magnusson et al. 1999; Kruis et al. 2000). The absence of solvent contamination in the prepared thin films and the uniformity of nanoparticles distribution are the advantages of physical approaches in comparison with chemical processes (Kruis and Rellinghaus 2000; Magnusson et al. 1999). Forster et al. (2012) investigated that copper nanoparticles with a size range of 4–50 nm could be generated in an arc furnace by the evaporation-condensation method. They also reported that the evaporation-condensation process is advantageous because it allows direct synthesis using pure metals as starting materials avoiding reactions of expensive and potentially poisonous precursors. One of the biggest challenges for the large scale commercial application is the development of a reliable method for the large scale synthesis of nanoparticles over a range of composition, uniform sixe and high monodispersity. For development of any nano device based on nanoparticles, large yield and controlled synthesis of nanoparticles is the major component. Jung et al. (2006) demonstrated that silver nanoparticles could be synthesized via a small ceramic heater that makes possible synthesis of small nanoparticles in high concentration, whereas Chu et al. (2007) reported a solution dependent high yield synthesis of cobalt-doped ZnO nanorods.

On the other hand some of the unique properties of laser make it a very important tool for nanofabrication. Laser radiation proved to be one of the most efficient physical methods for nanofabrication. The method consists of ablation of a target by an intense laser radiation on a solid target in a liquid, yielding to melting and then ablation of the material from target leading to the ejection of atoms and nanoparticles and nanostructures. In the last decade, laser ablation in liquids seems to be a unique and efficient technique due to the following advantages:

1. It can be applied unanimously with an almost unlimited variety of materials and solvents to generate nanoparticles.
2. Trouble-free collection of the particles compared with fabrication in gas.

3. It can yield about 100 % pure particles without using chemical precursors and have inherent stochiometry.
4. Nanoparticle colloids are not inhalable and thus lead to an improved occupational safety.
5. Availability of ablation parameters are there for controlling the size and shape of nanomaterials.

Silver nanoparticles could be synthesized by laser ablation of metallic bulk materials in solution (Mafune et al. 2000, 2001; Kabashin and Meunier 2003; Tsuji et al. 2003; Sylvestre et al. 2004). Singh and Gopal (2007) reported the synthesis of highly stable colloidal metallic zinc nanoparticles using pulsed laser ablation in an aqueous solution of suitable surfactant. It was also reported that silver nanoparticles synthesized in natural polymer such as Ct, Gt and St using laser ablation technique have more efficiency and stability (Zamiri et al. 2012). Kadhim et al. (2012) synthesized high purity gold nanoparticles at room temperature by using pulsed laser ablation in NaOH solution. Therefore, from the above discussion it can be assumed that pure and uncontaminated metal colloids for further applications can be prepared by this technique.

16.2.1.2 Chemical Approach

The chemical approach is mostly done for the commercial synthesis of nanoparticles. Different chemical methods, like reduction method, colloidal method, sonochemical method etc., have been adopted for the synthesis of nanoparticles, but, however, choice of the methods may vary with the material. The most common approach for synthesis of nanoparticles is chemical reduction by organic and inorganic reducing agents. Different reducing agents have been used for the reduction of metallic ions to produce metallic nanoparticles. In 1857, Michael Faraday for the first time reported the synthesis of colloidal gold using chemical reduction route (Khan and Rizvi 2014). Various workers have reported the use of chemical reduction method for the synthesis of stable sized metal nanoparticles (Song et al. 2004; Abou El-Nour et al. 2010; Ghorbani 2014). In chemical reduction method, protective agents are used as stabilizer to stabilize dispersive nanoparticles and protect them for avoiding agglomeration (Oliveira et al. 2005; Guzman et al. 2009; Kheybari et al. 2010; Dang et al. 2011; Usman et al. 2013).

Micro-emulsion/colloidal method is one of the recent and ideal techniques for the preparation of inorganic nanoparticles (Yu et al. 2010). This technique promises to be one of the versatile preparation method which enables to control the particle properties such as mechanisms of particle size control, geometry, morphology, homogeneity and surface area (Pileni 2003; Hu et al. 2009; Malik et al. 2012). The microemulsion method has been used to synthesize colloidal metals, colloidal Fe_3O_4, colloidal AgCl, nanocrystalline Fe_2O3, TiO_2, Al_2O_3, Pt Nanoparticle and starch nanoparticles (Boutonnet et al. 1982; Bandow et al. 1987; Ayyup et al. 1988; Lal et al. 1998; Chin et al. 2014; Martinez-Rodriguez et al. 2014).

Moreover, currently sonochemical method has been used extensively to generate nanoparticles of a much smaller size, with controllable morphologies and higher surface area than those reported by other methods. Zhu et al. (2000) reported the synthesis of about 3 nm size ZnSe nanoparticles by the sonochemical irradiation of an aqueous solution of selenourea and zinc acetate under argon. A variety of nanoparticles like Fe/Co alloy nanoparticles, Ag, iron oxide, colloidal silver nanoparticles and ZnO nanoparticles were also synthesized by using the sonochemical method (Li et al. 2003; Manoiu and Aloman 2010; Roshan et al. 2011; Azadeh et al. 2011).

16.2.1.3 Biological Approach

Biosynthesis approach is a current addition to the large repertoire of nanoparticles synthesis methods as it is not only inexpensive but also less cumbersome, time consuming, complicated and mostly non-toxic. This route for nanoparticle synthesis includes far less requirement of energy, less wastage of inputs and more practical control of constituent ingredients. Natural bio resources such as plants and microorganisms possess great potential in the synthesis of nanoparticles.

Table 16.1 summarizes different bacterial, fungal, viral, yeast and plant species that have been analysed for the intracellular or extracellular synthesis of several kinds of nanoparticles. However, the nanoparticles obtained from different species show broad distribution among particle size.

16.3 Application of Nanoparticles

Nanoparticles are of immense interest due to their extremely small size and large surface to volume ratio, which lead to both chemical and physical differences in their properties. Nanoparticles synthesized by various techniques exhibit size- and shape-dependent properties which have received special attention because they have found potential application ranging from biosensing and catalysts to optics, antimicrobial activity, computer transistors, chemical sensors, medical imaging, nano-composites, filters, drug and gene delivery system, etc. Table 16.2 summarizes the different types of nanoparticles, their applications along with their source of synthesis.

16.3.1 Application of Nanoparticles in Agriculture

With the growing realization that the conventional farming techniques would not increase a farm's productivity, attempts to use nanotechnology in agriculture have begun. Agriculture, in spite of being the backbone of third world countries, unfortunately is facing various global challenges like climate changes, increasing human population, urbanization, sustainable use of resources and environmental issues. Traditional agricultural practices lead to the degradation of the soil quality and contribute to the eutrophication of water ecosystem and lead to the increased fertiliser use, more irrigation and higher energy inputs to maintain the productivity on the degraded soil. Therefore, nowadays there has been significant interest in using nanotechnology in agriculture as it has a great potential in transforming conventional agricultural practices and food production with novel tools. Nanobiosensors and other smart delivery systems will also help the agricultural industry to fight against different crop pathogens and provide an efficient means to distribute pesticides and fertilizers (Derosa et al. 2010) in a controlled fashion with high site specificity. However, the current degree of understanding of nanomaterial fate and effects in agricultural systems is poor. Nanosensors development can help in determining the required amount of farm inputs by indicating the nutrient or water status of crop plants which makes the farmers to apply nutrients, water or crop protection (insecticide, fungicide or herbicide) only where necessary (Prasad et al. 2014). Moreover, nano-pesticides, nanofungicides and nanoherbicides are being used in agriculture (Owolade et al. 2008). Nano-labelled water filters have been used in remediation of waste sites in developed countries (Karn et al. 2009). Liu et al. (2006) reported that the use of nano-encapsulated fertilizers can regulate the release of fertilizer consumption depending on the requirements of the crops and minimize environmental pollution. Bhattacharyya et al. (2011) reviewed applications of nanotechnology in different fields like nano-food, nano-food packaging and nano-farming and also highlighted on their effects on ecological balance. In general, scientific application of nanotechnology has great potential to change agriculture scenario by increased productivity. This can only be achieved by allowing better management and conservation of inputs to plant production. Public awareness about the advantages and challenges of emerging nanotechnology and its products will lead to better acceptance of the technology. Thus, nanotechnology can be an indispensable and important part of the future agriculture and food industry.

16.3.2 Nanoparticles for Controlling Nematode Infection in Plants

Soil nematodes infecting plants are one of the most devastating parasites worldwide and cause crop damages worth billions of dollars. Nematodes attack plant root system and feed on the plant nutrients causing lose in the crop yield

Table 16.1 Different biological organisms used for the synthesis of nanoparticles

Strains exploited	Types of nanoparticle synthesized	Location of synthesized nanoparticles	References
Bacteria			
Pseudomonas aeruginosa	Gold	Extracellular	Husseiny et al. (2007)
Lactobacillus	Titanium dioxide	Intracellular	Jha et al. (2009)
Plectonema boryanum	Silver	Extracellular	Lengke et al. (2007)
Bacillus subtilis and Escherichia coli	Zinc	—	Meruvu et al. (2011)
Bacillus megaterium	Silver, Lead and Cadmium	Extracellular	Prakash et al. (2010)
Enterococcus sp.	Cadmium sulfide	Extracellular	Rajeshkumar et al. (2014)
Fungi			
Aspergillus flavus	Ag	Intracellular	Vigneshwaran et al. (2007)
Trichothecium sp.	Au	Intracellular	Ahmad et al. (2005)
Volvariella volvacea	Ag and Au	Extracellular	Philip (2009)
Penicillium fellutanum	Ag	Extracellular	Kathiresan et al. (2008)
Aspergillus terreus	Zinc	—	Baskar et al. (2013)
Fusarium oxysporum	Silver	—	Birla et al. (2013)
Viruses			
Tobacco mosaic virus (TMV)	SiO_2, CdS, PbS, and Fe_2O_3	—	Lee et al. (2002)
M13 bacteriophage	ZnS and CdS	—	Mao et al. (2003); Dameron et al. (1989)
Cowpea mosaic virus (CMV), an engineered CMV	Iron-platinum nanoparticle (30 nm diameter)	—	Shah et al. (2009)
Red clover necrotic mosaic virus	Au, $CoFe_2O_4$, and CdSe nanoparticles	—	Loo et al. (2007)
Cowpea chlorotic mottle virus (CCMV)	Gold nanoparticle	—	Slocik et al. (2005)
Tobacco mosaic virus (TMV)	Nanowire of nickel and cobalt	—	Young et al. (2008)
Yeast			
Yeast strain MKY3	Ag	Extracellular	Gardea-Torresdey et al. (2003)
C. glabrata	CdS	Intracellular	Kowshik et al. (2002)
Yarrowia lipolytica NCYC 789	Au	—	Nair et al. (2013)
S. cerevisiae	Sb_2O_3	Intracellular	Jha et al. (2009)
Rhodotorula mucilaginosa	Cu	Intracellular	Salvadori et al. (2014)
Plant extract			
Azadirachta indica (neem)	Ag/Au bimetallic	—	Yang et al. (2010)
Jatropha curcas L. latex	Pb	—	Santhoshkumar et al. (2011)
Cinnamomum camphora	Au and Pd	—	Joglekar et al. (2011)
Aloe vera	Au and Ag	—	Daisy and Saipriya (2012)
Nerium oleander	Cu	Intracellular	Gopinath et al. (2014)
Vitis vinifera	Se	—	Sharma et al. (2014)

and even death of the plants. Nematode infected plants are prone to the secondary infection from different bacteria and fungi which make the control of the plant parasite more important. There are very few effective methods to control plant nematodes, but they are not very effective. Most commonly used measure for the nematode infection in the plants is crop rotation or cultivation of the trap crops which are later destroyed by burning. Conventional breeding methods are also not very effective against nematodes as they require many years to have a resistant variety, and, moreover, in nature very few crop plants have got nematode resistant varieties. Traditional breeding practices, biological control methods and chemical nematicides can reduce nematode infection to some extent, but often don't provide long-term suppression of nematodes. Nanotechnology has the potential to change the entire scenario of plant pathology with the help of new tools developed for the treatment of plant diseases, rapid detection of pathogens using nano-based kits, improving the ability of plants to absorb nutrients, etc. Nanoformulations are seemed to be a safer and environment friendly outlook for plant disease management. Though there is surfeit of examples of using nanosensors as a detection tool for animal/human pathogen, in plant pathogen, nanotechnological application is still in its infancy.

Plant-parasitic nematodes were estimated as a potent plant pathogen by causing 12 % yield loss in various crops (Prabhu et al. 2009). But no effective nematicides are available due to their toxicity and less efficacy towards them. Therefore, nowadays, nanotechnological approach has been implicated against plant-parasitic nematodes as they have multisite mode of action against the nematodes and no phytotoxicity. Pluskota et al. (Pluskota et al. 2009) reported that the silica nanoparticles were capable of inducing degeneration of reproductive organs in *Caenorhabditis elegans*. It was reported that the mortality rate of invasive larvae of entomopathogenic nematodes depended on the concentration and the time of exposure to nanoparticles (Kucharska and Pezowicz 2009; Kucharska et al. 2011). There is also report on the toxicity performance of nanoparticles like titanium oxide, ZnO, Al_2O_3, silver and Fe_2O_3 against *C. elegans* nematode (Wang et al. 2009; Roh et al. 2010; Ellegaard-Jensen et al. 2012). Jo et al. (2013) reported the application of silver nanoparticles significantly reduced the nematode population and improved the turfgrass quality. Ardakani (2013) investigated the nematotoxicity of silver, silicon oxide and titanium oxide nanoparticles on second-stage juveniles (J2) of the root-knot nematode, *Meloidogyne incognita*, in laboratory experiments. In this experiment, it was seen that all treatments of AgNP and 0.02 % TiO_2NP completely controlled *M. iIncognita*. Cromwell et al. (2014) also reported that AgNP possess nematicidal activity against *M. incognita* that may provide an alternative to high-risk synthetic nematicides in turfgrass without phytotoxicity.

In addition to nematicidal effect of silver nanoparticles against root-knot nematodes which applies to the other genera of plant parasitic soil nematodes, these nanoparticles are also toxic to plant pathogenic fungi and bacteria. Node of action of these silver nanoparticles is not specific but is associated with disrupting the cellular mechanism at multiple level and severely effect membrane permeability, ATP synthesis and response to oxidative stress in these organisms (Roh et al. 2009; Ahamed et al. 2010; Lim et al. 2012). Siver nanoparticles are considered to be broad spectrum antimicrobial, antifungal and nematicidal agents. AgNP possess a very potential nematicidal activity that may prove an effective alternative to high-risk chemically synthesized nematicides and unreliable biological agents.

16.4 Conclusion

Nanotechnology has a great impact on biological sciences, and more and more nanomaterials are used in medicine, pharmacy, food industry and agriculture. Plant diseases caused by various agents are among the major factors limiting crop productivity throughout the globe. The adaptation of new emerging technologies such a nanotechnology in various fields of agriculture will

Table 16.2 Different types of nanoparticles and their applications

Sl No.	Area	Types	Source	Applications	Reference
1	Commercial	Magnetite	Canola oil	Removal of heavy metals such as As and Cr for cleaning polluted water.	Kumar (2014)
				Use as a green catalyst.	
				Used in target drug delivery to enhance the curative effect and minimize the adverse effects of an anticancer drug	
2		Silver/Gold	plant extract	Produce insecticides and insect repellants.	Adhikari et al. (2013)
				control the mosquito population	
3		Silver nanorods	Industrial waste of milk	Used to increase the shelf-life of raw milk without sacrificing the physical, chemical, and nutritive values of the milk	Sivakumar et al. (2013)
4		Solid lipid	nanostructured lipid carriers	Dermal application of cosmetics and pharmaceutics, i.e., controlled release of actives, drug targeting, occlusion and associated with it penetration enhancement and increase of skin hydration	Pardeike et al. (2008)
5		Hydroxyapatite	hydrated calcium nitrate and triethylphosphite ($P(OEt)_3$)	For the treatment and removal of heavy metals from industrial wastewater such as Pb(II) and Cd (II)	Foroughi and Zarie (2013)
6		copper oxide	carbon paste electrode	Determination of thiourea in fruit juice, orange peel and industrial waste water	Tian et al. (2013)
7		Silver and gold	cashew nut shell liquid	Antibacterial activity, minimum inhibitory concentration and minimum bactericidal concentration on bacteria associated with fish diseases	Velmurugan et al. (2014)
8		Palladium	Soybean (Glycine max) leaf extract	As catalysis in degradation of azo dyes	Patel et al. (2012)
9		Silver and gold	Blackberry, blueberry, pomegranate and turmeric extract	For the delivery of usefull oxidants and cancer chemo-preventive space agents based on curcuminoids	Nadagouda et al. (2014)
10		Silver	Black carrot root extract	For large scale production of AgNps	Abubakar et al. (2014)

(continued)

Table 16.2 (continued)

Sl No.	Area	Types	Source	Applications	Reference
11	Agricultural	Gold	*Salicornia brachiata* plant extract	Higher antibacterial activity	Ahmed et al. (2014)
				Medicine for the treatment of itches	
				Combination with ofloxacin shows superior bactericidal property	
				Efficient catalysis for reduction of 4-nitrophenol to 4 -aminophenol	
12		Gold	Fruit extract of *Terminalia arjuna*	Enhance germination of seed	Gopinath et al. (2014)
				improve the mass propagation of endangered medicinal plant	
13		Cellulose	Pineapple leaf	Reuse of agro waste	Santos et al. (2013)
		Nanocrystal		Improve pineapple cultivation, generate extra income for farmers and also help in agribusiness diversification	
14		Silver	Parthenium leaf extract	Utilization of weed	Parashar et al. (2009)
				Eco-friendly nanoparticles in bactericidal,	
				Wound healing and other medical and electronic applications	
15		Nanosilver	*Cassia auriculata* leaf extract	Inhibiting harmful fungi and bacteria present on seeds and as an alternative source of fertilizer that may improve sustainable agriculture	Parveen and Rao (2014)
16		Silver	Leaf and stem extract of *Piper nigrum*	Antibacterial activity against agricultural plants pathogens	Paulkumar et al. (2014)
17		Silver and silica	Chemically synthesized	In pest management program of *C. maculatus*.	Rouhani et al. (2012)
18		Silver	Shewanella algae bangarama (marine bacteria)	In agriculture and marine pest control	Babu et al. (2014)
				To control biofouling in marine ecosystem	

19	Therapeutic	Magnetic	Ferrous hydroxide	Treatment of inflammatory joint disease	Gupta and Gupta (2005)
			Ferrous + ferric hydroxide	Cellular labeling, cell separation, detoxification of biological fluids, tissue repair, drug delivery, magnetic resonance imaging, hyperthermia, magnetofection	
20		Silver	Bacteria, fungi and plant extract	Anti-inflammatory agents for various therapy	Brady et al. (2003); Lee et al. (2007); Prabhu and Poulose (2012)
				bioimaging	
				used in disinfectants	
21		Silver	Latex of *Calotropis gigantea*	Therapeutic application in context with nano drug formulation	Rajkuberan et al. (2015)
22		Silver	Latex and leaf extract of *Ficus sycomorus*	Controlling pathogenic bacteria with better dispersion and better efficiency in aqueous environment	Salem et al. (2014)
23		Ultra – small solid lipid nanoparticles	Pharmaceutical lipids	Enchance the pulmonary delivery and anti-virulence efficacy of novel quorum sensing inhibitors	Nafee et al. (2014)
24		Lipophilic	Microfluidic – generated precursor micro droplets	Medical imaging and therapy for cancer detection and treatment	Seo and Matsuura (2014)
25		Gold	Bax inhibiting peptide	Treatment of traumatic brain injury, spinal injury, neuronal and cardiac ischemic events.	Muroski et al. (2014)
26		Silver	*Dendrophthoe falcate*(L.f) Ettingsh	Against human breast cancer cells	Sathishkumar et al. (2014)
27		Quantam dots	Muran from *Halomonas Maura*	Safe fluorescent agent for invitro imaging and clinical diagnostics	Raveendran et al. (2014)
28		Gold	*Amarnthus spinosus* leaf extract	In drug delivery and molecular imaging such as magnetic resonance imaging (MRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT)	Das et al. (2012)

(continued)

Table 16.2 (continued)

Sl No.	Area	Types	Source	Applications	Reference
29	Environmental	Fe_3O_4 magnetic nanorods	*Punica Granatum* rind extract	Removal of Pb(II) from aqueous environment	Venkateswarlu et al. (2014)
30		Nanoscale iron particles	Fe(II) and Fe(III)	For the transformation and detoxification of a wide variety of common environmental contaminants, such as chlorinated organic solvents, organochlorine pesticides and PCBs	Zhang (2003)
31		Carbon	Carbon rich agricultural wastes	capability to detect various analytes of the environment	Sing Muk Ng (2014)
				portray good fluorescence property that enables the integration onto optical sensing transducers	
				acts as alternative for environmental monitoring.	
32		Silver	Polydimethylsiloxane	the ability to efficiently remove H_2S from mixed gas streams and offers a plethora of environmental, agricultural, biotechnology and energy conversion applications	Nour et al. (2014)
33		Silver	Trisodium citrate	Mineralization of pesticides in water.	Manimegalai et al. (2012)
34		PtRu Nanoparticles	hydrosilylation reaction	Act as a catalyst for direct methanol fuel cell	Huang et al. (2005)

revolutionize agriculture. The limited studies so far conducted on the application of nanotechnology for the improvement of the agriculture are sufficient enough to warrant potential use of this technology in agriculture. The research has shown that direct application of the various nanoparticles significantly suppressed the plant diseases caused by bacteria, fungi and nematodes. Different nanoparticles possess unique nematicidal activity that may provide an effective alternative to non-environmental friendly high-risk synthetic nematicides of unreliable biological control agents.

Acknowledgements The authors are thankful to Dr. D Ramaiah, Director, CSIR- North-East Institute of Science & Technology, Jorhat, Assam, India, and Dr. SC Nath, Chief Scientist, MAEP division for their consistent support to carry out this work. BSB is thankful to Council of Scientific & Industrial Research (CSIR), Government of India, New Delhi, for financing the network project – PMSI (BSC-0117).

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Understanding the Role of Nanomaterials in Agriculture

17

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Abstract

Nanotechnology offers immense opportunities for improvement in the quality of life through applications in agriculture and the food systems. Development of nanotechnology-based novel agro-products, viz., nanosensors, nano-fertilizers, nano-pesticides and nanoformulations of biocontrol agents, is currently a subject of intense investigation. A variety of nanomaterials has been recommended for use in agriculture, in order to help reduce the consumption of agrochemicals by use of smart delivery systems, minimize the nutrient losses and increase the yield through optimized water and nutrients management. Nanotechnology-derived devices have also been explored in the areas of plant breeding and genetics. Additionally, the agricultural products and/or by-products can be utilized as a source for developing bio-nanocomposites. Nevertheless, the potential advantages of nanotechnology applications in the agricultural sector are still marginal, and have not been commercialized to a significant extent, as compared to other industrial sectors. Researches in the area of agricultural nanotechnology are being extensively pursued in quest for the solutions to the agricultural and environmental challenges, such as sustainability, increased productivity, disease management and crop protection through innovative techniques for monitoring, assessing and controlling the agricultural practices. This chapter provides a basic knowledge about the role of nanotechnology in developing sustainable agriculture and environment, and eventually in the welfare of human society, at large, in the near future.

Keywords

Nanoparticles • Nano farming • Green synthesis • Nano-pesticides

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_17

17.1 Introduction

Significant developments in the agricultural sector have been witnessed in recent years with the rapid technological advancements and innovations, to address the challenging issues of sustainable production and food security. Indeed, the demand for food will be increasing with the passage of time, while the natural resources such as water, fossil fuel, land and soil fertility are gradually being depleted. Furthermore, the cost of production inputs, viz., pesticides, chemical fertilizers and micronutrients, is enhancing at an alarming rate. Therefore, to address these pertinent issues, advanced researches in the area of nanotechnology are underway for development of precision farming practices. This will lead to reduction in the production costs and maximize the output with a precise control at a nanometer scale. In this context, nanotechnology offers enormous potential for improvement in the quality of life through its applications in agriculture and the food system (Ditta 2012). Nanotechnology-based devices are being explored in the field of plant breeding and genetic transformation (Torney et al. 2007). Smart delivery systems for agrochemicals, which were otherwise sprayed, have been developed using nanomaterials as carriers for the delivery of active ingredients, with minimum losses and increased yields through optimized water and nutrient management (Gogos et al. 2012). Further, the agricultural produce or its waste like wheat straw and soy hulls could be effectively utilized by converting into bio-nanocomposites, with enhanced physical and mechanical properties, for bio-industrial purposes (Alemdar and Sain 2008). Researches in the area of agricultural nanotechnology are being pursued for almost a decade, searching for solutions to various challenges, such as sustainability, improved varieties and increased productivity. Several studies have revealed the increasing trend of both the scientific publications and patents in agricultural nanotechnology, especially for disease management and crop protection (Sastry et al. 2010; Gogos et al. 2012). Most likely, the knowledge of nanotechnology gained in other emerging sectors, such as electronics energy and medical sciences, could be effectively transferred or adopted for agricultural applications. Also, the improved fuel additives and lubricants can improve the performance and the carbon footprint of agricultural machinery. Furthermore, the improved packaging measures could benefit farmers by reducing the post-harvest loses and degradation of products before consumption. Apart from the progress achieved in environmental monitoring and drug delivery techniques (Chen et al. 2013), nanotechnology can also benefit the poultry and livestock sectors. Nanotechnology is poised to provide better solutions to multiple problems in agriculture and food sciences by offering novel approaches in preservation of raw materials and their processing for development of better quality plant and other food products. Thus, agricultural nanotechnology has a potential for (i) reducing the amount of pesticide consumption through nanocarriers via effective targeted delivery to the pests; (ii) making nano-fertilizers/ nutrients more available to nanoscale plant pores, resulting in greater nutrient use efficiency; (iii) adding nano-silicon to increase water uptake efficiency in plants; and (iv) developing nanobiosensors for slow release of fertilizers and other agrochemicals, and providing many more benefits.

17.2 Nano Farming: A New Perspective

Precision farming or agriculture has emerged in recent years with the developments in the field of wireless networking and miniaturization of the sensors for monitoring, assessing and controlling agricultural practices (http://www.lofar.org/p/Agriculture.htm). It relates to site-specific crop management and covers a wide range of pre- and post-production aspects of agriculture from horticulture to field crop production (Burrell et al. 2004; Mayer et al. 2004; Zhang et al. 2004). Recently, precision farming based on tiny microelectromechanical systems (MEMS) called 'smart dust' is regarded as a future nanotechnology for agricultural applications. Smart dust is comprised of sensors, robots and transponders

that operate on a wireless computer network and sense light, temperature, vibration, magnetism or chemicals through radio-frequency identification. They can be sprinkled across a field and linked to existing farming equipment used in precision agriculture and to a personal computer. For instance, ASTRON, the Netherlands Institute for Radio Astronomy, has developed new radio telescope of the LOFAR (Low Frequency Array) based on tens of thousands of antennas that are connected to each other with a large ICT infrastructure. LOFAR_Agro has applied it for measurement of the microclimate in potato crops and to combat phytophtora infection in the crop. Phytophthora is a fungal disease in potatoes, which can enter a field through a variety of sources. The infestation in crop depends strongly on the climatological conditions within the field (Wallin and Waggoner 1950). The decision support system (DSS) gathers the information from the meteorological station and the wireless sensors from the Agro Server to help farmers to combat phytophtora in the crop. The DSS alerts the farmer of most susceptible patches within the fields based on the information maps of temperature distribution within the fields, along with the weather forecast, and help develops a strategy on how to prevent or control the disease *(*http://www.lofar.org/agriculture/fighting-phytophtora-using-micro-climate*)*. In the near future, smart dust will help in monitoring the soils, crops and livestock in a more efficient manner and may contribute significantly in increasing the agriculture productivity by providing accurate information for quick and useful decisions.

17.2.1 Green Synthesis of Nanoparticles

Plants have been used for the biosynthesis of a variety of nanoparticles by spontaneous, economical, eco-friendly process of one-pot synthesis, suitable for large scale production (Huang et al. 2007). Green synthesis of nanoparticles by plants material involves the phytochemicals such as flavonoids, terpenoids, carboxylic acids, quinones, aldehydes, ketones and amides, which cause the reduction of ions (Prabhu and Poulose 2012). Numerous plants have been investigated for their role in the synthesis of nanoparticles, such as *Cinnamomum camphora* leaf (Huang et al. 2007); *Pelargonium graueolens* leaf (Shankar et al. 2003); *Azadirachta indica* leaf (Shankar et al. 2004); *Emblica officinalis* leaf (Ankamwar et al. 2005); Aloe vera leaf (Chandran et al. 2006); Alfalfa sprouts (Gardea-Torresday et al. 2003); Helianthus annus, Basella alba and *Saccharum officinarum* (Leela and Vivekanandan 2008); *Carica papaya* callus (Mude et al. 2009); *Jatropha curcas* leaf (Bar et al. 2009); *Eclipta* leaf (Jha et al. 2009); *Glycine max* (soybean) leaf (Vivekanandan et al. 2009); *Coriandrum sativum* leaf (Sathyavathi et al. 2010); *Syzygium cumini* leaf (Kumar et al. 2010); *Cycas* leaf (Jha and Prasad 2010); *Allium cepa* (Saxena et al. 2010); *Stevia rebaudiana* leaves (Varshney et al. 2010); *Solanum torvum* (Govindaraju et al. 2010); *Zingiber officinale* (Singh et al. 2011); *Capsicum annuum* (Li et al. 2007); *Dillenia indica* fruit (Singh et al. 2013); *Alternanthera sessilis* (Niraimathi et al. 2013); *Morinda citrifolia* (Suman et al. 2013); *Phytolacca decandra, Gelsemium sempervirens, Hydrastis canadensis* and *Thuja occidentalis* (Das et al. 2013) (Pinus desiflora); *Diopyros kaki, Ginko biloba*, *Magnolia kobus* and *Platanus orientalis* (Song and Kim 2009); and *Ulva fasciata* (Rajesh et al. 2011). It has been reported that alfalfa plants grown in an $AuCl_4^-$ rich environment absorb gold metal and the gold nanoparticles produced by the plant can be recovered mechanically from the harvest by dissolving the plant tissue. Also, the geranium leaves immersed in a gold-rich solution for 3–4 h have been reported to produce 10 nm sized gold particles shaped as rods, spheres and pyramids. Similarly, the uptake of silver by the alfalfa plant in silver-rich solid medium transformed silver to silver nanoparticles (Gardea-Torresday et al. 2003).

17.2.2 Nano-Fertilizers: An Efficient Resource for Nrop Nutrition

Targeted delivery and slow or controlled release of nanoformulations in response to environmental stimuli and biological demand increase nutrients use efficiency, reduces soil toxicity, minimizes the

potential negative effects of over dosage and reduces the frequency of the application (Naderi and Danesh-Shahraki 2013). The nutrients can be encapsulated inside nanoporous materials, coated with thin polymer film, or delivered as particles or emulsions of nanoscales dimensions (Rai et al. 2012). Increased food grain production depends upon proper irrigation, good quality seed and fertilizers. Imbalanced application of fertilizers, nutrient deficiencies and reduced level of soil organic matter are often very challenging and these issues can be addressed effectively by developing nano-fertilizer formulations with multiple functions. Nano-fertilizers, contrary to traditional methods of fertilizer application, make a gradual and controlled release of nutrient into soil, which may prevent autrification and contamination of water bodies and environment. Besides, significant increase in crop yields has been reported with the foliar application of nano fertilizers (Tarafdar 2012; Tarafdar et al. 2012a). Lately, the nanocomposites are being developed to supply the essential nutrients in suitable proportion through a smart delivery system. However, the supply of micronutrients as nano-formulations through soil-borne and foliar applications needs to be ascertained. Currently, the nitrogen use efficiency is low due to the loss of 50–70 % of the nitrogen being supplied in the form of conventional fertilizers. The novel nutrient delivery systems can exploit the porous nanoscale parts of plants and cause significant reduction in nitrogen loss by enhanced uptake. Tarafdar et al. (2012b) again suggested that the fertilizers encapsulated in nanoparticles can increase the uptake of nutrients.

Further, the nano clays and zeolites, a group of naturally occurring minerals with a honeycomb-like layered crystal structure, have also been used for increasing fertilizer efficiency (Chinnamuthu and Boopathi 2009). The main application of zeolites in agriculture is in nitrogen capture, storage and slow release (Leggo 2000). Millan et al. (2008) reported that urea-fertilized zeolite chips can be used as slow-release nitrogen fertilizers. Ammonium-charged zeolites have shown their capacity to raise the solubilization of phosphate minerals and thus exhibit improved phosphorus uptake and yield of crop plants. Similarly, the mixtures of zeolite and phosphate rock show the potential for slow-release fertilization of plants in synthetic soils by dissolution and ion-exchange reactions (Allen et al. 1993). Li (2003) demonstrated the possibility of using surfactant modified zeolite using hexa decyl trimethyl ammonium as fertilizer carrier to manage slow release of nitrate and other anions. Liu et al. (2006) suggested that coating and binding of nano and sub-nanocomposites are able to regulate the release of nutrients from the fertilizer capsules. Jinghua (2004) demonstrated that application of a nanocomposite consists of N, P, K, micronutrients, mannose and amino acids enhance the uptake and use of nutrients by grain crops. It has also been shown that fertilizer incorporation into cochleate nanotubes improves the crop yield (DeRosa et al. 2010).

17.2.3 Nano-Herbicides: An Efficient Weed Control Agent

Weeds are considered as a serious problem in agriculture as they significantly reduce the vigour and yield of crop. Nanotechnology provides a solution to the weed problem by application of nano-herbicides in an eco-friendly manner without causing any residual toxicity in soil and environment (Perez-de-Luque and Rubiales 2009). Owing to nanoscale dimensions, the nano-herbicide blends with soil particles and prevents the growth of weeds resistant to conventional herbicides (Prasad et al. 2014). Generally, the herbicides available in the market either control or kill the above-ground part of the weed plants, without affecting the underground parts like rhizomes or tubers, which results in regrowth of weeds. Therefore, herbicide molecules encapsulated with nanoparticles specifically for receptors on the weed roots could be developed for targeted interactions with root system (Joel et al. 2007).

17.2.4 Nano-Pesticides and Pest Control

Conventional pest controlling methods are based on large-scale application of over-the-counter pesticides, which not only make the crop production

more expensive but also cause environmental and water pollution. Therefore, the need for minimizing the amount of pesticides to save the environment and to reduce the cost involved in crop production is strongly realized (Sharon et al. 2010). This could be achieved by increasing the retention time of pesticides without compromising efficiency. Persistence of pesticides in the initial stage of crop growth helps in bringing down the pest population below the threshold level, and consequently provides effective control for a longer period of time. In this context, nanotechnology proves to be a functional approach to improvise the insecticidal value. The USEPA (United States Environmental Protection Agency) is considered to be the first regulatory authority to have recognized the role and significance of nano-pesticides, and granted a conditional registration for the first nano silver pesticide (USEPA 2011). Indeed, the efficacious approach is 'controlled release of the active ingredient' that may greatly improve the efficacy with much lesser pesticide input and associated environmental hazards. For instance, 'Halloysites' (clay nanotubes) have been developed as cost-effective carriers of pesticides. These nanoparticles have been shown to greatly reduce the amount of conventional pesticide use and have extended the release time with better contact and minimum impact on the environment (Allen 1994). Further, the availability of nano-structured catalysts may increase the efficiency of pesticides and insecticides and also reduce the dose level required for plants (Joseph and Morrison 2006). Liu et al. (2006) have reported that the porous hollow silica nanoparticles (PHSNs) stacked with the pesticide validamycin can be effectively used for controlled release of pesticide. Also, the nanosilica has been studied to control agricultural insect pests (Ulrichs et al. 2005). By physio-sorption, the nano-silica gets strongly attached to insect cuticular lipids and eventually kills the insect (Ulrichs et al. 2005).

Lately, the nano-encapsulated broad-spectrum pesticides have been marketed under the trade name of Karate® ZEON to control the insect pests of soybeans rice and cotton (http://tirmsdev.com/Syngenta-Crop-Protection-Inc-Karate-with-Zeon-Technology). It releases the active chemical lambda-cyhalothrin, when it comes in contact with the leaves. Similarly, another nano-insecticide with the trade name 'gutbuster' releases its contents under alkaline environment in the stomach of insects (Prasad et al. 2014). Several studies have suggested the development of new polymer-based nanoformulations with less harmful plant-protection products in combination with biodegradable polymers. Such polymers mainly consist of polysaccharides (e.g., chitosan, alginates and starch) and polyesters (e.g., poly-ε-caprolactone and polyethylene glycol). In recent years, there has been an increase in demand for biodegradable materials of biological origin such as beeswax, corn oil, ecithin (Nguyen et al. 2012) or cashew gum (Abreu et al. 2012). These eco-friendly matrices can be applied in organic crop production with no toxic effects. There are certain natural substances, which also exhibit pesticidal properties but are unstable and can easily undergo premature degradation (Macías et al. 2004). Therefore, polymer-based nanoformulations in the form of nanospheres, nanogels or nanofibers could serve as better alternatives and offer more advantages. In view of increasing use of nanoparticles, the USEPA is contemplating to release regulation for handling the issues pertaining to nano-pesticides. The Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) Scientific Advisory Panel, in consultation with EPA, embarked on the evaluation of nanometal pesticide products (FIFRA-SAP 2009).

17.2.5 Nano-Antimicrobials for Phytopathogens

Nanoscale materials are emerging as novel antimicrobial agents due to their high surface area to volume ratio, which increases their contact with microbes and their ability to permeate cells (Morones et al. 2005; Kim et al. 2007). Nano silver is one such example, which is known to attack a broad range of biological processes in microorganisms and disrupt the cell membrane structure and functions (McDonnell and Russell 1999; Sondi and Salopek-Sondi 2004). It also inhibits gene expression for the proteins associated with ATP production (Yamanaka et al. 2005). Also, the

polymer-based copper nanoparticles have been investigated for their antifungal activity against plant pathogenic fungi (Cioffi et al. 2004). Silica-silver nanoparticles have also been reported to be effective antimicrobial agents against the plant pathogenic *Rhizoctonia solani, Pythium ultimum, Botrytis cinerea, Magnaporthe grisea* and *Colletotrichum gloeosporioides* (Park et al. 2006). Antifungal and antibacterial action of nanoparticles has been demonstrated against a variety of plant pathogenic fungi such as *Raffaelea* sp., *Bipolaris sorokiniana, Magnaporthe. Grisea, Fusarium, Phoma* and many other Gram-negative and Gram-positive bacteria (Kim et al. 2009; Gajbhiye et al. 2009; Esteban-Tejeda et al. 2010). Besides this, the nano-based products have been used for the control of pumpkin disease and powdery mildew (Lamsal et al. 2011). The infecting pathogens on the leaves disappear within 3 days after nanoformulation is sprayed. Growth of fungal hyphae and conidial germination could be significantly inhibited by nano-based products especially of silver and copper nanoparticles. Thus, the nano-herbicides, nano-fungicides and nano-pesticides have a tremendous scope in agriculture. There nanoformulations or nano-emulsions can be effectively used in preservation of pre- and post-harvest agricultural produce (Rickman et al. 1999; Zahir et al. 2012).

17.2.6 Nanotechnology and Integrated Pest Management (IPM)

Nanotechnology has a good scope in the IPM due to the insect pest controlling ability of nanomaterials. Nanoparticles have shown to be effective against a verity of plant pathogens and insect pests. Several different formulations of insecticides, pesticides and insect-repelling chemicals are reported (Esteban-Tejeda et al. 2010; Zahir et al. 2012). It is now possible to deliver any desired chemical into the plant tissues for eliciting the host plant defence against the pest insects (Torney 2009). For instance, the porous hollow silica nanoparticles loaded with validamycin work as effective transfer system for water soluble pesticides that can be released under controlled conditions (Liu et al. 2006). A wide range of agricultural insect pests can be controlled by the use of nano-silica (Ulrichs et al. 2005). Similarly, the nanoparticles coated with polyethylene glycol and garlic oil have been shown to exhibit biocidal activity against adult stage of *Tribolium castaneum*, a red flour beetle in stored grain pest (Yang et al. 2009). Thus, nano-emulsions are regarded as efficient pesticide formulations effective against several agricultural insect pests (Gao et al. 2007).

17.2.7 Categories of Nanoparticles

Nanoparticles can be categorized into two broad groups, i.e., organic and inorganic nanoparticles. Organic nanoparticles are mainly carbon nanoparticles (fullerenes, carbon nanotube, graphenes, etc.), whereas the inorganic nanoparticles may be magnetic nanoparticles, noble nanoparticles (gold and silver) or semiconductor nanoparticles (titanium oxide and zinc oxide). The inorganic nanoparticles have attracted more attention due to their superior material properties with versatile functions. The nano size, rich functionality and good biocompatibility of nanoparticles make them a suitable carrier for targeted drug delivery and controlled release (Xu et al. 2006). Synthesis of nanoparticles is of significance in nanotechnology due to variability in size, shapes, chemical composition, crop controlled dispersity and their potential applications in the agricultural sciences, for the better crop productivity and disease-free long-term post-harvest storage and preservation.

17.2.8 Inorganic Nanoparticles

17.2.8.1 Aluminium

Nanoalumina dust has been proposed to protect stored grains (Stadler et al. 2010). The insecticidal activity of nanoalumina dust comparable to the doses has been reported to be comparable to the recommended doses of commercially available

insecticidal dusts. Stadler et al. (2010) suggested the insecticidal activity of nanoalumina on insect pests *Sarocladium oryzae and Rhyzopertha dominica*. Nanoalumina is regarded as a good alternative to products based on diatomaceous earth. However, the mode of action of nanoalumina has yet to be elucidated. Further studies are required to optimize the product in terms of the mineral composition of the dust and the type of formulations, in order to ensure efficacy for a range of insect species under varying environmental conditions.

17.2.8.2 Copper

Mondal and Mani (2012) reported that a nanoformulation of copper has been shown to suppress the growth of bacterial blight on pomegranate at concentrations of 0.2 mg/L, which is 4-fold lower than the recommended dose of copper oxychloride (2500–3000 mg/L). There is a need for testing nanoformulations under a range of conditions that are as realistic as possible.

17.2.8.3 Silver

Nano silver, being one of the most extensively used nanoparticles, exhibits the broad-spectrum inhibitory and bactericidal effects. The in vitro studies have demonstrated a dose-dependent growth inhibition of plant pathogens with nanosilver (Kim et al. 2012). Their possible use as coatings for fruit bags (Chun et al. 2010) and treatments to cut flowers (Liu et al. 2009a; Solgi et al. 2009) indicate their possible benefits over synthetic fungicides. Kim et al. (2008) have also demonstrated the antifungal activity of colloidal nanosilver against rose powdery mildew caused by *Sphaerotheca pannosa* Var *rosae*. Till date maximum numbers of patents have been filed for nano silver for preservation and treatment of diseases in the agriculture field. The International Center for Technology Assessment (ICTA) submitted a petition to EPA requesting for regulation of nano silver usage in products as a pesticide under the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA). Silver is now an accepted agrochemical replacement. It is being used as foliar spray to stop fungi, moulds, rot and several other plant diseases. Nano silver kills unicellular microorganisms by inactivating enzymes having metabolic functions in the microorganisms by oligodynamic action (Kim et al. 1998). It is also known to exhibit superb inhibitory effects on algal growth. Silver in ionic state is known to exhibit high antimicrobial activity (Kim et al. 1998; O'Neill et al. 2003; Thomas and McCubin 2003). However, ionic silver is unstable due to its high reactivity and thus gets easily oxidized or reduced into a metal with no antimicrobial activity. Silver as a metal or oxide is stable in the environment, but due to its low antimicrobial activity it is used in relatively large quantities, which is not economical. Therefore, Park et al. (2006) developed a new composition of nanosized silica silver for control of various plant diseases.

17.2.8.4 Nano Silica

Silicate is reported to exhibit preventive effects on pathogenic microorganisms causing powdery mildew or downy mildew in plants (Lamsal et al. 2011). Besides, it also promotes the physiological activity and growth of plants and induces disease and stress resistance in plants (Garver et al. 1998; Kanto et al. 2004). Since the effect of silica varies with the physiological environment, it has not been registered as an agrochemical.

17.2.8.5 Titanium Dioxide

The antimicrobial activity of titanium dioxide is well recognized. Several studies have suggested that titanium dioxide exposure to crops can suppress the bacterial and fungal pathogens (Norman and Chen 2011). Nanoscale titanium dioxide either alone or doped with silver or zinc is effective against the bacterial spot disease in tomatoes (Paret et al. 2013a) and roses (Paret et al. 2013b). Greenhouse and field trials (Paret et al. 2013a, b) demonstrated that titanium dioxide/zinc can significantly reduce the bacterial spot severity compared to untreated controls. Some phytotoxicity may occur upon repeated applications, which could be avoided by using electrostatic sprayers instead of conventional sprayers (Paret et al. 2013a). In general, the titanium dioxide/zinc formulation exhibits relatively lower ecological and toxicological risks, compared to currently used copper-based treatments.

17.2.9 Biodegradable Polymers

Polymers such as cellulose, chitin, starch, polyhydroxyalkanoates, polylactide, polycaprolactone, collagen and other polypeptides are naturally synthesized by the organisms. Based on the nature of their synthesis, they are classified as (i) agro-polymers, such as starch or cellulose; (ii) microbial polymers, such as polyhydroxyalkanoates (PHAs); (iii) chemical polymers, such as polylactic acid (PLA) obtained from agro-resources; and (iv) polymers obtained from fossil resources. All these polymers are easily degradable by the microorganisms and cellular enzymes (Kaplan et al. 1993; Chandra and Rustgi 1998).

17.3 Smart Delivery System

One of the important applications of nanoparticles is their use as 'smart' delivery systems. Particularly, the use of nanocapsules has a huge scope in agriculture (Liu et al. 2002; Cotae and Creanga 2005; Pavel and Creanga 2005; Joseph and Morrison 2006). A typical example is the gene transfer by bombardment of DNA-absorbed gold particles to generate transgenic plants in a species-independent manner (Christou et al. 1988). Torney et al. (2007) have reported the efficient delivery of DNA and chemicals through silica nanoparticles in plant cells. Adak et al. (2012) recorded that amphiphilic copolymers, synthesized from poly (ethylene glycols) and various aliphatic diacids as nano-micellar aggregates, can be used to develop controlled release formulations of imidacloprid (1-(6 chloro-3-pyridinyl methyl)-N-nitro imidazolidin-2-ylideneamine) through encapsulation technique. Thus, high solubilization power and low critical micelle concentration of these amphiphilic polymers may increase the efficacy of formulations. Some common polymers, both synthetic and natural, that have been studied for smart delivery of insecticides are listed in Table 17.1.

Table 17.1 Nano products developed for agriculture use

Nano products	Active ingredients	Polymer matrix	Reference
Capsule	Neen Seed Oil	Alginate-glutaraldehyde	Kulkarni et al. (1999)
Capsule	Bifenthrin	Polyvinylpyrrolidone	Liu et al. (2008)
Capsule	B-Cyfluthrin	Polyethylene glycol	Loha et al. (2012)
Capsule	Deltamethrin	Polyethylene	Frandsen et al. (2010)
Capsule	Carbaryl	Carboxymethylcellulose	Isiklan (2004)
Capsule	Itraconazole	Acrylic acid-Bu acrylate	Goldshtein et al (2005)
Capsule	Etofenprox	Chitosan	Hwang et al. (2011)
Spheres	Carbaryl	Glyceryl ester of fatty acids	Quaglia et al. (2001)
Fiber	Pheromones	Polyamide	Hellmann et al. (2011)
Particle	Azadirachtin	Carboxymethyl chitosan	Feng and Peng (2012)
Particle	Imidacloprid	Chitosan-poly(lactide)	Li et al. (2011)
Particle	Chlorpyrifos	polyvinylchloride	Liu et al. (2002)
Film	Endosulfan	Starch-based polyethylene	Jana et al. (2001)
Granules	Imidacloprid	Lignin	Fernandez-Perez et al. (2011)
Micelle	Carbofuran	Polyethyleneglycol	Shakil et al. (2010)
Gel	Cypermethrin	Methyl methacrylate	Rudzinski et al. (2003)
Gel	Aldicarb	Lignin	Kok et al. (1999)
Powder	Novaluron	Anionic surfactants	Elek et al. (2010)
Resin	Pheromones	Vinylethylene	Wright (1997)
Clay	Imidacloprid	Alginate-bentonite	Fernandez-Perez et al. (2011)

17.3.1 Controlled Release of Agrochemicals from Nanocarriers

The nanomaterials that have been used for controlled release of agrochemical include nanosphere, nanogel, nanotubes and micelle formulations, as specified below.

17.3.1.1 Nanospheres

Nanospheres are aggregates in which the active compound is homogeneously distributed into the polymeric matrix. They are spherical particles of size between 10–200 nm in diameter and exhibit some novel size-dependent properties in comparison to larger spheres of the same material. They can be formed by dissolution, entrapment, encapsulation or attachment of chemicals and drugs with the matrix of polymers. Nanospheres can be amorphous or crystalline in nature and possess the ability to protect the chemicals from enzymatic and/or chemical degradation (Singh et al. 2010).

17.3.1.2 Nanogels

Nanogels are considered as better carrier than nanospheres for the reason that they are insoluble in water and thus less prone to swelling or shrinking with changes in humidity (Bhagat et al. 2013). They can significantly improve the loading and release profiles and avoid the occurrence of bursts or potential leaks (Paula et al. 2011). Owing to these advantages, the nanogels have been recommended, as per the organic farming standards (Kok et al. 1999), for delivery of pheromones and essential oils. Pheromones are considered to be highly specific and eco-friendly biological control agents, but their deployment requires slow release and protection from decomposition under ambient conditions. Bhagat et al. (2013) proposed the immobilization of pheromones within a nanogel without using any toxic cross-linkers or antioxidants. Evaporation of the pheromones in the nanogel gets significantly reduced, and the efficacy could be increased up to 33 weeks compared to only 3 weeks in case of pure active ingredients (Bhagat et al. 2013). The efficacy of a nanogel formulation of the essential oil of *Lippia sidoides* has also been reported to be better than free oil. Brunel et al. (2013) suggested the use of pure chitosan nanogels to improve the performance of antifungal treatments based on copper. The advantages of using a nanogel over a solution include easier handling, improved distribution on the leaves and the long-term release of copper on to leaves or into the soil without comparing its antifungal properties. Formation of the copper (II)–chitosan complex is pH dependent. Since most fungi tend to reduce the pH of their surrounding environment, therefore the release of copper (II) can be easily triggered by the growth of the pathogen. A strong synergistic effect between chitosan and copper in inhibiting the growth of *Fusarium graminearum* has been reported (Brunel et al. 2013).

17.3.1.3 Nanotubes

Nanotube devices served as excellent candidates for electrical sensing of individual biomolecules when integrated with other chemical, mechanical or biological systems (Chopra et al. 2007). Nanotube electronic devices have been shown to function very well under extreme biological conditions such as saline water (Liu et al. 2009a, b). Indeed, there are practical difficulties in reliable, rapid and reproducible nanofabrication of complex arrays of nanotubes; however, such devices have the potential to revolutionize exact diagnosis, drug delivery and livestock disease and health management, as well as in the identification and site-specific control of plant pests and diseases (Perez-de-Luque and Rubiales 2009).

17.4 Nanobiosensors

Nanobiosensors are analytical devices, where immobilized layer of a biological material is in contact with a sensor that analyses the biological signal and converts it into electrical signal (Gronow 1984). Biosensor offers a new analytical tool with major applications in environmental, clinical diagnostics and agriculture. In agriculture, the nanobiosensors can be effectively used for sensing a wide variety of fertilizers, herbicides, pesticides, insecticides, pathogens, moisture and

soil pH, and their controlled use can support sustainable agriculture for enhancing crop productivity (Rai et al. 2012).

17.4.1 Role of Biosensors in Agriculture

Excessive use of agrochemicals has lead to the elevated levels of herbicides, pesticides and heavy metals in agricultural soil. In order to monitor their status in soil and also to forecast the possible occurrence of soil disease, regular monitoring through the specific biosensors needs to be done. Biological diagnosis of soil using a biosensor means to study the reliable prevention and decontamination of soil. The basic principle of soil diagnosis with the biosensor is to estimate the relative activity of good and bad microbes in the soil based on quantitative measurement of differential oxygen consumption by soil microorganisms. Accurate sensors need to be developed as miniaturized portable devices and remote sensors, for the real-time monitoring of large areas. Field use of biosensor can reduce the time required for microbial testing and immunoassays, and also for detection of contaminants in water supplies, raw food materials and food products. Electronic nose (E-nose) is one such example for identification of different types of odours based on the pattern of response across an array of gas sensors. E-nose consists of gas sensors, composed of nanoparticles such as ZnO nanowires (Xu et al. 2008). Biosensors provide high specificity and sensitivity, rapid response, user-friendly operation and compact size at a low cost (Amine et al. 2006). Mendes et al. (2009) have reported the biosensor for the detection of the fungus *Phakopsora pachyrhizi* that causes Asian rust or Soybean rust, using the SPR (Surface Plasmon Resonance) technique. Amine et al. (2006) have also reported a biosensor for the detection of aflatoxin in olive oil.

In recent years, significant advances have been made towards the synthesis of colloidal semiconductor quantum dots (QDs), particularly II–VI compounds such as CdSe, CdS and CdTe (Park et al. 2007; Reiss et al. 2009). These highly visible luminescent nanomaterials are very promising for various applications in optoelectronics and biological labelling (Kaufmann et al. 2007; Walker et al. 2010). The optical properties of QDs per se and in conjugation with other entities have been extensively studied for their role in agricultural production. Pesticides/herbicides and growth promoting hormones have been widely used in agricultural production and their residues accumulate in various agricultural products and soils. Therefore, efficient and reliable methods for detecting residual pesticides and other agrochemicals were developed exploiting QDs for highly sensitive and selective detection.

17.5 Nanoparticle–Soil Interactions

Interaction of nanoparticles with the environmental components such as plants, microorganisms and soil have been extensively studied (Abhilash et al. 2012; Bakshi et al. 2014; Mohanty et al. 2014). Once the nanoparticles find their way into the soil environment, their fate, transport, bioavailability and consequent toxicity are largely affected by the soil physico-chemical properties (Shoults-Wilson et al. 2011; Cornelis et al. 2012; Benoit et al. 2013). Comprehensive information on the occurrence, activities and effects of nanoparticles on the agro-ecosystem is depicted in Fig. 17.1. The factors such as soil texture, pH, cation exchange capacity and soil organic matter govern the transport, mobility and sorption of nanoparticles in the soil (Oromieh 2011; Benoit et al. 2013). Oromieh (2011) and Benoit et al. (2013) have demonstrated that the soil pH and cation exchange capacity significantly affect the bioavailability of silver nanoparticles and silver metal in soil. At higher pH, the soil exhibits a greater cation exchange capacity due to which Ag ions are absorbed onto the soil surface, which reduces their bioavailability. Also, the soil organic matter affects the sorption and mobility of nanoparticles. High organic contents of soil promote the strong binding of nanoparticles

to the soil, and thereby retard their mobility, availability for biological uptake and subsequent toxicity (Shoults-Wilson et al. 2011). Furthermore, physico-chemical properties of soil and nanoparticles such as size, shape and surface charge are believed to exert important control on dissolution, agglomeration and aggregation of nanoparticles. Interestingly, enhanced ionic strength and divalent cations are reported to promote silver nanoparticle aggregation and retention in soil (Lin et al. 2011; Thio et al. 2012). Cornelis et al. (2013) have suggested that hetero-aggregation of silver nanoparticles with natural soil colloids significantly reduce their mobility. However, agglomeration of polyvinylpyrrolidone (PVP)-silver nanoparticles has not found to be influenced by increasing ionic strength, which reflects the importance of stabilizing agents.

17.5.1 Nanoparticles Interaction with Soil Bacteria

The broad-spectrum antimicrobial properties of nanoparticles, particularly the nano silver, against human and plant pathogens have been extensively reported (Shahverdi et al. 2007; Kim et al. 2009; Musarrat et al. 2010). The interaction of nanoparticles with soil microbiota and the plausible mechanism of cyto and genetic toxicity are represented in Fig. 17.1. However, their impact on soil biota is still not well understood. Certain studies have suggested the adverse effect of silver nanoparticles on denitrifying bacteria, which disrupts the process of denitrification in soil (VandeVoort and Arai 2012). Also, the effect of nanoparticles on *Pseudomonas stutzeri* (denitrifier), *Azotobacter vinelandii* (nitrogen fixer) and

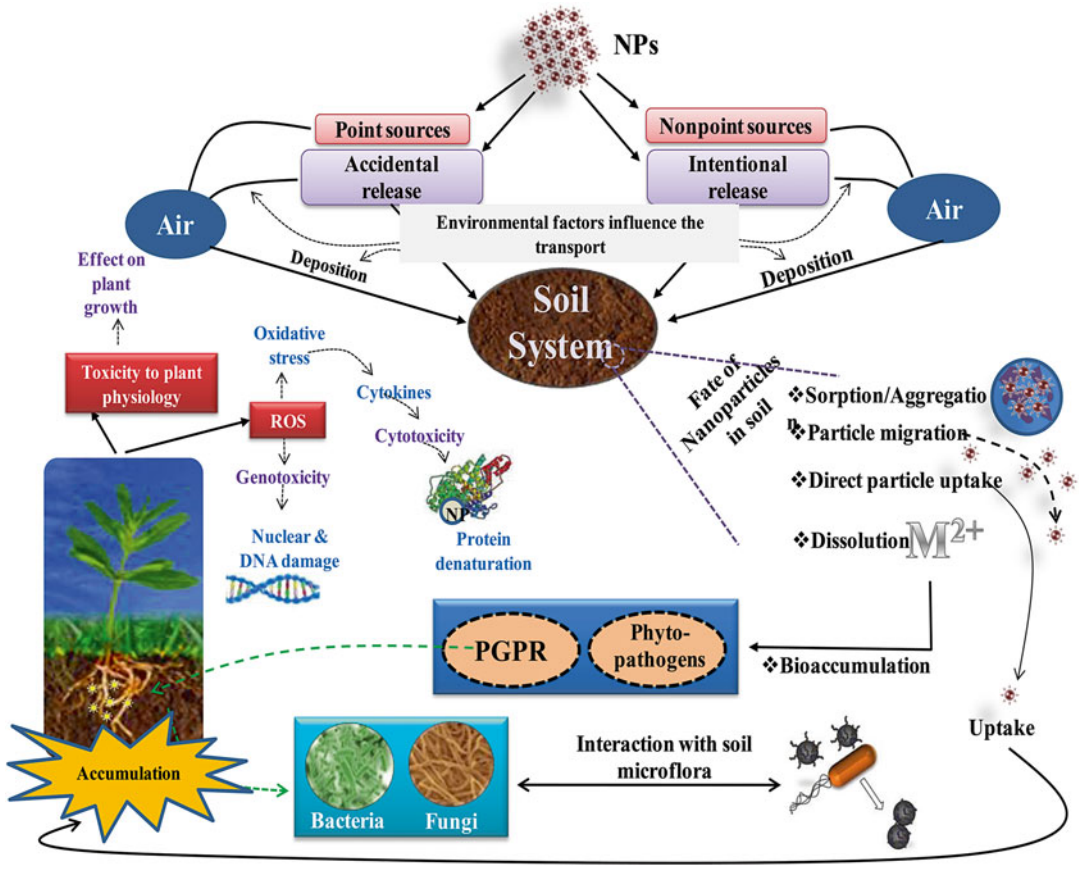

Fig. 17.1 Occurrence, activities and effects of nanoparticles in the agro-ecosystem

Nitrosomonas europaea (nitrifier) have been reported (Yang et al. 2013). Shahrokh et al. (2014) have demonstrated that nano silver at low doses exerts no adverse effect on nitrate reductase activity of *Rhizobium* and *Azotobacter*. However, size-dependent toxicity of nanoparticles has been demonstrated by Choi and Hu (2008), who have suggested that nanoparticles of size < 5 nm exhibit more toxicity to nitrification bacteria. On the contrary, Zhang et al. (2014) reported no significant impact of the long-term exposure of nano silver at concentrations of 0.10 μg/mL on microbial community structure and nitrifying bacterial community in an activated sludge. Studies have reported an increase in the copy number of the silver-resistant gene silE, which may change the population dynamics (Silver 2003). Also, an increase in diversity of nirK denitrifiers (nirK encodes the copper nitrite reductase) has been reported with increasing concentration of nano silver in soil, whereas the gene copy number and denitrification activity have been found to be decreased (Throbäck et al. 2007). Besides microbial diversity, the microbial community functions also get influenced, simultaneously, by nano silver exposure (Silver 2003). Hansch and Emmerling (2010) suggested a dose-dependent effect of silver nanoparticles on soil microbial biomass and enzyme activities. However, no significant effect on microbial biomass nitrogen and enzymatic activities is reported on C, N and P cycling in soil. Similarly, exposure to zinc oxide nanoparticles (ZnO-NPs) also has shown to exert adverse effect on plant development (Lin and Xing 2007). For instance, the growth of garlic raised under hydroponic conditions gets retarded at a ZnO-NP concentration as low as 15 μg/ mL, with dose-dependent effects found up to 50 μg/mL (Child et al. 2007). ZnO-NPs are also reported to reduce cucumber biomass in hydroponic cultures (Dimkpa et al. 2012), whereas the growth of wheat, bean, corn and rye grass has been attenuated in sand or liquid growth systems (Parker et al. 2005; Wang et al. 2009). In addition to reduction in root elongation, stimulation of lateral roots occurs in wheat, which causes a change in root architecture upon ZnO-NPs treatment (Jackson and Taylor 1996). Similarly, the exposure to CuO-NPs has also demonstrated negative impact on growth and DNA integrity in case of raddish, rye grass and buckwheat (Lok et al. 2006; McQuillan et al. 2012). A study in tomato suggested the role of CuO-NPs as fungicides against plant pathogens with little or no deleterious effect on plant performance (Nel et al. 2006). Toxic effect of CeO_2-NPs has been reported in wheat and pumpkin (Kloepper et al. 1980). These nanoparticles were also found to induce significant antioxidative enzyme activity and prevented membrane peroxidation and leakage of cytoplasmic membrane in maize (Lodewyckx et al. 2002). Also, the TiO_2-NPs have been shown to inhibit maize leaf growth and transpiration (Xiu et al. 2012) and result in impaired growth of wheat (Kahru et al. 2008). Tomato root and stem elongation, as well as biomass production, has also been shown to be inhibited by TiO_2-NPs and mitigate the growth of root-knot nematodes infesting the plants (Lewinson et al. 2009). However, in spinach plant, the TiO_2-NPs caused improved physiological and growth responses due to increase in ribulose-1,5-bis-phosphate carboxylase/oxygenase activity and chlorophyll production, responsible for enhanced photosynthesis (Miller et al. 2009; Loper et al. 2012). The effects of TiO_2-NPs on *Lepidium sativum* (cress) varied with soil type and have exhibited both positive and negative growth outcomes at varying concentrations (Li et al. 2008).

17.5.2 Conclusion and Future Perspective

Nanotechnology is a promising technology with the potential to engender colossal changes in food and agricultural sectors. Extensive research on the application of nanomaterials in agriculture is expected but with a caveat for environmental security and food safety. Indeed the risk assessment of the nanomaterials and nanoformulations developed for use in agriculture is still not well defined. Undoubtedly, nanotechnology-based applications can increase production and allow better management and conservation of inputs. However, the extensive use of nanomaterials has

raised critical issues regarding their disposal and other associated risks. Extensive studies are warranted to understand the mechanism for nanomaterials toxicity and their impacts on environment and human health. Furthermore, the innovative agro-nanotech products are facing difficulties in market outreach, making agriculture still a marginal sector for nanotechnology. Perhaps this is due to relatively high production costs of nanotech products, indistinct technical benefits and legislative uncertainties. Therefore, it is important to create awareness about the potential advantages of nanotechnology in agriculture for general public interest and acceptance.

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Nanoparticles: The Next Generation Technology for Sustainable Agriculture

18

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Abstract

Agri-nanotechnology has the potential to transform the agricultural practices. Nanoparticles of interest can be produced both by various physical and chemical methods. The biogenetic production of nanoparticles is now of high interest due to simplicity of the procedures and their versatility. Several species of bacteria and plants are able to synthesize nanoparticles or help in the process of their production. Implementation of nanoparticle-based smart delivery system and nanosensors holds the promise of controlled release of agrochemicals and site-targeted delivery of various macromolecules needed for improved plant disease resistance, efficient nutrient utilization and improved plant defence in an environment-friendly manner. Nanoparticle-mediated plant transformation has the potential for genetic modification of plant improvement.

Keywords

Agri-nanotechnology • Biogenic • Nanosensors • Biocontrol • Inoculants

18.1 Introduction

The word 'nano' was originally derived from the Greek word 'nanos' meaning dwarf, which refers to the size of 10^{-9}. Nanotechnology is the cutting-edge track of research for development of sustainable agriculture system. The nanoparticles (NPs), which are essential parts of nanotechnology, can be naturally produced from agricultural soil and plants. The exploitation of the natural biosynthetic machinery for the production of nanoparticles will transform nanotechnology towards green nanotechnology.

The next green revolution will be based on precision farming, which aims to maximize output (i.e., crop yields) while minimizing input (i.e., fertilizers, pesticides, herbicides, etc.) through monitoring environmental variables and applying targeted action. Precision farming makes use of

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_18

nanosensor and smart delivery systems. So, nanotechnology will be an unavoidable part of the next green revolution with implementation of nano-based smart sensors and smart delivery systems. Nanotechnology can improve the existing crop management techniques. Conventionally, agrochemicals are being applied to crops by spraying and/or broadcasting and/or drenching. Due to leaching, photolysis, hydrolysis or microbial degradation, a limited quantity of agrochemicals, usually below minimum effective dose, reaches the target site and thereby repeated application of the chemicals is mandatory for optimum growth and development of crops which in turn pollute the environmental resources such as soil, water and air. Usually only a very low concentration of chemicals, which is much below the minimum effective concentration required, has reached the target site of crops due to problems such as leaching of chemicals, degradation by photolysis, hydrolysis and by microbial degradation. Hence, repeated application is necessary to reach the goal, which might adversely affect natural resources such as soil and water. Nanotechnology offers the use of smart delivery system for agrochemicals wherein encapsulated agrochemicals possess all essential stuffs such as effective concentration, time-controlled release in response to certain stimuli, enhanced targeted activity and less ecotoxicity with safe and easy mode of delivery, which in turn avoid frequent application (Green and Beestman 2007; Wang et al. 2007; Boehm et al. 2003). In this chapter we will discuss biological production of nanoparticles and different applications of nanotechnology in the agriculture sector.

18.2 Sources of Nanoparticles

Nanoparticles (NPs) can be derived from natural and anthropogenic sources. The natural processes that produce NPs can be photochemical reactions, volcanic eruptions, forest fires, simple erosion and plants and animals. Recently plants and microorganisms have emerged as efficient biological source for nanoparticle production and represent green source of nanoparticles.

18.2.1 Bio-production of Nanoparticles

Production of nanoparticles by biological systems such as microorganisms and plants is of high interest due to simplicity of the production process and eco-friendly nature as compared to various physical and chemical processes. Several species of bacteria and plants are able to synthesize nanoparticles or help in the process of their production (Ankamwar et al. 2005).

18.2.1.1 Plants as Bio Nanoparticle Production Unit

Among various biological systems exploited for production of nanoparticle, plants are the best green route for the synthesis of various metal nanoparticles because they possess wide variety of metabolites. The prime requirement for plant-based production of nanoparticles is that the metal of interest should be present in the growth medium of plants and should be efficiently transported through the plant root cells. From the medium, nanoparticles enter into the plant through cell wall. The sieving properties are of cell wall range from 5 to 20 nm (Fleischer et al. 1999), which selects the size of nanoparticles or their aggregates to pass through and reach the plasma membrane (Moore 2006). Further during endocytosis nanoparticles may also cross the membrane using transport carrier proteins or through ion channels. Here they accumulate at high rate and are further modified by plant processes and distributed throughout the plant. For example, gold nanoparticles were formulated inside live alfalfa plants and *sesbania* seedlings grown in gold enriched media (Sharma et al. 2007). The uptake and translocation of nanoparticles across root cells depend on the type of metal ions and plant species in which several active and passive transport processes are involved.

A number of plants are being currently investigated for their role in the synthesis of nanoparticle. Gold nanoparticles with a size range of 2–20 nm have been synthesized using the live alfalfa plants. Nanoparticles of silver, nickel, cobalt, zinc and copper have also been synthesized inside the live plants of *Brassica jun-*

cea (Indian mustard), *Medicago sativa* (Alfa) and *Helianthus annus* (Sunflower), of which *Brassica juncea* performed better for synthesis and accumulation of nanoparticles (Bali et al. 2006). Certain plants are known to accumulate higher concentrations of metals compared to others and such plants are termed as hyper accumulators. The amount of nanoparticle accumulation in plants also varies with reduction potential of ions and the reducing capacity of plants that depends on the presence of various polyphenols and other heterocyclic compounds present in plants as well as microorganisms associated with them (Huang et al. 2007; Egorova and Revina 2000). Phytochemicals such as terpenoids, flavones, ketones, aldehydes, amides and carboxylic acids are found to play a key role in plant-assisted reduction of metal nanoparticles. Among the plants growing in different habitats, xerophyte *Bryophyllum* sp. was found to contain an anthraquinone emodin, which could undergo redial tautomerization and leads to the formation of silver nanoparticles. The silver ions were found to be reduced within some metallophytic plants and then after distribution as silver nanoparticles within cellular structure. Some wetland plants and endomycorrhizal fungi transform copper into metallic nanoparticles at soil-root interface and thereby reduce copper toxicity in contaminated soils (Gardea-Torresdey et al. 2005). Leaf extracts and dead tissues of various plants can also be used for biological synthesis of nanoparticles. Recently, gold nanoparticles have been synthesized using the extracts of *Magnolia kobus* and *Diopyros kaki* leaf extracts. Temperature also plays a key role in synthesis of different shapes of nanoparticles, lower temperature polydisperses particles whereas high temperature supports formation of smaller spherical particles (Song et al. 2009). The terpenoids with functional groups of alcohol, ketones, aldehyde and amines play an important role in the stability of the nanoparticles. Several research groups synthesized different shapes and morphologies of (nanotriangles, nanoprisms, octahedral) gold particles using leaf extract of tamarind, geranium, neem, *Hibiscus rosa sinensis*, coriander, *Magnolia Kobus* and *Dyopiros Kaki* as well as *Emblica officinalis* fruit extract, Aloe vera extract, mushroom extract and even honey (Popescu et al. 2010). Plants are considered as better candidates for biological synthesis of nanoparticles as water-soluble phytochemicals require shorter incubation time to reduce metal ions as compared to microorganisms (bacteria and fungi).

18.2.1.2 Microorganisms as Bio Nanoparticle Production Unit

Microorganisms including bacteria, fungi and algae are capable of accumulating nanoparticles within their cellular structures. One obvious example of the microbial production and accumulations of nanoparticle by microorganism is diatoms (brown algae) that have exoskeletons made up of 50–100 nm sized silica nanoparticles. Diatoms carefully design and control natural nanostructures formation. Silicon is the basic structural component and silicic acid is the precursor that synthesizes nanoparticles, within a few hours near or below the room temperatures. Silicic acid transporters aid in its transport into the cell where they accumulate at very high concentrations. These silicon precursors can be transported into specific vesicles named silicon deposition vesicle located at the vicinity of cytoplasmic membrane. It is shown that some proteins are involved in the Si polymerization process. Diatoms shells when treated with magnesium vapours at high temperature lead to Mg-Si oxide replica. The process was shown to be compatible with other metals and this is a significant step towards application of diatoms in nanotechnology.

Nanoparticles can be precipitated within microbial cells when incubated in the medium containing the metal. Gold nanoparticles could be precipitated within the bacterial cells by incubation of the cells with Au^{3+} ions (Beveridge and Murray 1980). Klaus et al. (2001) synthesized silver nanoparticles using *Pseudomonas stuzeri* AG 259 isolated from silver mine by incubating it in silver nitrate solution. The silver nanoparticles get accumulated within periplasmic space of the bacteria. Synthesis of magnetic nanoparticle by magnetotactic bacteria such as *Magnetosirillium*

magneticum within magnetosomes has been reported. Under anaerobic condition, sulphate-reducing bacteria *Desulfovibrio desulfuricans* NCIMB 8307 produce palladicum nanoparticles, and synthesis of spherical aggregates of 2–5 nm of sphalerite ZnS nanoparticles has also been reported by sulphate-reducing bacteria (Mandal et al. 2006). *Clostridium thermoaceticum* and *Klebsiella aerogens* can synthesize CdS nanoparticles. *Rhodopseudomonas capsulate* can synthesize gold nanowires (He et al. 2008). Extracellular secretion of enzymes by cyanobacteria offers the advantage of getting large quantities of nanoparticles of size 100–200 nm in a relatively pure state, free from other cellular proteins. Ali et al. (2011) reported that cyanobacterium *Oscillatory willei* NTDMO1 reduces silver ions and stabilizes the silver nanoparticles by a secreted protein and thereby synthesizes silver nanoparticles extracellularly. Similarly, yeast has been used successfully in the synthesis of CdS and PbS nanoparticles. Kowshik et al. 2003 have shown that *Torilopsis* species is able to synthesize nanoscale PbS (intracellularly) when exposed to aqueous Pb^{2+} ions. Similarly, yeast strain *Schizosacharomyces pombe* are used to prepare CdS quantum dots. Similarly, silver-tolerant yeast strain MKY3 can synthesize high concentration of silver nanoparticles (Kowshik et al. 2003).

Among all types of microorganisms, fungi, mainly *Verticillium* sp., *Aspergillus flavus*, *Aspergillus furnigatus*, *Phanerochaete chrysoparium* and *Fusarium oxysporum*, are considered as most efficient for biosynthesis of metal and metal sulphide containing nanoparticles. *Verticillium* sp. brings about reduction of metal ions extracellularly, which results in the formation of gold and silver nanoparticles with approximate size of 5–20 nm. Nithya and Ragunathan (2009) proposed a two-step procedure of nanoparticle synthesis by *Vericillum* spp.; in the first step, Ag^{+} ions get adsorbed on the surface of fungal cells followed by reduction of silver ions by fungal enzymes. Shahi and Patra (2003) synthesized bioactive nanoparticles using lichen fungi (*Usneea longissima*) in laboratory conditions. The extracellular enzyme hydrogenase was found to be the main factor responsible for the ability of *Fusarium oxysporum* to synthesize nanoparticles. Hydrogenase is having outstanding redox potential and thereby acts as an excellent reducing agent in metal reduction (Nithya and Ragunathan 2009). Another best electron shuttle for nanoparticle biosynthesis is hydrochinones released by microorganisms for nanoparticle. The fungi *Aspergillus flavus*, *Aspergillus furnigatus*, *Phanerochaete chrysoparium* and *C. versicolar* produce stable silver nanoparticles when immersed in aqueous silver nitrate solution (Bahamas and Disouza 2006).

18.3 Nanoparticle-Based Smart Delivery System for Agriculture

Recently, nanotechnology is gradually moved from the experimental into the practical areas. The development of slow/controlled release of fertilizers, pesticides and herbicides based on nanotechnology has become critically important for upholding the progress of eco-friendly and viable agriculture. Nanotechnology has provided the feasibility of exploiting nanoscale or nanostructured materials as agrochemical carriers or controlled-release vectors for development of smart delivery system to enhance nutrient and active ingredient use efficiency and reduce costs of cultivation while protecting the environment in the long run (Cui et al. 2010; Chinnamuthu and Boopathi 2009). Many mechanisms such as encapsulation and entrapment, surface ionic and weak bond attachments may be used to store, protect, deliver and release required payloads of agrochemicals in crop production processes. Nanoparticles improve stability of the agrochemicals against degradation in the environment which in turn increases its effectiveness and reduces the quantity of the chemicals. This reduction helps in addressing agricultural chemicals run-off and alleviates the environmental consequence. The nanoscale delivery vehicles may be designed to attach to plant roots or the surrounding soil structures and organic matter (Johnston 2010). Controlled release mechanisms allow the active ingredients to be slowly taken up, hence,

avoiding temporal overdose, reducing the amount of agricultural chemicals used, lowering the risk on non-target organisms and minimizing the input and waste.

18.3.1 Nanofertilizers

Fertilizers play an axial role in enhancing agricultural production. Only 30–40 % of the total applied fertilizers can be used by crops for their growth, the remaining is lost form the ecosystem through evaporation, leaching or degradation. This will reduce the efficiency of fertilizers and increase the cost of cultivation. Moreover, indiscriminate use of fertilizers will pollute our natural resources to the extent that creates hazard to all living forms in the ecosystem including soil, water and air. In spite of this, it is known that yields of many crops have begun to decrease as a result of imbalanced fertilization and decrease in soil organic matter. To enhance nutrient use efficiency and overcome the long-lasting problem of pollution, nanofertilizer might be the best alternative, being synthesized in order to control the release of nutrients depending on the requirements of crops and believed to be more efficient than conventional fertilizers (Liu et al. 2006). Slow-release fertilizers releases nutrients at a slower rate throughout the crop growth and, hence, plants are able to take up most of the nutrients without any loss. Nanofertilizers allow careful discharge linked to time or environmental condition. Nanofertilizers also improve soil health by decreasing toxic effects of fertilizer overuse (Suman et al. 2010).

Nanofertilizers are mainly produced by encapsulation of fertilizers within a nanoparticle. There are mainly three techniques to encapsulate fertilizers within nanoparticles:

1. The nutrient can be encapsulated inside nanoporous materials
2. Coated with thin polymer film
3. Delivered as particle or emulsions of nanoscale dimensions (Rai et al. 2012).

In addition, nanofertilizers will combine nanodevices in order to synchronize the release of fertilizer-N and -P with their uptake by crops, so preventing undesirable nutrient losses via direct internalization by crops, and avoiding the interaction of nutrients with soil, microorganisms, water and air (DeRosa et al. 2010). Slow and steady release of nutrients from the fertilizers can be assured by coating fertilizer particles within nanoparticle membranes. Chinnamuthu and Boopathi (2009) reported enhanced uptake of nutrients by grain crops from nanocomposite containing macro (N, P, K) and micronutrients. Natural minerals such as nano clays and zeolites are also used to increase fertilizer use efficiency (Chinnamuthu and Boopathi 2009). The crystalline layer of these minerals can be filled up with macro and micronutrients so that the nutrients get slowly released upon requirement. Zeolites are generally used for capture, storage and slow release of nitrogen in agriculture (Leggo 2000). These types of nano-coated fertilizers also help in reduction of soil, water and air pollution as nutrient release from the absorbed form (zeolite) is slower than the routinely used ionic form of fertilizers. Zeolite chips with urea (Millán et al. 2008) can be used for slow release of nitrogenous fertilizers. Surfactant-modified zeolites are suitable absorbents for nitrate and have potential to be used as fertilizer carrier for controlled release of nitrate and other anions. Moreover, the zeolite particles containing ammonium can also help in increasing uptake of phosphorous by raising the capacity of phosphate solubilization which in turn increases the crop yield. Allen et al. (1993) reported that mixture of zeolite and rock phosphate can ensure slow release fertilization by dissolution and ion-exchange mechanisms.

Encapsulation of fertilizers within nano- and sub-nanoparticles by coating or binding can aid in regulation of nutrient release from fertilizers (Liu et al. 2006). Nanocomposites with zinc-aluminium layer confirm controlled release of plant growth regulators (DeRosa et al. 2010). The effects of low/controlled-release fertilizers cemented and coated by nanomaterials, clay-

polyester, humus-polyester and plastic starch were studied on crops with wheat (Liu et al. 2006; Zhang et al. 2006), and results showed that nanocomposites gave 99 % of wheat seed germination as well as higher emergence and growth of seedlings. Recently, carbon nanotubes were shown to penetrate tomato seeds (Fernandez and Eichert 2009) and zinc oxide nanoparticles were shown to enter the root tissue of ryegrass (Eichert et al. 2008), suggesting possibility of development of novel nutrient delivery system with nanoscale porous materials for plant surfaces.

More recently, research on nanofertilizer delivery systems that can react to environmental changes is in progress. The final goal is production of nanofertilizers that will release nutrients in a controlled manner (slowly or quickly) in reaction to different signals such as heat, moisture, etc. Biotic mineralization of nitrogen from soil organic matter and phosphorous from organic matter as well as inorganic soil colloids is accomplished by release of carbonaceous compounds in to rhizosphere by crops under nutrient limiting conditions. Using such root exudates nanobiosensors to be used with nanofertilizers can be prepared (Al-Amin Sadek and Jayasuriya 2007; Sultan et al. 2009).

18.3.2 Nanocides (Pesticides and Herbicides)

The active ingredients (AI) of many conventional pesticides and herbicides have limited water solubility and thus require a delivery system for their application in the field. Moreover, such AIs are also harmful to non-target organisms and also aid in development of resistance in target organisms; many of the formulations are available in the market to overcome above mentioned limitations of AIs, but such alternatives are very unstable and prone to undergo premature degradation. Such limitations can be overcome by using nanoformulations. Technologies such as encapsulation and controlled release methods have revolutionized the use of pesticides and herbicides. Nowadays pesticides and herbicides that contain nanoparticles within the 100–250 nm size range are available and they dissolve in water more effectively than existing ones, which in turn increase their activity. Some of the nanopesticides and herbicides are available as water- or oil-based nanoemulsions having uniform suspension of pesticide or herbicide which can be mixed with gels, creams, liquids, etc. Generally neem oil (Anjali et al. 2010; Jerobin et al. 2012; Xu et al. 2010), garlic essential oil (Yang et al. 2009), Artemisia arborescens L essential oil (Lai et al. 2006) and Lippia sidoides oil (Abreu et al. 2012) are used as target substances. Various scientists suggested use of nanodelivery systems for pheromones (Bhagat et al. 2013; Hellmann et al. 2011), capsaicin from chili peppers (Bohua and Ziyong 2011) and Lansiumamide B extract from the seeds of *Clausena lansium* (Yin et al. 2012).

Nanoformulations of pesticides or herbicides are reported to be more effective as compared to either the pure active ingredients (AI) or commercial formulations. This could possibly be the result of a higher bioavailability and increased uptake of active ingredients with nanoparticles, compared to the AI. Increased uptake of AI by target organism is necessary, but it should be achieved without its hazardous effect on to non-target organisms including human beings and animals. The bioavailability of AI of nanocides depends on the carrier properties and target organisms. As the nanocides are of relatively larger sizes, their direct uptake is not possible. It has, for instance, been shown that chitosan (a polysaccharide frequently used as a polymer carrier for nanopesticides) can change the bioavailability of the chiral herbicide dichlorprop (Wen et al. 2010). The location of AI within the nanoparticle's polymeric matrix is important to protect it from photodegradation (Qing et al. 2013). Moreover, soil microorganisms can reach up to AI molecules located at the surface of the nanoparticles and the AIs, which are located within the core portion, are not accessible for microorganisms and thereby we can say that availability of AI is also dependent on its location and distribution within the nanoformulation. Difference between the efficiency of AI within different types of nanoformulations and for different organism is highly dependent on the char-

acteristic of nanoformulation and the organism (Kumar et al. 2013; Pradhan et al. 2013).

Nanoencapsulation of agrochemicals within biodegradable materials helps in safe and easy handling of concentrated AI by farmers. Nanoencapsulated AI of herbicides can effectively penetrate the cuticle and facilitates slow and controlled release of active ingredients upon contact with the target weed. Surface modified hydrophobic nanosilica has been successfully used for control of insect pests (Barik et al. 2008; Rahman et al. 2009) as it gets absorbed into cuticular lipids of insects and damages the protective wax layer which results in to death of insect by desiccation (Athanassiou et al. 2007; Mewis and Ulrichs 2001). Such nanobiopesticides are more acceptable as it causes less environmental pollution as well as safe for plants as compared to chemical pesticides. Li et al. (2007) studied the effect of nanosphere formulations on cotton plants infested with aphids. The ability of nanoformulation to penetrate through the plant and reach the sap and thereby exerting systemic effect has been studied wherein nanosphere formulations have more effectively controlled pest infestation at all doses as compared to control due to their increased systemicity. Li et al. (2007) proposed a best use of hollow nanoparticles of silica having wall thickness of about 15 nm with inner hollow diameter of 4–5 nm. This type of nanotubules protects pesticides from degradation by ultraviolet light by protecting them from direct exposure to sun rays. Such type of hollow nanoparticles is generally used as carrier for pesticides and certain drugs like avermectin and protects it from photodegradation and also allows controlled and sustained release of drugs from carrier materials. Nanoparticles and nanotubules are considered to have a futuristic potential of being used in agriculture as carriers for active ingredients of pesticides, herbicides as well as fertilizers for assuring need-based release of photosensitive components. Nanoparticulate delivery systems are considered as boon for the agrochemicals having small sized active ingredient of diameter [1–5 nm] and found to effectively controll various fungal disease like powdery mildew (Park et al. 2006). Till date meagre efforts have been made to develop targeted delivery system with nanoparticles wherein drug molecules can be dispersed specifically over the infected tissues; so, these types of studies needs to be concentrated on. Another application of nanoparticles is the introduction of organic wood preservatives and fungicides to wood products to reduce or halt wood decay (Liu et al. 2001, 2002a, b, 2003). These all together suggest that nanoparticles are next generation agro technology which aids the targeted delivery of agrochemicals with improved efficiency.

18.3.3 Nanogenetic Manipulation of Agricultural Crops

Nanobiotechnology plays a key role to manipulate the genes using nanoparticles, nanofibres and nanocapsules in modern agriculture (Radu et al. 2004; Torney et al. 2007; McKnight et al. 2003). Nanomaterials can act as transporter to carry a larger number of genes as well as stimulants of gene expression. It also aids in controlled release of genetic material throughout time in plants (Nair et al. 2010). Nanotechnologies pave the way to lead plant genetic engineering to the next level down atomic engineering. Seed DNA can be rearranged to obtain different plant properties, viz., colour, growth season and yield, using atomic engineering (Miller and Kinnear 2007). Nanomaterials are used to transport number of genes as well as chemicals that trigger gene expression in plants. Nanofibre arrays deliver genetic material to cells quickly and efficiently. Controlled biochemical manipulations in cells have been achieved through the integration of carbon nanofibers that are surface modified with plasmid DNA (Miller and Kinnear 2007). The successful delivery and integration of plasmid DNA was confirmed from the gene expression. This process has a similarity with microinjection method of gene delivery (Segura and Shea 2001; Neuhaus and Spangerberg 1990; Bolik and Koop 1991), making it feasible in the plant cells in which the treated cells could be regenerated into whole a plant that would express the introduced trait. DNA can be tied up on carbon nanofibers

without integration in the host genome but it allows transcription of tied gene and thereby it does not allow the transmission of modified traits to the next generation. This is considered as advantageous trait of the method as currently practiced genetic engineering techniques do not allow one time modification of the cells. It has been demonstrated possibility of using fluorescent labelled starch-nanoparticles as plant transgenic vehicle. Here, the nanoparticles produce instantaneous pore channels within plant cell wall, cell membrane and nuclear membrane with the help of ultrasound, which, thereby, allows the transport of genes within the plants. By this method it is possible to integrate various genes at the same time on nanoparticles. The imaging system fluorescence microscope enables researchers to monitor real time movement of genes and expression of the transferred genes along their path towards the nuclei. Success of nanoparticle-mediated DNA delivery to regenerative calli and soft tissues is dependent on successful generation of pores by suitable agents. Use of plasmid DNA coated silver nanoparticle treatments has been successfully practiced in petunia wherein plasmid DNA was incubated along with ethylene glycol (Rad et al. 2013). It is also possible to design nanobiosensors that can detect pollen load that contaminates genetic purity of many wind pollinated crops and thereby enable us to conserve genetic purity. Such bio-nanosensors *Clostridium thermoaceticum* and *Klebsiella aerogens* enable us to detect pollen contamination and thereby reduce the same for maintaining genetic purity. This method also prevents contamination of pollen of genetically pure crops with genetically modified crops. Generally, plant diseases are transmitted by seeds and many times stored seeds are lost by various diseases. The problem of this solution is nanocoating of seeds using elemental forms of Zn, Mn, Pa, Pt, Au and Ag. Such coated material when introduced on the seeds protects the seeds and also fulfils the minor element requirement of seeds to some extent.

Moreover, quantum dots (QDs) technique, comprising of fluorescence marker coupled with immuno-magnetic separation, was found to be useful to separate diseased seeds from healthy ones (Su and Li 2004). Imbibition of seeds within the nanocapsules containing specific bacterial strains enables us to reduce seed rate and improve crop performance. This type of smart seed can be dispersed over a mountain range for reforestation and their germination is programmed. As these seeds are coated with nano membranes, they can sense the availability of water and germinate then after. In the future, research initiatives can be started for aerial broadcasting of seeds surrounded with magnetic nanoparticle as well as detecting the moisture content during storage. It allows us with the opportunity to take appropriate actions to reduce the seed damage (Chinnamuthu and Boopathi 2009). The delivery of DNA and its activators by surface functionalized mesoporous silica nanoparticles (MSNs) that penetrate plant cell wall is the best example of precise manipulation of gene expression at single cell level. Mesoporous silica nanoparticles stimulate the plants to accept the DNA through cell wall. They deliver the genes coated with the nanoparticles and allow the gene transfer as well as activation of the genes in controlled fashion, without any toxic side effects. Use of mesoporous silica nanoparticle for gene delivery has been first demonstrated by Torney et al. (2007) for genetic manipulation of tobacco and corn plants. Honeycomb MSN system having 3 nm pore size was reported to be actively involved in transport of DNA and chemicals into isolated plant cells and intact leaves. In this technique MSNs containing gene of interest and its chemical inducer were coated with gold nanoparticles that protect the gene and chemical stimulant from being leached out. Removal of gold nanoparticle cap under controlled release condition and release of chemicals trigger gene expression in plants. It has been discovered that surface modification of MSNs with triethylene glycol is essential for penetration within the plants in the experiments wherein protoplasts were incubated with fluorescently labeled MSNs. Adsorption of plasmid DNA on MSN surface is also enabled by surface modification of nanoparticles with triethylene glycol. The plasmid DNA gets released after entering the protoplasts, the plasmid DNA was released from the MSNs after

entering into the protoplast and the expression of green fluorescent protein (GFP) marker encoded in the DNA was detected by microscopy in the cells. The advantage of this method is requirement of small quantity of DNA to detect marker expression which seems to be 1000 folds less as compared to conventional gene delivery methods. In the present era, gene gun and particle bombardment are popular tools for DNA delivery into plant intact plant cells in which particles used for gene delivery are generally made up of gold as they readily adsorb DNA and are non-toxic to cells. Deng et al (2001). MSN-based gene delivery system has a great promise to revolutionize protoplast-based gene expression. The main limitation of using MSN is their lighter weight, which makes them difficult to use with gene gun. This limitation can be overcome by capping MSN with heavy weight gold nanoparticles that aid in acceleration of their momentum during delivery. This method of using gold capped MSNs for gene transfer through gene gun was successfully practiced in intact tobacco and maize tissues (Klein et al. 1989). The main advantage of the method is delivery of DNA and effector molecules at the targeted specific site. In this way nanoparticle-based gene transfer in to plants differs from conventional methods of plant genetic engineering such as electroporation, microinjection, etc. Looking to this future thrust regarding MSN includes pore enlargement which provides possibilities of target-specific delivery of proteins, nucleotides and chemicals in plant biotechnology.

18.4 Nanosensors

Nanosensors, based on nanotechnology, are emerging as powerful tools to track, detect and control plant pathogen. The bioanalytical nanosensors either use biology as a part of the sensor or are used for biological samples (Scott and Chan 2003). Recently, the nanosensors are being used in conjunction with GPS system for real time monitoring of cultivated fields. Such network of wireless nanosensors can be formulated in cultivated fields, which enables the users to monitor soil condition and crop growth throughout the growing season. Precision farming can be best practiced using nanosensors, which aim to enhance productivity by minimum inputs, so information provided by nanosensors can help farmers to take better decisions (Joseph and Marrison 2006). Nanosensors may detect contaminants, pests, nutrient content and plant stress due to drought, temperature or pressure. They can improve the grower's ability to determine the best time of harvest for the crop, the health of the crop and questions of food security such as microbial or chemical contamination of the crop. They may also potentially help farmers increase efficiency by applying inputs only when necessary. Nanosensors, when inserted inside the cultivated fields, will provide essential information about essential data, which leads to precise use of agro inputs to get higher yield and thereby helps in precision farming (Scott and Chan 2003). The information provided by nanosensors includes optimum time for watering, fertilization, pesticide and herbicide application as well as time for planting and harvesting the crops depending on the physiology of soil and environmental conditions. Presently, agrochemicals, *viz*., fertilizers, pesticides, antibiotics and nutrients, are applied through spray or drenching. Generally plant protectants such as pesticides are applied before incidence of disease or after the visible symptoms have been developed. Nanodevices are intended to treat plant health problems such as diseases or malnutrition before the visible appearance of symptoms on a large scale. Nanoscale devices are intended to treat disease by its targeted action on affected area. Nanosensors comprising of immobilized bio receptors selective for target analyte molecules are called nanobiosensors. Applications of nanobiosensor include detection of urea, glucose, pesticides, etc., as well as microorganisms/pathogens (Rai et al. 2012). In agriculture and food sector nanobiosensors are also important for quantification of small amounts of contaminants like viruses bacteria, toxins, pollutants, etc. Using nano biotechnology, smart sensors are designed that possess high sensitivity and allow quicker response to environmental stimuli once incorpo-

rated in the equipment. Proficient detection, monitoring and detection of pathogen invasion, infection, nutrition requirement, *Clostridium thermoaceticum* and *Klebsiella aerogens* uptake and contamination have been developed. Nanosensors enable detection and eradication of infectious diseases in plants before incidence of visible symptoms, thereby reducing heavy economic losses which otherwise result in yield losses if kept untreated. Nanoparticle-based smart delivery systems help in controlled delivery of nutrients, pesticides, probiotics and neutraceuticals. Photosystem II containing biosensors can bind several groups of herbicides. Moreover nanobiosensors containing PSII isolated from photosynthetic organisms can be used to monitor chemical pollutants and provide a chance for set up of a low-cost, easy-to use technology to detect specific herbicides and a wide range of organic compounds before their disposal in to the environment (Rai et al. 2012). For measurement, two sensor detectors for relative activity of beneficial microbes and pathogenic microbes are inserted in soil suspension prepared in the buffer solution; the oxygen consumption data for both types of microbes were detected. After comparison of the two data, one can easily detect presence of prevalent microorganisms in the soil. Besides this, prediction of disease outbreak before its occurrence is also possible, which shows that biosensors provide us a novel diagnostic technology for detection of soil conditions by semi-quantitative approach. Nanosensors are portable instruments that allow precise quantitative monitoring and can overcome the deficiency of present sensors. Nanosensors that enhance the capacity to detect time of crop harvest and crop health have capacity to be an integral part of Controlled Environmental Agriculture (CEA).

18.5 Conclusion

Presently, agrochemicals such as fertilizers, pesticides, etc., are applied through spray or drenching to soil or plants. Nanoparticles are capable of detecting and treating any disease and malnutrition before visualization of symptoms. Nanoscale devices ensure delivery of agrochemicals to the targeted sites and thereby known as 'smart delivery system'. Such smart delivery system facilitates timely and controlled release of agrochemicals to the target site as the devices are self-regulated and preprogrammed and also comprised of capability of monitoring effect of delivered agrochemicals. Plants and their extracts provide a biological system for eco-friendly synthesis of metallic nanoparticles.

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Challenges in Regulation and Registration of Biopesticides: An Overview

19

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Abstract

Indiscriminate use of chemical pesticides in agriculture and allied sectors have contributed to soil, water and environmental pollution leading not only to human and animal illnesses but also biodiversity loss. The late 1980s, thus, opened new avenues for alternative pest management practices. Biopesticides derived from biological sources were tried, and, over the years, they have become an integral part of pest management practices, thereby minimizing the use of chemical pesticides. However, to protect the farmers, registration requirement of biopesticides has been made mandatory to ensure safety to human health, beneficial non-target organisms and environment. Countries promoting biopesticides constitute various regulatory bodies including committees/ boards/special authorities in order to oversee the regulatory affairs for biopesticides. These regulatory bodies formulate the dossier requirements for biopesticides and update the dossiers from time to time based on local and international needs. Robust and stringent but user-friendly regulatory norms would ensure availability of quality and safe biopesticide formulations.

In this review, current regulatory mechanisms operative in India, associated issues and probable modalities to address them have been discussed.

Keywords

Biopesticides • Biosafety • Registration • Regulation • Microbes • Inoculants

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D.P. Singh et al. (eds.), *Microbial Inoculants in Sustainable Agricultural Productivity*,
DOI 10.1007/978-81-322-2644-4_19

19.1 Introduction

After the introduction of the Green Revolution, commercial agriculture adopted the use of chemical pesticides during the late 1960s for management of emergent pests. However, over time, for reducing the losses due to these biotic stresses to the barest minimum and also due to lack of knowledge of specificity of the molecules against different insect pests and pathogens, the farming community started indiscriminate use of pesticides. This has led to development of resistance in the insects and pathogens to these molecules (James 1989; Robert 2013). Further, this indiscriminate use has resulted in polluting the soil, water and environment and harmful toxic effects to the end user (Nobuhiko et al. 2014; Venkateswarlu et al. 2000). After realizing these side effects of chemical pesticides, research was refocused towards integrated management of biotic stresses where chemical pesticides were given last priority (Jitendra et al. 2015). As an alternative, new avenues of pest management practices were developed, which included cultural practices, host plant resistance and biological control to counter industry-acclaimed issues like pest resurgence/resistance to chemical pesticides, residue restrictions for exports and appropriate registration protocols.

Thus, in recent years, biopesticides derived from plants, animals, microbes and other natural substances have become an integral part of pest management practices to minimize the use of chemical pesticides. Though biopesticides may not totally replace the chemical pesticides, increasing awareness about organic agriculture and green environment among the producers and consumers compelled the agricultural input manufacturing industries across the globe to produce biopesticides on a commercial scale. Since biopesticides are included under pesticides category, their registration has been made mandatory (Pawar 2001). The topic of registration and regulations in general may not be an interesting subject matter to read/learn. In the current chapter, the concept is simplified for better understanding along with an in-depth discussion on the need for developing improved regulatory procedures, important bottle necks for registrations and possible solutions to design and develop standard protocols for improving current biopesticide regulation and registration practices.

19.2 Regulation for Biopesticides

In India, it is mandatory to register biopesticides in order to ensure their safety to human health, beneficial non-target organisms and environment. The developed and developing countries have put forth several dossiers for registering biopesticides (OECD 1996, 2002; FAO 1988; Leahy et al. 2014). Countries promoting biopesticides set minimum dossier requirements when compared to dossiers required for conventional pesticides, since biopesticides are generally less toxic. Different tier systems are adopted across countries for regulating and registering any substance or mixture of substances like biochemical – and microbial – pesticides intended for controlling the pests. Countries like USA have included genetic material for pest management used for developing transgenic plants also under biopesticides (Smyth and McHughen 2012).

Most of the countries are keen to promote biopesticides by easing the registration and dossier requirements. The rising consumer desire for quality organic food produce is creating a need to regulate and register biopesticides at the highest standards. However, biopesticide registration is still a challenge in several countries especially for small and medium enterprises (SMEs), which significantly contribute to the production of biopesticides in developing countries. Since the majority of biopesticide products are not 'standalone' molecules, SMEs are unable to spend more on capital-intensive registration and dossier procedures due to their limited investments.

The regulatory authorities for registration and scrutiny of microbial biopesticides data need highly qualified and experienced subject experts. Due to the lack of in-depth scientific insight, the regular registration experts handling conventional pesticide data are having limitations in handling the complex biopesticide data. Good laboratory practice (GLP) compliance is becoming a key

factor for the universal acceptance of biopesticide data. Statutory requirement for registration also varies from country to country.

Several efficient biopesticides did not reach the status of what they are supposed to, though neem and microbial-based Bt formulations have been partly successful. In spite of strict regulatory and dynamic guideline changes, implementing authorities in countries like India and elsewhere treat biopesticides equivalent to conventional chemical pesticides. This is a major hurdle for the registration and market penetration of biopesticides.

Biopesticides derived from microbes and biochemical components of naturally occurring substances are mostly regulated. Genetically modified plants were also brought under biopesticide act in USA and other European countries (Smyth and McHughen 2012). In USA, Environmental Protection Agency (EPA) regulates the biopesticides generated using genetically modified organisms (GMOs). The other biological pest management mechanisms like kairomones, allomones, pheromones, apnimones, predators, parasites and parasitoids are not covered under regulation (Table 19.1).

19.3 Data Requirements for Biopesticide Registration

The majority of nations promoting biopesticides have their own regulatory guidelines. These guidelines have also been posted on their respective websites. Biopesticides registration also needs data on 'technical' (technical ingredient of the formulation like Azadirachtin in case of neem formulation and delta-endotoxin in case of Bt formulations) and 'formulation' (the technical ingredients along with carrier, adhesives, surfactants, spreaders, etc.) similar to conventional pesticides. The dossier required for the regulatory authorities is expected to be generated for both 'technical' and 'formulation' stages of biopesticides.

The protocol and dossier requirements of biopesticide registration vary slightly from country to country including Europe and OECD countries. To register biopesticides worldwide, data such as identification and description of the organism/ingredients, biological properties, bioefficacies in the laboratory/screen house and field, safety/ecotoxicity studies, toxicology, packaging, etc., are required. Readers can verify the respective regulatory authority's websites for complete information on dossier requirements. In addition to the listed guidelines, authorities can also insist on additional studies that are not listed in the protocol.

19.4 Regulatory Mechanisms for Biopesticides

Countries promoting biopesticides constitute various regulatory bodies including committees/boards/special authorities in order to oversee the regulatory affairs of biopesticides. These regulatory bodies formulate the dossier requirements for biopesticides and update the dossier from time to time based on local and international needs. Country-wise select regulatory authorities are listed in Table 19.2.

Biopesticide regulatory mechanisms differ from country to country. A principle dossier requirement is based on the country's local need and advancement of scientific knowledge in the respective region. For example, in India, there is a two-tier system for biopesticide registration. Tier-I [9(3b)] approves provisional registration for 2 years with certain conditions and Tier II [9(3)] sanctions permanent registration after submission of specific additional data. The data generated under Tier-I provisional registration in India would be sufficient for some countries to sanction a permanent registration for a biopesticide. In contrast, in USA, the regulatory agency EPA requires additional data even after meeting Tier-II data requirements in India. Similarly, CIBRC in India insisted additional data on safety of *Paecilomyces lilacinus* against human in Tier II during 348 and 349 meeting in 2014. This indicates the complexity involved in biopesticide registration among various regulatory bodies worldwide. More importantly, majority of the countries do not insist on data generated through

Table 19.1 Regulatory status of products under pesticide regulation act

Source	Descriptions	Examples
A. Products under pesticide regulations		
Biochemical	Components derived from plant/ animal origin with pesticide properties Substances that are naturally occurring Stimulants that induce the plant defence mechanisms	Neem formulations containing Azadirachtin *Triptericium wilferdii* Hook GTW – Plant extract – Glycosides having Triptolide, Tripdiolide and Tripterolide Spearmint Extract – Spearmint (*Mentha Spicata*) Oil containing L-Carvon, Limonin and Pines American Wormseed Oil, Chenopodium Oil, ECANA and ECANA Mimic Eucalyptus leaf extract Containing 1, 8 cineole (eucalyptol) Extract of *Chenopodium ambrosioides* near ambrosioides and extract of *chenopodium ambrosioides* near ambrosioides (Mimic) Bitterbarkomycin (Plant extract of *Apocynum venetum*) 1a, 2a, diacetoxyl-8A,15 dissobutyl acyloxyl-9a-benzyloxy-4A; 6A dihydroxy-A-dihydrolignaloefuran Squamocin – Seed extract of Plant *Annona squamosa* Linn (Custard apple)
Microbial	Any eukaryotes like fungi, protozoa, algae, etc., with pesticide properties Any prokaryotes like bacteria with pesticide properties Obligate pathogens with self replicating genetic material with pesticidal properties	*Bacillus thuringiensis* var *kurstaki* *Bacillus subtilis* Kuhn *Gliocladium* spp *Pseudomonas* spp *Myrothecium verrucaria* *Trichoderma* spp *Beauveria bassiana* *Metarrhizium anisopliae* var *acridum* *Nomuraea rileyi* *Verticilium lecanii* *Hirsutella* spp *Verticilium chlamydosporium* *Streptomyces griseoviridis* *Streptomyces lydicus* *Ampelomyces quisqualis* *Candida oleophila* *Fusarium oxysporum (non pathogenic)* *Burkholderia cepacia* *Coniotyrium minitans* *Agrobactarium radiobacter* strain 84 *Agrobactarium tumefaciens* *Pythium oligandrum* *Erwinia amylovora* (Hairpin protein) *Phlebia gigantea* *Plaecilomyces lilacinus* *Penicillium islanidicum* *Alcaligenes* spp. *Serratia marcescens* GPS 5 (Bacteria) *Photorhabdus luminescences akhurustii* Strain K-I *Chaetomium globosum* *Aspergillus niger*-strain AN27 Grannulosis Viruses (GV) Nuclear polyhedrosis virus (NPV) Bacterial Extract (Physiological Extract of Bacteria and species of Blue Green Algae) Glutamic Acid content as 'Marker'

(continued)

Table 19.1 (continued)

Source	Descriptions	Examples
Transgenic plants	Plant Incorporated-Protectants (PIPs) are the incorporation of genetic material having the pesticide property into the plant (GM crops)	*Bt* cotton, *Bt* brinjal, *Bt* soyabean
B. Products not covered under pesticides regulation		
Semiochemicals	Pheromones: Synthetic chemical substance(s) that attract/ deter insect pests with minimum or no risk to environment Kairomones: Chemical substance(s) that are beneficial to the releaser and harmful to the receiver with minimum or no risk to environment Chemical substance(s) that are beneficial to the receiver and harmful to the releaser with minimum or no risk to environment	Pheromones for monitoring and/ or as sex disruptants for *Helicoverpa armigera, Spodoptera litura, Scirpophaga incertulas* and *Leucinodes orbonalis.*
Parasites and Predators	Macro organisms that predate/ parasite and reduce the population of the pest	Nematodes, *Trichogramma chilonis, Chrysoperla carnea*, etc.
PGP	Microorganisms either individually or in combination promote(s) the growth of the plant with no pesticide properties	*Bacillus polymixa, actinomycetes, etc.*
Biofertilizers	Microoragnisms that mobilize/ solibilize nutrients required for plant growth	*Pseudomonas* spp. *Glomus fasciculatum* *Bacillus* spp. *Azospirillum* spp. *Azotobacter* spp. *Rhizobium* spp. and Arbuscular mycorrhiza.

laboratories adopting universal good laboratory practices (GLPs). With the absence of uniform protocol requirement and laboratory standards, it is becoming difficult to register biopesticides for small and medium enterprises (SMEs).

19.5 Is biopesticide a Threat to PGP?

Under EPA, biopesticide regulation does not include plant growth promoting rhizobacteria (PGPR) and semiochemicals. It also exempts special pesticides that are having minimum risks. However, several other countries do not have precise regulations on semiochemicals like insect sex pheromones and PGPR. In some cases, though the regulatory bodies do not register them under biopesticides, the implementing states may insist on regulations on these products. This leads to several challenges for the large-scale promotion of PGPR, etc., that is not covered under biopesticides. Spurious 'biochemical pesticides' laced with conventional pesticides and/or intermediates are becoming a real threat to genuine PGPRs. There is great need for initiating a brain storming session between various regulatory agencies across the globe to arrive at clear guidelines for registering and regulating PGPR and semiochemicals. Fortunately, there are no ambiguities observed on promoting specific macro-organisms like predators/ parasitoids except release of exotic organisms.

Table 19.2 List of regulatory agencies/bodies that started enforcing biopesticide registration in various countries

Country	Year	Authority
India	1999	Central Insecticide Board and Registration Committee (CIBRC)
USA	1961	United States Environmental Protection Agency (US EPA)
Italy	1993	Italian Ministry of Agriculture Food and Forestry
Japan	1981	Food and Agriculture Material Inspection Center (FAMIC)
Australia	1987	Australian Pesticides and Veterinary Pesticide Authority
Brazil	1990	Ministry of Agriculture and Food Supply
Switzerland	1964	The Federal Office for Agriculture (FOA)

19.6 Is Microbial-Based Biopesticide over Regulated?

In the era of spurious biopesticides flooding the market (biopesticides spiced with conventional pesticides or their intermediates), regulatory bodies bring stringent protocols for biopesticides. However, on the other hand, plant growth promoting rhizobacteria (PGPR) and bio-fertilizers (bio-inoculants and composts) have been extensively promoted with acceptable regulatory procedures. Though microbial-based biopesticides, PGPRs and bio-fertilizers have single or multiple microorganisms with similar methods of application, the regulation is very stringently implemented only for biopesticides. Is microbial-based biopesticide over regulated since it is having a suffix pesticide? The positive side of regulation is that it ensures accessibility of safe and quality biopesticide formulations with lawful commercial trade protocol and acceptable performance.

Owing to the safety and less toxic effect of biopesticides over chemical pesticides, manufacturers and research experts in India and elsewhere always claim that biopesticide registration is over regulated. For example, biopesticide generated from microbes meant for soil application needs data monitored on parameters such as pH, other microbial contaminants and pathogens. It also needs to be scrutinized for safety to earthworms and/ or other beneficial arthropods. The composts, *viz.*, vermicompost, city composts and cane composts, have also been advocated extensively to enrich the soil organic carbon. The microbial load recorded in the vermicompost is provided in Table 19.3 in order to compare it with the biopesticide formulation recommended for soil application.

Table 19.3 Comparison between a biopesticide formulation and vermicompost

Biopesticides	Vermicompost
Permitted for single organism/strain Eg: *Pseudomonas fluorescens* 2×10^8 or *Trichoderma viride* 2×10^6 (CFU/g)	Reported to contain multiple microbial organisms/ strains including bacteria (19×10^8), actinomycetes (9×10^4), fungi (19×10^3), phosphate solubilizers (176×10^4) cellulolytic bacteria (14×10^3) fluorescent pseudomonads (3×10^4) nitrifying bacteria (0.3×10^6) and denitrifying bacteria (3.68×10^6) (CFU/g) (Daniel et al. 2013)
Contamination permissible limit 1×10^3(CFU/g)	No check for contamination
Dosage ranged between 1 kg and 5 kg per ha	10–25 t per ha
Strictly no human pathogens	Depending on the raw material possibilities of the pathogens presence are high
pH to be maintained	No such parameters are followed
Highly Regulated	Not regulated under Environmental Protection Act.

Note: Though biopesticides and compost cannot be compared, the tabulated content indicated here is to give an overview of two different formulations meant for soil application

In India, where government is actively promoting biopesticides, regulatory bodies take about 2–5 years in awarding permanent registration to biopesticides. There is no strict time frame specified for awarding biopesticide registration. In contrary, obtaining registration for chemical pesticides in India under the category 9(4) is very simple and cost effective. It costs less than 5 USD. In brief, obtaining registration for chemical pesticides in India under 9(4) is much faster and cost effective than obtaining registration for

biopesticide under 9(3), which costs about 10–15 thousands USD.

The present implementation of biopesticide registration is about 10–25 years old. However, the experts who handle conventional chemical pesticides often struggle to understand the intricacies of biopesticides. This is often becoming true in case of microbial biopesticides. Toxicology experts may be unaware about the basic biology of species and speciation. Some of the common errors of judgment that crop up in biopesticide registration are

- Potency of the formulation
- Mis-identity of biopesticide organism sharing the same genus name with known human/plant pathogens
- Methodologies adopted for scientific studies
- Comparison of results with conventional pesticide studies
- Non-availability of standard protocols in selected cases and
- Viewing biopesticides as equalent to conventional pesticides

19.7 Refinement of Regulation in Global Perspective

Recommendation for the withdrawal of organochlorine-based conventional pesticides like endosulfan, monochrotophos, phorate and carbofuran due to their toxic nature and select nicotinoids for their safety against honey bee has always raised concern about intensive use of conventional chemical pesticides in spite of strict regulations and dossier studies (Gill et al. 2012; Williams et al. 2015). Consumers need more safe non-chemical pesticide formulations. The demand and awareness is also increasing gradually for pest management with no or reduced conventional pesticides. Therefore, it is essential to have the following constructive policies for the speedy development of biopesticides at a global level:

- Internationally accepted uniform guidelines for biopesticide registration.
- Simplification of dossier requirement focusing biopesticides.
- A separate registration committee for biopesticide under biopesticide acts by not including them under insecticide act.
- Appointment of appropriate technical experts for biopesticide registration. More importantly, a separate team or division having biopesticide expertise is essential for promoting biopesticides.
- Fast track registration procedures with specified time frame.
- Waiving of select data requirements for the subsequent applicant in case if the biopesticide dossier of the strain is already approved. This is applicable mainly for microbes obtained from public domain.
- Implementing tier system in case of toxicological evaluation when there is a real necessity for chronic studies.
- Insisting multinational conventional pesticide companies to promote a minimum 15–25 % of biopesticides equivalent to their conventional pesticide market. This needs to be considered as social responsibility for companies promoting conventional pesticides.
- Providing financial assistance to SMEs for data development and registrations.
- Tax incentives for domestic/import/export of biopesticides.
- Acceptance of published data in peer-reviewed journals for label expansion.
- Development of guidelines and registration for biopesticides having microbial consortia in order to compete on efficacy with conventional pesticides.
- Create task force for checking spurious 'biopesticides' laced with regulated/ non-regulated chemical ingredients. The spurious formulations bring down the trust of consumers on biopesticides.

19.8 Conclusion

Biopesticides, though passive right now, would take the centre stage within two decades when more scientific studies reveal the adverse effects of

conventional pesticides on humans, animals and the environment. The present dossier requirements are dynamic and differ from country to country. Due to heavy expenses and complexity involved, majority of SMEs are unable to bear the expenses of data generation and registration of biopesticides. There is a need for the development of an appropriate internationally accepted tier system dossier. Able technical expertise and a separate division or authority for biopesticide registration in several countries is the need of the hour.

Diverse formulations and easy availability to the consumers are the two important factors for promoting biopesticide use. Regulators should work towards promoting cost-effective, stable and bio-effective biopesticide formulations with single or consortia of technical ingredients in order to reduce the overdependence on conventional chemical pesticides. This would ensure availability of quality and safe biopesticide formulations across the globe for the benefit of the end users leading to a cleaner environment.

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