Naveen Kumar Arora · Samina Mehnaz
Raffaella Balestrini *Editors*

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Editors
Naveen Kumar Arora
Department of Environmental Microbiology
School for Environmental Sciences,
Babasaheb Bhimrao Ambedkar University
Lucknow, Uttar Pradesh, India

Raffaella Balestrini
Istituto per la Protezione Sostenibile delle Piante (IPSP)
Consiglio Nazionale delle Ricerche (CNR)
Torino, Torino, Italy

Samina Mehnaz
Department of Biological Sciences
Forman Christian College (A Chartered University)
Lahore, Pakistan

ISBN 978-81-322-2777-9 ISBN 978-81-322-2779-3 (eBook)
DOI 10.1007/978-81-322-2779-3

Library of Congress Control Number: 2016939419

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer (India) Pvt. Ltd.

Preface

Plant growth-promoting (PGP) microorganisms help the plants in a number of ways. In the last few years, several efforts have been made by the researchers to highlight the applicative potential of these beneficial soil microorganisms providing nutrients and protection to the plants. The role of such microbes in sustainable agriculture has been well documented. These microorganisms, such as plant growth-promoting rhizobacteria (PGPR) or plant growth-promoting fungi (PGPF), can be the best alternatives for chemicals (fertilizers and pesticides). Although the development and use of bioformulations with these beneficial microorganisms have seen an upward trend in the last few years, in broader perspective, the growth has not been up to mark, particularly so in the developing countries. There is a great concern for green and safe food, but a bigger question is on food security for all. We know that to fit in the shoes of chemicals, a lot more has to be done for the development of bioformulations which are reliable and as effective as in lab conditions.

To be successful, a formulation should provide the right number of beneficial microbes in good state to the soil or rhizosphere or the plant. In present-day scenario where the soils are becoming non-fertile or barren by the day, it is important that useful soil microorganisms are augmented in the fields. It is particularly important that the deficient soils are enriched with useful microorganisms such as *Rhizobium*, *Azotobacter*, PGP fluorescent pseudomonads, arbuscular mycorrhizal (AM) fungi, etc. This can be only achieved if the end users, i.e., the farmers, have confidence in the bioformulations. For this achievement, the quality of the current bioformulations has to be enhanced, involving the use of the latest technologies and understanding the ecological requirements, the mechanisms involved in the interaction with roots, and the habitat where they are going to be introduced. It is important to find out the loopholes and address them so as to enhance the credibility of the bioformulations in future. However, already we are seeing a drift or change. The bioformulations being developed now are multifaceted, involving consortia of useful microbes and also including secondary metabolites which aid the establishment in rhizosphere and help root colonization. Successful inclusion of microbial metabolites such as flavonoids, phytohormones, and lipochitooligosaccharides (LOCs) in bioformulations is showing the way. Although in recent years several improvements have been done, research in this field should be enhanced.

For further development, a fruitful partnership between industry-academia-government/regulatory authorities and the end users is required. All these issues have been elaborately discussed in the volume so as to give directions for future research and development of a consistent and globally successful product.

The book consists of 16 chapters contributed by experts from around the globe. The experts are involved in the development of bioinoculants by utilizing diverse PGP microorganisms. The book includes and discusses the history of growth of bioformulations. The process of development of bioformulations, the constraints faced, and the requirements for the future have been discussed in detail. The tome also describes the present-day market scenario of the bioformulations, including both biofertilizers and biopesticides, and how improvements can be done in the future. The diverse roles of bioformulations in reclamation of stressed and marginal soils are also discussed. Recovery of stressed and polluted soils with the help of bioformulations is a very important aspect for the future research and applicative projects. This will not only increase the arable land but will make our planet greener and also help in enhancing productivity for food security. Overall it can be said with conformity that the future of bioformulations is bright; the only thing is to achieve the goals and targets as swiftly as possible, so as to enhance the market share of these green alternatives.

The volume is a unique compilation on microbial products available and to be formulated in future for sustainability of agriculture and soils. The tome will be extremely useful for the researchers involved in the development of bioformulations/bioinoculants, agricultural sciences, microbiologists, biotechnology-related industries, end users, and regulatory authorities around the globe. The book will also be helpful for graduate and postgraduate students and faculty pursuing their career in the field of bioformulation development and use.

The editors are thankful to all the authors who have contributed in this book and have provided excellent knowledge on diverse aspects of the topic. The contributors have really made the book exceptional and novel in itself. The editors would also like to thank Dr Mamta Kapila, Senior Editor, Springer (India), for her support. It is because of her sustainable approach that such a product will see the light of the day, which will go some way to make this planet greener.

NKA is obliged to Prof RC Sobti, Vice Chancellor, BBA University, Lucknow, UP, India, for the encouragement and support. NKA would also like to thank his team of research scholars, namely, Jitendra Mishra, Rachna Singh, Sakshi Tewari, Maya, Shweta, Jai Prakash, and Sushma Verma, for helping in the compilation of the manuscript. Last but never the least, NKA is indebted to his wife Preeti and kids Pranay and Nav for bringing calmness and fulfillment.

Lucknow, Uttar Pradesh, India — Naveen Kumar Arora
Lahore, Pakistan — Samina Mehnaz
Torino, Italy — Raffaella Balestrini

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About the Editor

Dr. Naveen Kumar Arora, Ph.D., Microbiology, Associate Professor in Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow, Uttar Pradesh, India, is a renowned researcher in the field of Environmental Microbiology and Biotechnology. His specific area of research is rhizosphere biology and PGPRs. He has 38 research papers published in premium international journals and several articles published in magazines and dailies. He is editor of the books *Plant Microbe Symbiosis: Fundamentals and Advances and Plant Microbes Symbiosis: Applied Facets*, published by Springer. He is member of several national and international societies and reviewer of several international journals. He has delivered lectures in conferences and seminars around the globe. He has a long-standing interest in teaching at the PG level and is involved in taking courses in bacteriology, microbial physiology, environmental microbiology, agriculture microbiology, and industrial microbiology. He has been advisor to sixty seven postgraduate and four doctoral students. Recently he was awarded for excellence in research by the Honorable Governor of Uttar Pradesh and recieved "Young Achievers Award" from Asian PGPR Society. Although an academician and researcher by profession, he has a huge obsession for the wildlife and its conservation and has authored a book, *Splendid Wilds*. He has a website www.naveenarora.co.in dedicated for the cause of wildlife and environment conservation.

Dr. Samina Mehnaz, Ph.D., Microbiology, is a Professor and Chairperson of the Department of Biological Sciences, Forman Christian College (A Chartered University), Lahore, Punjab, Pakistan. She is a researcher in the field of Microbiology. Her specific area of interest is rhizosphere microbiology, specifically PGPRs and their secondary metabolites. She has 34 research papers published in international journals and 4 book chapters (as a single author) published by Springer. Currently, she is an editor of *Archives of Phytopathology and Plant Protection* (Taylor & Francis). She is a reviewer of several international journals and is the continuous winner of the research productivity award since 2010, by Pakistan Council for Science and Technology. She is an Alexander von Humboldt (AvH) Fellow (Germany) and Natural Sciences and Engineering Research Council (NSERC) Fellow (Canada). She has been advisor to nine post graduate and three doctoral students and one post-doctorate fellow.

Raffaella Balestrini, Ph.D., Biology and Biotechnology of Fungi, is Senior Researcher at the Institute for Sustainable Plant Protection, CNR. Her researches mainly focus on different aspects of plant-symbiotic fungi interactions (endo- and ectomycorrhizae). The researches she currently developed, using molecular and cellular approaches including confocal microscope, in situ hybridization, and laser microdissection (LMD), are genomics and functional genomics in mycorrhizal fungi; cellular and molecular interactions between plants and mycorrhizal fungi, with particular attention to plant and fungal cell wall modifications; plant-mycorrhizal fungi interactions to improve plant tolerance to abiotic stress; and transcriptome profiles of specific cell types involved in mycorrhizal symbiosis. She is also involved in the black truffle *Tuber melanosporum* genome project as the person in charge of the annotation of cell wall-related genes thanks to the experience in gene expression and cell biology studies in mycorrhizal associations, in post-genomic activities. She has worked with research groups from national and international institutions in joined research projects, as mirrored by the several papers in collaboration. She is referee for several international journals and project proposals. She has 68 publications in international journals and 6 book chapters to her credit.

Part I

Introduction

1 Bioformulations for Plant Growth Promotion and Combating Phytopathogens: A Sustainable Approach

Jitendra Mishra and Naveen Kumar Arora

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Abstract

The role of microbes in sustainable agriculture has provided new insights to agro-economy, and one of the direct benefits is the lesser reliance on chemical fertilizers and pesticides as the continuous application of these chemicals not only showed detrimental effects on agro-ecosystems but also resulted in health risks to humans and animals. In last few years, the development of microbial bioinoculants for enhancing plant growth and disease eradication has emerged as an alternative, but a broader aspect of their application as formulatory product has remained in infancy especially in developing countries. At the economic and social level also, this green strategy is facing hurdles and lags far behind their competitors, the synthetic fertilizers and pesticides. Most of the times it has been found that bioformulations available for a particular crop do not give good results equivalent to those in the laboratory conditions. Such and related constraints are major challenges of this greener approach. Various workers all over the world are continuously engaged in developing formulation products which could be easier to use, show enhanced activity toward phytopathogens, and may cover more target crops. Whole process of bioformulation development, from screening of microbe to product development and its implementation, need to be reviewed.

J. Mishra • N.K. Arora (✉)
Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow 226025, Uttar Pradesh, India
e-mail: nkarora_net@rediffmail.com

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_1

In this article several aspects of formulation development have been critically discussed, and the main motive is to describe types of bioformulations being used, their efficacy in the field, and reasons which limit their wider application in field.

1.1 Introduction

Sustainable agriculture is proving as one of the toughest jobs in these days. There is no uniformity in agriculture practices all over the world, but one thing which is more or less common is the use of chemical pesticides and fertilizers. Around the globe about 890 synthetic chemicals are approved as pesticides, whereas marketed products are estimated to be approximately 20,700 and among them organophosphorus insecticides are the biggest group (Stenersen 2004). The use of pesticides is not evenly distributed among various crops, and data indicates that 93 % of all row crops such as corn, cotton, and soybeans are treated with some type of pesticide, whereas percentage of forage crop is less than 10 (Pimentel 1993). Albeit synthetic chemicals enjoy a great reputation in enhancing crop productivity and checking several plant diseases but are also proving as environmental havoc (Fenske and Day 2005; Colt et al. 2007), in recent years their role in damaging agro-ecosystems is very well known. The scenario in developing countries is worse and studies show that although these regions use only 20 % of the world's agrochemicals, they suffer 99 % of deaths from pesticide poisoning (Kesavachandran et al. 2009). Among them farmers are the main victims and occupational exposure is very high with lack of technical education (Konradsen et al. 2003; Coronado et al. 2004; Khan et al. 2009a). According to the World Health Organization (WHO), approximately 20,000 workers die from pesticide exposure every year (Pimentel et al. 1992). Studies conducted show that most of the agricultural pesticides now being used can have detrimental effects on human health (Reigart and Roberts 1999) that include rashes, headaches, nausea and vomiting, disorientation, shock, respiratory failure, coma, and, in severe cases, death (Moses et al.1993), whereas long-term exposure can cause cancer and neurologic and reproductive problems (Sanborn et al. 2007). Although human health is of much concern, at the same time excessive use of chemical pesticides is also deteriorating the environment and mostly aquatic systems, wildlife, and sensitive ecosystems are very much disturbed (Stoate et al. 2001; Berny 2007). Pesticides pose a drastic change in the richness of soil microbial diversity (Dorigo et al. 2009), and large-scale killing of beneficial microbes is more common in agricultural lands or the crops which are treated by their repeated use (Papp et al. 1992). Moreover it has also been found that microbial diversity is mainly affected by the type of pesticide used (Johnsen et al. 2001), and data suggest that diverse pesticides may have varied effects on residential microbial population (Spyrou et al. 2009).

Another major dependency of our agriculture is on synthetic fertilizers, used and widely accepted to maintain soil fertility and crop yields. In the last few years, a great number of long-term experiments were initiated to examine the effects of fertilization on soil fertility (Mitchell et al. 1991; Whitbread et al. 2003) and these studies concluded that in one way the use of fertilizers was necessary, and its continuous application increased the concentrations of soil organic matter (SOC), total nitrogen (TN), and other nutrients in plow layers compared with the initial value at the beginning of the experiment (Huang et al. 2010), but there were also results showing that the continuous use of fertilizers is responsible for the decline of soil quality and productivity (Kumar and Yadav 2001; Yang 2006). Recently in a study, Liang et al. (2013) also showed that excessive application of nitrogen and phosphorus fertilizers induces soil acidification and phosphorus enrichment during vegetable production. Microbial composition, population, and functions are also affected by fertilizers applied, and some workers showed that microbial activity of soil microbes increased

(Mandal et al. 2007; Ge et al. 2008), whereas other experiments confirmed little or no effect on soil microbial diversity and activities when organic or inorganic fertilizers are applied (Nakhro and Dkhar 2010). Allison and Martiny (2008) also reviewed that microbial composition is, in the majority of cases, sensitive to elevated CO_2, mineral fertilization, temperature changes, and C amendments. The sensitivity of soil microbial communities is also a matter of interest as it likely differs between unmanaged and agricultural ecosystems (Daniel and Kate 2014). That is why there is an emergent need of developing a greener and safer alternative for increasing crop productivity and disease control at the global level, and the use of microbe-based bioformulations is an open choice for achieving the goal of sustainability (Mishra et al. 2015).

Microbe-based formulations also known as bioformulations are more robust than synthetic chemicals as the formulation product of a single microbe may involve direct interactions with pathogens, and numerous mechanisms take part in disease suppression and plant growth promotion (Rodrigo et al. 2011). The smartness of these bioagents can be surmised by the fact that a particular strain in the vicinity of a plant is capable to control disease without producing lasting effects on the rest of the microbial community or other organisms in the ecosystem (Howarth 1991). A specific group of such useful microbes are the plant growth-promoting rhizobacteria (PGPR), characterized by their innate capability to colonize the root surface (Kloepper and Schroth 1994). PGPR are endowed with several traits that assist plant growth promotion (PGP) (Lugtenberg and Kamilova 2009). PGP activities which are very much of interest are phytohormone production (Strzelczyk et al. 1994; Suzuki et al. 2005; Tank and Sarafa 2009), secondary metabolite production such as hydrogen cyanide (Lorck 2004) and siderophores (Neilands 1995; Tian et al. 2009), ammonia production and nitrogenase activity (Glick 2012), phosphate solubilization (Whitelaw 2000; Igual et al. 2001), and several others. Amid PGPR *Rhizobium* spp., *Bradyrhizobium* spp., *Mesorhizobium* spp., *Pseudomonas* spp., *Bacillus* spp., *Azotobacter* spp., and *Azospirillum* spp. are of great interest, and now their use in making bioinoculants to promote plant growth is being promoted at the global level (Saharan and Nehra 2011). A high-impact research is being done on screening novel species of soil beneficial microbes and their use in developing microbial inoculants for combating phytopathogens and enhancing crop productivity (Hafeez et al. 2006; Glick 2012; Ahemad and Kibret 2014). Although the gradual success of such beneficial microbes in agro-ecosystems is indicating that our goal of sustainable agriculture is not a formidable task, more challengeable is to design such a state-of-the-art bioformulation technique that not only gives environmentally friendly easy-to-use product but also assures good results in field so as to displace the harmful chemicals. Besides this global market and registered companies are also trying to reduce manufacture cost which will definitely help farmers to opt these microbe-based products as suitable alternatives for their crop production. This review addresses the current status of bioformulations, their availability in the market and shortcomings, and future of these greener alternatives.

1.2 Brief History

The use of microbial inoculum in PGP and eradication of phytopathogens has been reported in historical events that took place in the field of soil microbiology (Davison 1988; Ehrlich 1990; Insam 2001). Although to describe all such events is a hectic task and also not the theme of this review (for this reviews by Coyne (1996) and Insam (2001) can be seen), some of the discoveries which strengthen the field of biofertilizers and biocontrol are being discussed here. In this series if we envisage the development of microbial biofertilizers, French chemist Boussingault first recognized that leguminous plants could use nitrogen (N_2) from the air and gave process detail in his report in 1838 (Wilson 1940). Again this finding was tested by Hellriegel and Wilfarth in Germany by performing an experiment and demonstrating

that pea plants (*Pisum sativum*) in association with bacteria in their root nodules are capable of using nitrogen (Bottomley 1912). Very soon in the United States of America (USA), the first patent "Nitragin" was registered for plant inoculation with *Rhizobium* sp. for providing nitrogen source to the crop (Nobbe and Hiltner 1896). The occurrence of cyanobacteria in rice fields was first identified by Fritsch (1907), but their role in soil nitrogen supply was demonstrated by De (1939). Although the use of microbial inoculum in N_2 fixation was successful, the role played by other beneficial microbes was to be discovered. Gerretsen was studying Mn deficiency problem in oat and during his study he also pointed out problem of phosphate uptake by plants and first demonstrated the capability of rhizospheric bacteria in solubilizing insoluble phosphates (Mulder 1967), and later other workers also confirmed microbe-mediated solubilization of phosphates (Sperber 1957; Goldstein 1986; Subbarao 1988). During the 1960s beneficial effect of mycorrhiza in nutrient uptake was also recognized and very soon they were utilized as biofertilizers (Roger and Mosse 2004). Application of potassium (K)-solubilizing microorganisms (KSM) for increasing K availability in soils was also reported by some workers (Zahra et al.1984; Vandevivere et al. 1994; Sheng and Lin 2006). Although these events are of prime importance, a holistic approach of soil-inhabiting beneficial bacteria was first revealed by Kloepper and Schroth by identifying the potential of PGPR (Kloepper and Schroth 1978). These rhizospheric bacteria were capable of affecting plant growth both directly and indirectly, and further research showed that various crops treated by plant growth-promoting microbes not only enhanced crop productivity but also minimized the use of synthetic chemicals (Adesemoye et al. 2009). Later for large-scale use, their mass production in the form of appropriate bioformulations was required, which included the development of formulations which should be stable, have a long shelf life, and give the desired results when applied in the field with an appropriate delivery system at the lowest effective dose.

Historical trends that opened opportunities in developing biopesticides were first started when Agostino Bassi in Italy discovered that a fungus, *Beauveria bassiana*, caused an infectious disease in the silkworm (Bassi 1835). After this splendid discovery, some workers including V. Audouin in France, J. LeConte in the United States, and E. Metchnikoff in Russia also started to think that pathogens might prove to be effective agents for controlling crop pests (LeConte 1874; Steinhaus 1975). This early work was just the beginning, and some grandeur discoveries were waiting as Elie Metchnikoff was trying to control grain beetle, *Anisoplia austriaca* (Russian cereal crops were in great economic loss due to this pest) (Steinhaus 1956, 1975; McCoy et al. 1988). Indulging himself in beetle behavior and life cycle, he found a fungus that he referred to as green muscardine and named *Entomophthora anisopliae* (now known as *Metarhizium anisopliae*) that was able to kill beetles. Another milestone was the discovery of *Bacillus thuringiensis* (Bt). In 1901 a Japanese biologist Shigetane Ishiwata discovered this bacterium and gave a new facet to microbial biocontrol potential. Since then Bt has been used routinely in a variety of control programs, and after its commercial success in 1938, it was sold as a biopesticide named "Sporeine" for the first time in France (Aronson et al. 1986). In the 1950s it entered in the United States. Shortly *Bacillus popilliae* was used as another variety of bacterial biopesticide (Lord 2005). The first genetically engineered Bt crop, Bt field corn, was registered with the United States Environmental Protection Agency in 1995 (USEPA 1999). This was only the beginning as in 1965 the first fungal product "Boverin" was developed in the former USSR. This product was based on *B. bassiana*, discovered by Agostino Bassi, and used to control the Colorado potato beetle and the codling moth (de Faria and Wraight 2007). Interest in using pseudomonads for biocontrol was also initiated in the 1970s and in early 1980s (Burr et al. 1998),

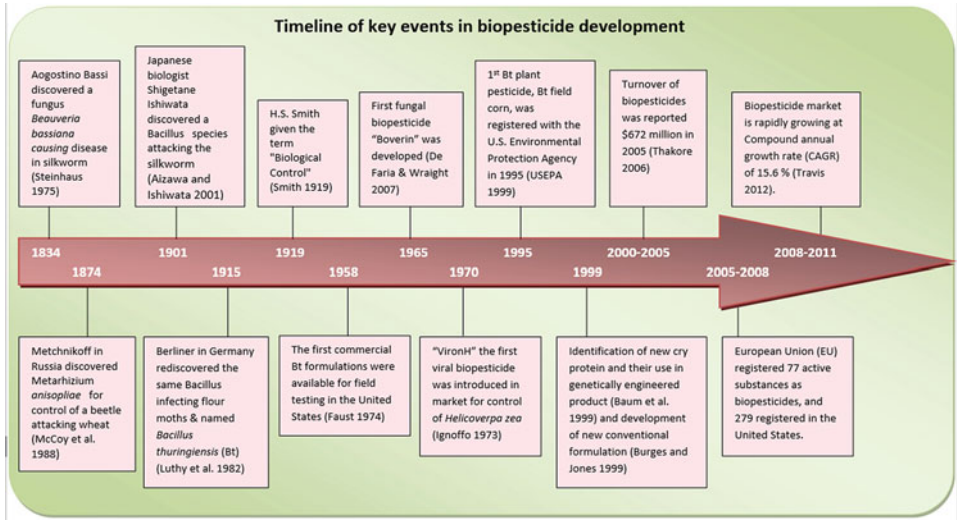

Fig. 1.1 Time line events of biopesticide development

and later reports confirmed the role of antagonistic fluorescent pseudomonads in suppression of take-all disease of wheat and barley (Smiley 1979; Weller 1988). The role of viruses in biocontrol was first reported in 1940, but it was in the 1970s when "VironH" was for the first time introduced in the market for the control of *Helicoverpa zea* (Ignoffo 1973). Major events in biopesticide history are given in Fig. 1.1.

1.3 Concept of Bioformulation

Typically a formulation is a mixture of an active ingredient in a formulated product with inert (inactive) substances (http://npic.orst.edu/factsheets/formulations.html). However regarding bioformulation we see that there is no uniform definition available and various authors define it in their own way. Burges and Jones (1998) stated bioformulation comprises aids to preserve organisms, to deliver them to their targets, and once there to improve their activities, whereas Arora et al. (2010) define the term bioformulation to preparations of microorganism(s) that may be partial or complete substitute for chemical fertilization/pesticides. But any operative definition must include an active ingredient, a carrier material, and an additive. The active ingredient is mostly a viable organism; it may be live microbe or spore and its survival during storage is very essential for successful formulation development (Auld et al. 2003; Hynes and Boyetchko 2006). Suitable carrier material is inert that supports active ingredient (cells) and assures that the cells are easily established in or around the plant and provide better chances of enhancing plant growth or killing target pest. Carrier materials also increase the shelf life of the product (Burges and Jones 1998). Some inert carrier materials are fine clay, peat, vermiculite, alginate, and polyacrylamide beads, diatomaceous earth, talc, vermiculite, cellulose (carboxymethyl cellulose), and polymers specially xanthan gum (Digat 1989). Additives such as gums, silica gel, methyl cellulose, and starch protect from harsh environment conditions and improve physical, chemical, and nutritional properties of formulations (Schisler et al. 2004; Hynes and Boyetchko 2006).

1.4 Types of Formulation Available

Broadly two types of bioformulations are available, liquids and solids (Burges and Jones 1998), although in these days there are so many other types of bioformulation available and being used all over the world.

1.4.1 Solid Formulations

Solid formulations include granules (GR), microgranules (MG), wettable powders (WP), wettable/water-dispersible granules (WG, WDG), and dusts (Larena et al. 2003; Abadias et al. 2005; Guijarro et al. 2007a). They are produced by adding binder, dispersant, wetting agents, etc. (Tadros 2005; Brar et al. 2006; Knowles 2008).

1.4.1.1 Granules (GR)

Granules are dry particles and contain active ingredient, binder, and carrier. Concentration of active ingredients in granules is 5–20 % (Brar et al. 2006). On the basis of particle size, they are classified as coarse particles (size range 100–1000 μm) and microgranules (size range 100–600 μm). The granules should be noncaking, non-dusty, and free flowing and should disintegrate in the soil to release the active ingredient. They are usually safer having no risk of inhalation and mostly used in soil treatment. Granular formulations are more concerned with storage and increased shelf life (Callaghan and Gerard 2005). Most commonly used granules are wheat meal granules (Navon 2000), corn meal baits, granules formed with gelatinized cornstarch or flour (Tamez et al. 1996), gluten (Behle et al. 1997), cottonseed flour and sugars (Ridgway et al. 1996), gelatin or acacia gum (Maldonado et al. 2002), sodium alginate (Guijarro et al. 2007b), diatomaceous earth (Batta 2008) and semolina (durum) wheat flour (Andersch et al. 1998). MET52 ®, a granular bioformulation of *M. anisopliae* var. anisopliae strain F52, is widely used in biocontrol of black vine weevil (*Otiorhynchus* spp.) larvae in soft fruit and ornamental crops (Ansari and Butt 2012). Sterile rice is used as organic carrier, whereas alginate prill is being utilized in "SoilGard" preparation. This granular formulation contains *Trichoderma virens* as active ingredients and marketed by Certis LLC for eradication of soilborne diseases caused by *Pythium*, *Rhizoctonia*, and *Fusarium*. Selection of different carriers may affect activity of active ingredients in field conditions. In a study Mejri et al. (2013) measured bioherbicidal activity of deleterious rhizobacterium *Pseudomonas trivialis* X33d by taking two granular formulations and found that semolina–kaolin (pesta) showed higher brome suppression activity in wheat field in comparison to kaolin–talc-based granular formulation, whereas BioShield™, formulated as a granule containing *Serratia entomophila*, is sold in New Zealand for control of grass grub larvae in established pasture (Young et al. 2010).

Although granular formulations are very effective, their application is also limited due to inactivation of active ingredient in ultraviolet (UV) light. In a study by Bailey et al. (1996), Bt product used to control apple moth caused by *Epiphyas postvittana* lost more than half of its activity within a day on exposure to sunlight, whereas BioShield, a *Serratia entomophila* containing granular formulation, is very sensitive to UV light and osmotic and desiccation stress and requires subsurface application (Johnson et al. 2001). Some UV protectants such as Tinopal, Phorwite, Intrawhite, and Leucophor; uric, folic, 2-hydroxy-4-methoxy-benzophenone, p-aminobenzoic, 2-phenylbenzimidazole-5-sulfonic acids; and dyes such as Congo red, methyl blue, safranin, brilliant yellow, and buffalo black may overcome UV inactivation of organism when added in formulation medium or coated on formulation product (Warrior et al. 2002; Cohen and Joseph 2009). Stilbene-derived optical brighteners are also more effective in baculoviruses containing formulation as these absorb UV radiation and emit visible blue wavelengths and enhance the infectivity (Goulson et al. 2003). Recently Fernandes et al. (2015) reviewed tolerance of selected entomopathogenic fungal strains to UV radiation.

1.4.1.2 Wettable Powders (WPs)

Wettable powders (WPs) are one of the oldest types of formulations. They consist of 50–80 % technical powder, 15–45 % filler, 1–10 % dispersant, and 3–5 % surfactant by weight to

achieve a desired potency formulation (measured in international units) (Brar et al. 2006). These dry formulations are of much interest as they are readily miscible with water and can be easily added to a liquid carrier, normally water, just before its application. WPs have a longer shelf life and by controlling moisture content, their shelf life may exceed 18 months. Longer shelf life is also related to their firm marketplace. Agricultural materials and industrial waste by-products such as wheat bran–sand mixture, sawdust–sand–molasses mixture, corn cob–sand–molasses mixture, bagasse–sand–-molasses mixture, organic cakes, cow dung–sand mixture, compost/farm manure, inert charcoal, diatomaceous earth, and fly ash (Table 1.1) can also be used to prepare powder formulations (Khan et al. 2007). Recently Cheng et al. (2015) prepared a WP containing 60 % *B. cereus* freeze-dried powder, 28.9 % diatomite as carrier, 4 % sodium lignin sulfonate as disperser, 6 % alkyl naphthalene sulfonate as wetting agent, 1 % K_2HPO_4 as stabilizer, and 0.1 % β-cyclodextrin as ultraviolet protectant, and in his preliminary study, they found this formulation was effective in biocontrol of postharvest disease in comparison to chemical used. Woo et al. (2014) reviewed current application of *Trichoderma*-containing products in agriculture, and it was found that 55.3 % of *Trichoderma* formulations are commercialized as WPs.

1.4.1.3 Wettable/Water-Dispersible Granules (WG, WDG)

Wettable/water-dispersible granules (WG, WDG) are also known as dry flowables. They have been designed to make WPs more user and environmental friendly, non-dusty, free-flowing granules quickly dissolving in water. They contain wetting agents and dispersing agents similar to those used in WPs, but the dispersing agent is usually at a higher concentration. Like WPs, WDG also show excellent shelf life. WDG formulations have wider role in nematode control and capture 90 % of the total market available for nematode-based products. Antagonistic fungus, *Ampelomyces quisqualis*, is used to control powdery mildew caused by several pathogenic species in grapes, tomato, apples, strawberries, and cucurbits, formulated as WDG (Falk et al. 1995). Chumthong et al. (2008) produced water-soluble granules containing *Bacillus megaterium* for biological control of rice sheath blight and showed that these granule formulations exhibited good physical characteristics, such as high-water solubility and optimal viscosity, suitable for spray application.

1.4.1.4 Dusts

Dusts are also one of the oldest formulation types and contain very finely ground mixture of the active ingredient (usually 10 %) with particle size ranging from 50 to 100 μm. Although they have been used since a long time and in some instances more effective in killing (Ifoulis and Savopoulou-Soultani 2004), there have always been handling and application problems associated with dusts (Harris and Dent 2000). Dust containing beauverial protein extract (weighing about 5 kDa) is also being used in biocontrol. Biofox C has been formulated as dust containing nonpathogenic *F. oxysporum* and used in basil, cyclamen, tomato and carnation (Kaur et al. 2010)

1.4.2 Liquid Formulations

Liquid formulations are also known as flowable or aqueous suspensions and consist of biomass suspensions in water, oils, or combinations of both (emulsions) (Schisler et al. 2004). A typical liquid formulation contains 10–40 % microorganisms, 1–3 % suspender ingredient, 1–5 % dispersant, 3–8 % surfactant, and 35–65 % carrier liquid (oil or water) (Brar et al. 2006). Liquid formulation may be of the following types.

1.4.2.1 Suspension Concentrates (SCs)

SCs are produced by adding solid active ingredient(s) with poor solubility in water and satisfactory stability to hydrolysis (Tadros 2013). SCs are diluted in water before use. Their storage

Table 1.1 Some of the WP bioformulations

Name of product	Category of bioformulation	Target organism	Carrier material	Application method	Name of country	References
Gypchek	Mycoinsecticides	Gypsy moth (*Lymantria dispar*) Multicapsid nuclear polyhedrosis virus (LdMNPV)	Virus infected gypsy moth larvae processed to produce a finely ground powder	Applied using aerial or ground application equipment	USA	Reardon and Podgwaite (1992)
Boverin, Naturalis, Boverosil, Trichobass-P, and L BioGuard Rich Mycotrol ES® Mycotrol O®	Mycoinsecticides	*Beauveria bassiana*	Talc or other inerts such as perlite, kaolin, bentonite, starch	Used to protect agricultural, fruit, decorative, and flowering plants from beetle and moths	Russia, India, South Africa, Former USSR, Spain	de Faria and Wraight (2007)
Metaquino	Mycoinsecticides	*Metarhizium anisopliae* var. *anisopliae*	Autoclaved rice or wheat bran	Aerial applications used to control spittlebugs in sugarcane and pastures	Brazil	Marques et al. (1981)
Bio-Blast	Mycoinsecticides	*Metarhizium anisopliae*	Autoclaved rice	Termites	USA	Wraight and Carruthers (1999)
Biolisa Kamikiri, Melocont-Pilzgerste,	Mycoinsecticides	*Beauveria brongniartii* (Sacc.)	Barley kernels or clay granules	Used to control coleopteran, homopteran, lepidopteran, and dipteran pests in flowers, vegetables, oil palms, and other crops	Japan, Austria, Italy	Hajeck et al. (2006)
Bio-fungus	Fungicide	*Trichoderma* spp.	Barley kernels or clay granules	Fungi causing wilt, take-all, root rot, and wood decay	Denmark	Monte (2001)
Trichoject, Trichoseal, Trichoprotection ®	Fungicide	*Trichoderma harzianum* and *Trichoderma viride*	Autoclaved corn or rice seed	*Chondrostereum purpureum* and other soil and foliar pathogens	New Zealand	Burges and Jones (1998)
Trichodex	Fungicide	*T. harzianum*	Autoclaved corn or rice seed	As above	Israel	Monte (2001)
Binab®	Fungicide	*T. harzianum*	Autoclaved corn or rice seed	Control *Botrytis*, *Fusarium*, *Gaeumannomyces*, *Pythium*, *Rhizoctonia*, *Sclerotinia*, *Sclerotium*, *Verticillium*, and wood-rot fungi	Denmark, USA, Israel	
Binab T		*T. harzianum and T. polysporum*	Autoclaved corn or rice seed	As above	Sweden	
Aspire®	Fungicide	*Candida oleophila*	Skimmed milk	Postharvest decay of citrus fruits, apples, and pears caused by *Penicillium* sp. and *Botrytis* sp.	USA	Droby et al. (1998)

Yield Plus	Fungicide	*Cryptococcus albidus*	Skimmed milk	As above	South Arica	http://www.anchor.co.za/
Sporidex®	Fungicide	*Pseudozyma flocculosa*	Skimmed milk	Powdery mildew on greenhouse roses and cucumbers	Canada	Punja and Utkhede (2003)
DiTera™	Nematicide	Killed *Myrothecium verrucaria*	Wheat bran, talc powder, silica	Plant parasitic nematode specially root-knot nematodes (*Meloidogyna Incognita*)	USA	Warrior et al. (1999)
BlightBan® A506	Bactericide	*Pseudomonas fluorescens A506*	Talc and peat	Postharvest decay in almond, apple, cherry, wettable powder	Canada	Stockwell and Stack (2007)
Bio-Save 10 LP, Bio-Save 100, and Bio-Save 1000	Fungicide	*Pseudomonas syringae* strain. ESC-100	Talc and peat	Postharvest decay in citrus and pome fruit	USA	
Collego®	Bioherbicide	*Colletotrichum gloeosporioides* f. sp. aeschynomene	Undisclosed	Controls northern joint-vetch (*Aeschynomene virginica*	USA	

and solubility can be improved by addition of surfactants and various additives. Farmers generally prefer suspension concentrates to wettable powders because they are non-dusty and easy to measure and pour into the spray tank.

1.4.2.2 Oil-Miscible Flowable Concentrate (OF)

OF is stable suspension of active ingredient(s) in a fluid intended for dilution in an organic liquid before use (Singh and Merchant 2012).

1.4.2.3 Ultralow Volume (ULV) Suspension (SU)

They are suspension ready for use through ULV equipment. ULV are aerial or ground spray equipment and generate extremely fine spray (Singh and Merchant 2012).

1.4.2.4 Oil Dispersion (OD)

OD is a stable suspension of active ingredient (s) in water-immiscible solvent or oil (Michereff et al. 2009). ODs have validated a growing importance over the past decade. Recently Mbarga et al. (2014) developed a soybean oil based formulation and found that *Trichoderma asperellum* containing OD had great potential for the control of cacao black pod disease with increased half-life of the conidia in comparison to aqueous suspension. Some protective measures are required with regard to handling fungi containing OD formulations. As in prolonged storage, active ingredient (conidia) may be settled out of suspension or densely compacted in the bottom of the container (Butt et al. 2001).

Some of the *Trichoderma* containing liquid formulations used in biocontrol are Trichojet, Enpro-Derma, and Trichorich-L (Woo et al. 2014). Oil-based formulations have been proven better in foliar spray and considered effective in enhancing the activity of entomopathogens (Feng et al. 2004). Oil evaporates much less, so it remains in contact for greater time and can be applied as an emulsion (oil in water) (Luz and Batagin 2005) or in some cases as an invert emulsion (water in oil) (Batta 2007).

1.4.3 Encapsulation

Encapsulation involves coating or entrapping microbial cells within a polymeric material to produce beads which are permeable to nutrients, gases, and metabolites for maintaining cell viability within the beads (John et al. 2011). Based on the size of the polymeric bead produced, two types of techniques, i.e., macroencapsulation (size ranging from few millimeters to centimeters) and microencapsulation (size ranging from 1 to 1000 μm, generally less than 200 μm), are used (Nordstierna et al. 2010). Macroencapsulation techniques are advantageous than microencapsulation (for further details on microencapsulation review by Rathore et al. 2013 can be seen). Encapsulation provides good protection to active ingredient from harsh environmental factors. Currently, gelatin, starch, cellulose, and several other polymers are used for encapsulation of active ingredients (Amiet-Charpentier et al. 1998; Park and Chang 2000; Cheze-Lange et al. 2002). Protection may enhance to some extent by coating capsule with dyes (Cohen et al. 1990). For further detail on encapsulation, chapter by Schoebitz et al. can be seen from this very book.

Although both liquid and solid formulations have been extensively used in agrosystems, dry formulations are generally preferred over wet formulations because they provide extended shelf life and are easier to store and transport (Burges and Jones 1998). The development of a bioformulation is proving a hectic job and earlier work done in this field is not sufficient. The increasing demand for developing new formulations to replace chemical pesticides and fertilizers has created interest amongst entrepreneurs in this field, and they are funding various projects for the development of cheaper and effective technology. Some technological advances in development of Bt-based products have provided substantial aid in its commercial production. For example, Micellar-enhanced ultrafiltration (MEUF) is a technique being used to separate dissolved organic compounds like thuringiensin from aqueous streams (Tzeng

et al. 1999). Similarly in situ product removal (ISPR) involves biochemical product removal during fermentation process and successfully applied in removal of Bt toxin proteins (Agrawal and Burns 1996), whereas cross-flow microfiltration (CFM) has been utilized for extraction of all kinds of proteins and harvest of recombinant yeasts (Persson et al. 2004)

1.5 Current Scenario/Market Trends

Microbe-based formulations for plant growth enhancement and for eradication of phytopathogens are being used all over the world (Leggett et al. 2011; Naderifar and Daneshian 2012; Gašić and Tanović 2013), but the data related to their use around the globe is fragmented. One probable reason is the discrepancy of the terminology used. Most of the developing countries are using the word "biofertilizers," whereas in the rest of the world, "bioinoculant" for enhancing crop yields is being used, but in both cases either materials derived from living organisms or organism itself are used (Vessey 2003; Chen et al. 2006) for increased absorption of nutrients in plants that assist in soil fertility and crop productivity. Many growers around the world are now routinely applying biofertilizers and biopesticides for different crops. European biofertilizer market is the most developed and widespread among all other regions and is estimated to grow from around $2566.4 million in 2012 to $4582.2 million by 2017, at a calculated annual growth rate (CAGR) of 12.3 %, from 2012 to 2017 (PRWEB 2014). In North America, biofertilizer market was highest in 2012 and is projected to grow at a rate of 14.4 % during the period of 2013–2018 (Micro Market Monitor 2015). Europe, together with North America, accounted for over 50 % of the global revenue (Grand View Research 2015). China the leading worldwide producer of rice, wheat, and many vegetables including onions and cabbage is also promoting biofertilizer use (Grand View Research 2015). There are 151 biofertilizer production units operated by government and nongovernment agencies in India (Mahajan and Gupta 2009). Among all, nitrogen-fixing biofertilizers were the most widely used, accounting for over 78 % of the global demand in 2012 (Agro news 2014). Table 1.2 provides details of some of the biofertilizers used around the globe.

In the field of biocontrol, the most successful biopesticides are Bt based and represent about 95 % of total microorganisms used (Bravo et al. 2011). Globally 322 products of Bt are generating annual revenue of $210 million (CAB International Centre 2010). But the use of other biopesticides (fungal and non-Bt) is also increasing. Recently market research survey has been carried out by various agencies to collect information about biopesticides, but reliability of such reports is of much concern and raised many questions. One of the main causes is that the criterion involved in market research may vary because some firms and agro-industries include subcategories such as microbes, biochemicals, plant growth regulators, plant-induced protectants (PIPs), insect growth regulators, essential oils, and pheromones, in the term biopesticide, whereas others use only the products of microbial origin. Gelernter (2007) endeavored to represent figures of sale in Europe and North America and estimated it to be $200 million in 2003. According to Thakore (2006), the turnover of biopesticides was $672 million in 2005, but there was no description of category included. A report by Harwood et al. (2007) showed global biopesticide market to be about $280 million in 2007. In this report true microbial agents were included. Business consultancies such as BCC Research (2010) and CPL (2006) are actively involved in direct marketing survey, so probably collecting much reliable data, and concluded that the global biopesticide market is growing at a rate of 10 % per year. Global industry analysis (2015) estimated that the annual biopesticide market could exceed $2.5 billion by 2015. Other market research reports by BCC on biopesticide represent the total sale of biopesticide to $1.2 billion in 2008 and $1.6 billion in 2009. This is planned to upturn to $3.3 billion in 2014 and $10 billion in 2017 (Marrone 2007). The

Table 1.2 List of some of the biofertilizers used around the globe

Name of country	Name of company	Name of product	Active ingredient	Use on the crops	References
Brazil	Embrafós, Institute of Biological Phosphate (IFB), Biophosphate of Brazil, Liderfós	BioAtivo	(Nitrogen fixation and phosphate solubilization)	Beans, maize, rice, sugarcane, soybean, eucalyptus, citrus, tomatoes, cotton, forage crops, and carrots	http://ifb.agr.br/biotecnologia/beneficios-do-bio-ativo/
Central Russia	PitBioTech, JSC "Industrial Innovations"	Bamil and Omug	*Bacillus*, *Micrococcus*, and *Clavibacter*	Barley, wheat, beetroot, onion, garden radish, carrot, potato, marrow leguminous and nonleguminous	Kutyova et al. (2002) and Zhigletsova et al. (2010)
		Ekud	*Bacillus* and *Staphylococcus hominis*		
		Pudret	*Bacillus* and *Staphylococcus*		
		Azotovit	*A. chroococcum*		
		Bactophosphin	*Bacillus mucilaginosus*		
Southern and Eastern Russia	Natural resources LLC EM Technology	Rizotorphin	*Rhizobium* spp.	Field pea, soybean,chickpea, broad bean, narrow-leafed lupin, tomato, pepper, brinjal, sorrel, asparagus, estragon, etc.	Karimov and Zaripov (2007), Balashov et al. (2008), and Fedotova et al. (2009)
		Rizoagrin	*Agrobacterium radiobacter*		
		Azotobacterin	*Azotobacter chroococcum*		
		Ekophit	*A. chroococcum*		
		Agrofil®	*Agrobacterium* sp.		
		Flavobacterin®	*Flavobacteria* spp.		
		Mizorin®	*Arthrobacter mysorens*		
		Baikal EM-1	Lactic acid, nitrogen fixation bacterias, photosynthetic bacteria, and yeast		
Japan	The Tokachi Federation of Agricultural Cooperatives (TFAC, Tokachi Nokyoren)	Mamezo	*Rhizobium* sp.	Soybeans, azuki beans, and *Phaseolus* beans	García-Fraile et al. (2015)
		R-Processing seeds	Leguminous seeds inoculated with *Rhizobia* sp.		
		Hyper-coating seeds	Rhizobia within the capsule of calcium carbonate)		
	EMRO (EM Research Organization)	EM•1® and EM Bokashi	*Lactobacillus plantarum*, *L. casei*, *L. fermentum*, *L. delbrueckii*, *Streptomyces cerevisiae*, and *R. palustris*	Soybeans, azuki beans, and *Phaseolus* beans	http://emrojapan.com/page/89-howtomakebokashi/
	CBF China Bio-Fertilizer AG	Xin Sheng Li	(*Bacillus mucilaginosus* and *B. subtilis*) a potassium-solubilizing bacteria and a phosphorus-solubilizing bacteria		

	Central Glass Co., Idemitsu Kosan Co., and Osaka Gas Co.,	VAM		Maize, alfalfa, onion	Ogawa (1989), Tawaraya et al. (1998), and Karasawa et al. (2001)
USA	TeraGanix, Inc	EM·1®	Lactic acid bacteria, yeast, and photosynthetic bacteria	Most plants (flowers, veggies, trees, etc.)	http://www.teraganix.com/EM-1-Microbial-Inoculant-Microbial-Inoculants-p/1000.htm
	Novozymes LLc	Nitragin Gold	*Rhizobium meliloti*	Alfalfa pea, lentil, soybean, corn, sorghum, sugar beet, winter wheat, canola sunflower,mustard	http://www.bioag.novozymes.com/en/products/unitedstates/biofertility/Pages/default.aspx
		TagTeam	Combines the best in rhizobia strains with *Penicillium bilaii*		
		JumpStart	*P. bilaiae*		
		JumpStart® LCO	*P. bilaii* and LCO Promoter Technology		
Argentina	Rizobacter S.A.	Rizoliq LLI	*Rizobacter* sp.	Legumes and cereal crops	http://www.rizobacter.com/argentina/productos/
		Signum	*Bradyrhizobium* sp.		
		Rizo Liq TOP	*B. japonicum*		
Australia	Mapleton Agri Biotec Pty Ltd	TwinN	*Azotobacter* (soilborne species), *Azospirillum* (endophytic species), and free-living nitrogen-fixing bacteria, together in a freeze-dried form)	Legumes and cereal crops	http://www.twinn.com.au/
	Vanadis Bioscience Pty. Ltd.	Catapult™	VAM plus 2 species of *Bacillus*		http://www.vanadisbioscience.com/
Hungary	Phylazonit Kft	Phylazonit-M	*B. megaterium* and *Azotobacter chroococcum*	Rice, maize	FNCA (2006)
India	T. Stanes & Company Limited	Symbion-N	*Azospirillum*, *Rhizobium*, *Acetobacter*, and *Azotobacter*	Cereals, legumes, and vegetable crops	http://www.tstanes.com/products.html
		Symbion-P	*B. megaterium* var. *phosphaticum*		
		Symbion-K	*Frateuria aurantia*		
		Symbion-S	*Thiobacillus thiooxidans*		
		Symbion-VAM	*Glomus fasciculatum*		
	Camson Bio Technologies Limited	CALOBIUM	*Rhizobium* sp.	Legume crops, cereal crops, vegetable crops	http://www.camsonbiotechnologies.com/products/bio_fertilizers_and_stimulants.htm
		CALMONAS	*Pseudomonas* sp.		
		CALSPIRAL	*Azospirillum* sp.		
		CALZOTO	*Azotobacter* sp.		
		CALTASH	K solubilizer		
		CALPHOROUS	PSB		

(continued)

Table 1.2 (continued)

Name of country	Name of company	Name of product	Active ingredient	Use on the crops	References
	Ajay Biotech	BIOPHOS	PSB	Various crops	http://www.ajaybio.in/prodpro.htm
	Gujarat State Fertilizers and Chemicals	Sardar Biofertilizers	*Azotobacter*, *Azospirillum*, and PSB	All types of crop	http://www.gsfclimited.com/bio_fertilizers.asp?mnuid=3&fid=32
Cuba	National Institute of Agricultural Sciences	EcoMic®	*Glomus fasciculatum*	Rice, cotton, soybean, corn, coffee, sorghum	Penton et. al. (2011), and Ortega (2007)
	National Program Project Agricultural Biotechnology of Cuba	FOSFORINA®	*P. fluorescens*	Tomato	
	Institute of Ecology and Systematic of Cuba)	Micofert®	*Glomus aggregatum*, *G. manihotis*, *G. spurcum*	Sugarcane, tomato, onion, garlic, potato, banana, mandarin, malanga, yam, grains, forest	
Mexico		Micofert	*G. intraradices*, *G. etunicatum*, *Gigaspora* sp.	Coffee, papaya, corn, onion, avocado	Cobos (2005)
		BuRIZE1	*Glomus intraradices*	Alfalfa, apple, tomato, orange, avocado,	BioScientific (2008)
		Myconate1	Isoflavanone and phormononetin compounds	Corn, soybean	Lara (2008)
Colombia		Mycobiol1	*Glomus* sp., *Entrophospora colombiana*, *Acaulospora mellea*	Banana, cassava, lettuce, tomato, cape gooseberry (*Physalis peruviana*)	www.corpoica.org.co
		FOSFOSOL1	*Penicillium janthinellum*	Rice	Moreno-Sarmiento et al. (2007)
		Dimargon1	*Azotobacter chroococcum*	Rice, cotton	
Malaysia		UPMB 10	*Bacillus sphaericus*	Oil palm	FNCA (2006)
		MYCOgold	Species of 4 genera of AMF	Banana	Malaysian Agri Hi Tech (2008)
Pakistan	Government	BioPower	Multi-strain of N_2 fixing	Cotton, maize, rice, sugarcane, and wheat	Kennedy et al. (2004)
	Microbial Biotechnologies	Ferti-Bio	Multi-strain of N_2 fixing, P-solubilizing and growth hormone-producing bacteria	Rice, wheat, corn, cotton, sugarcane, and vegetables	

region-based research report states that the United States represents the largest region of biopesticides worldwide, whereas Europe represents the fastest-growing regional market for biopesticides showing annual average growth rate (AAGR) of 15.0 % (Industrial Equipment News 2011). Asia-Pacific is also coming up in the market, where biopesticide sales were projected to reach US $362 million in 2012. Latin America has the smallest increase of all the regions. The market was nearly $70 million in 2005 and remained only $88 million in 2010, an AAGR of 5.0 % (Industrial Equipment News 2011).

1.6 Available Bioformulations

Although bioformulations have a widespread use in agriculture, they are exclusively recognized for their effective role in the field of biocontrol and in biofertilization (Trabelsi and Mhamdi 2013), and here we have focused on both the aspects.

1.6.1 Formulations for Nutrient Uptake

In the last few years, the use of microbial inoculants is realized as an effective way of providing nutrients to plants since it would substantially reduce the use of chemical fertilizers, and hence there are an increasing number of biofertilizers that are commercially produced for various crops (Berg 2009; Trabelsi and Mhamdi 2013). Bioformulations containing microbes for better availability of nutrient are being described here.

1.6.1.1 Nitrogen (N)

N is considered as an essential plant macronutrient required in large quantities (1–3 % on a dry-weight basis) (Kraiser et al. 2011), but only a small proportion of the nitrogen fertilizers supplied to agricultural systems is utilized (Vitousek et al. 2009). According to an estimate, around 50 % is used by the crops, 25 % is emitted to the atmosphere, 20 % runs off into aquatic systems, and only 5 % is stored in the soil (Galloway 2005; Garnett et al. 2009). This is why without considering environmental impact, the amount of synthetic nitrogen applied to crops has risen dramatically, from 12 to 104 Tg/year in the last few years (Mulvaney et al. 2009). Biological nitrogen fixation (BNF) is one way of converting elemental nitrogen into plant-usable form (Gothwal et al. 2009) and has considerable advantages from ecological and economical point of view (Sainju et al. 2003), but the capability of N fixation is limited and exclusively restricted to most of the phyla of bacteria and in methanogenic archaea (Young 1992). Symbiotic fixation of nitrogen within nodules of vascular plants is done by two major groups of bacteria not phylogenetically related, rhizobia (leguminous association) and *Frankia* (nonlegume association) (Franche et al. 2009). Legumes constitute the third largest family of flowering plants (Polhill and Raven 1981) accounting for approximately 27 % of the world's crop production (Graham and Vance 2003) including important crop legumes: soybean (*Glycine max*), peanut (*Arachis hypogaea*), mung bean (*Vigna radiata*), chickpea (*Cicer arietinum*), lentil (*Lens culinaris*), common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), and alfalfa (*Medicago sativa*). About 200 species of non-legume plants distributed among 24 genera in eight angiosperm families are nodulated by *Frankia* (Huss-Danell 1997). BNF produces roughly 200 million tonnes of nitrogen annually (Peoples et al. 2009) which saves farmers millions of dollars in fertilizer costs yearly. In a study Townsend and Howarth (2010) argue that humans are now fixing nitrogen at twice the rate of natural processes. There have been many workers which confirmed the role of rhizobia in sustainable crop production and envisaged that agronomic practices using rhizobial inoculum may ensure adequate nitrogen to legumes instead of N fertilizers (Gupta 2004; Arora et al. 2010). Both leguminous seed and soil can be treated by application of legume inoculants containing live rhizobia (Martinez-Romero 2003; Deaker et al. 2004). Legume inoculants may consist of one strain or may

comprise two or more strains effective on that particular host and commercially produced in powder or granular and liquid formulations (Lupwayi et al. 2006). Conventionally peat is mostly used as a carrier material in legume inoculation production (Albareda et al. 2008). But cell number is dependent on environmental conditions and rhizobial species used. In a study Albareda et al. (2008) found that growth and survival of inoculated strains were as high in peat as when compost cork and perlite were used as carrier materials. Other locally available carrier materials such as coal, bagasse, coir dust, etc., are also used (Albareda et al. 2008).

Although direct applications of rhizobia as bioinoculants have been very successful, more recent application of Nod factors or lipophilic chitin oligosaccharides (LCOs) has also showed substantial impacts on crop yield in soils containing very small populations of rhizobia (Kidaj et al. 2012). In the United States, LCO Promoter Technology® (a technique of using the beneficial effect of Nod factor molecules) has been widely popularized by Novozymes for promoting growth in economic crops, such as soybean, peanuts, and alfalfa. A liquid formulation of non-associative free-living nitrogen fixers such as *Azotobacter* and *Azospirillum* including cyanobacteria has been also marketed in various countries and showed a significant increase in crop production (Vendan and Thangaraju 2006). For sustainable rice production, southern China, Vietnam, India, and Bangladesh are also using free-floating water fern *Azolla* in which cyanobacteria *Anabaena azollae* symbiotically fixes nitrogen (Pabby et al. 2003). Recent use of endophytic nitrogen-fixing bacteria as biofertilizers is also reported, and members of *Azoarcus*, *Achromobacter*, *Burkholderia*, *Gluconacetobacter*, *Herbaspirillum*, *Klebsiella*, and *Serratia* have been identified as potent endophytic nitrogen-fixing strains (Rothballer et al. 2009; Franche et al. 2009).

1.6.1.2 Phosphate

Plants obtain phosphorus as phosphate anions from the soil solution. Phosphate is being considered as probably one of the least available plant nutrients found in the rhizosphere due to its inorganic fixation and formation of organic complexes (Eswaran et al. 1997). The phosphorus content in average soils is about 0.05 % (w/w) of which only 0.1 % is available to plants (Achal et al. 2007). It has been found that the application of phosphatic fertilizers do not validate the need of the plant as a consequence of organic and inorganic fixation. Nearly 80 % of applied phosphorus may be unavailable to plants (Holford 1997). About 5.7 billion hectares of land worldwide has been reported to be phosphate deficient (Batjes 1997; Vassilev and Vassileva 2006). Biological processes in the soil, such as microbial activity, tend to control the mineralization and immobilization of organic conversion of the insoluble forms of phosphorus to an accessible form by plants (orthophosphate), which is an important trait of phosphate-solubilizing bacteria (PSB) and arbuscular mycorrhizal fungi (AMF) (Fankem et al. 2006, Khan et al. 2007). In the last few years, the development of microbial inoculum containing phosphate-solubilizing microbes (PSM) gained attention of agriculturists (Fasim et al. 2002). Application of PSM, either individually or in combined form, remained successful for increasing yield of soybean (Fernandez et al. 2007), maize (Hameeda et al. 2008), wheat (Minaxi et al. 2013), mung bean (Jha et al. 2011), and chickpea (Singh and Prakash 2012). Although phosphate-solubilizing ability of PSM was discovered very early (Mulder 1967), their commercialization in the form of bioformulations was not very successful (Rodriguez and Fraga 1999). It has been observed that quality management is essential for the development of reliable and contaminant-free bioproducts, whereas in field conditions performance is hampered by environmental variables including salinity, pH, moisture, temperature, and climatic conditions of the soil (Khan et al. 2009b). Among PSB-based biofertilizers, products having *Pseudomonas* spp., *Bacillus* spp., *Aspergillus* spp., and *Penicillium* spp. are predominantly used (Sharma et al. 2013). Phosphobacterin is one of the oldest, *Bacillus megatherium* containing biofertilizer

(Smith et al. 1961), and approximately 10 million hectares were treated with the product in Russia in 1958 (Brown 1974). In India P Sol B® has wide application in agriculture and contains *Pseudomonas striata* (NCIM 2847). FOSFOSOL® is a phosphatic biofertilizer containing *Penicillium janthinellum* and being used at very large scale in Colombia (Moreno-Sarmiento et al. 2007).

1.6.1.3 Potassium

For balanced plant growth, K uptake is as important as N and phosphorus. A lot of vital functions of plant are directly or indirectly potassium dependent. This macronutrient takes part in enzyme activation of several physiological reactions including protein synthesis, photosynthesis, starch synthesis and also helps in resistance to diseases and insects (Rehm and Schmitt 2002). Although 2.5 % of the lithosphere is of K, its actual soil concentrations vary widely, ranging from 0.04 to 3 % (Sparks and Huang 1985). Soil potassium is available to plants in four different pools: (i) soil solution, (ii) exchangeable K, (iii) fixed K, and (iv) lattice K (Syers 1998). Amongst all four, soil solution and exchangeable K are directly available for plant uptake, but for a rapidly growing crop with enough K, their acquisition by this alone is not adequate (Philip et al. 2013) and require external application in the form of potassic fertilizers. At global level after the United States, China, and Brazil, India ranks fourth in total consumption of potassium fertilizers (Investing News Network 2015). It has been found that "non-exchangeable" K in soils is solubilized by the release of organic acids by some bacteria that increase the K^+ concentration in the soil solution (Meena et al. 2014). Their ability of solubilizing K-bearing minerals such as micas, illite, and orthoclase is of much interest in developing bioinoculants with the ability to provide soluble K to plants (Sheng and Lin 2006). In some countries, especially in China and South Korea, K biofertilizers have been tested (Basak and Biswas 2008). Most of the work in developing K biofertilizers involves utilization of those PSB which can also solubilize K-bearing minerals (Ahmed and El-Araby 2012). Recently *Frateuria aurantia* has been recognized as very effective K-mobilizing bacterium and used in commercial production of Symbion-K, Biosol-K, and K Sol B® biofertilizers.

1.6.1.4 Iron

Almost all life forms require iron in the form of various proteins and pigments (Escolar et al. 1999). In soil its concentration ranges from 7000 to 500,000 mg kg^{-1} mainly in the insoluble Fe (III) (ferric) form which hydrolyzes readily to give Fe $(OH)_2^+$, Fe $(OH)_3$, and Fe $(OH)_4^+$. Although in soil ferric iron dominates, plant uptake of iron occurs in ferrous (II) form and availability of both form of Fe depends on pH and oxygen level in the soil (Fageria et al. 1990). It has been found that the microorganisms inhabiting around growing roots drop redox potential in the rhizosphere because of the microbial oxygen demand, and this serves to increase concentrations of Fe (II) ions for plant uptake (Nikoloic and Romheld 1999). This microbe-mediated iron uptake is facilitated by a low molecular weight iron chelators termed siderophores (Garibaldi and Neilands 1956). There are so many siderophores identified from rhizospheric region (Saha et al. 2015), but those produced by pseudomonads are known for their high affinity to the ferric ion (Meyer 2000), for example, pyoverdines produced by fluorescent pseudomonads show affinity for Fe^{3+} with a stability constant of about 10^{32} (Meyer and Abdallah 1978). Such a high concentration of siderophores produced by plant growth-promoting microbes confers them competitive advantage in comparison to other microorganisms (Saha et al. 2015). Microbe-mediated iron uptake has been identified as potential tool for providing efficient nutrient uptake to plant, and many studies showed application of microbial inoculants having capability to chelate iron even at low concentrations to enhance plant productivity (Fageria 2009). One more attribute of good siderophore-producing microorganisms is that they may suppress soil-borne fungal pathogens by scavenging available

iron and rendering it unavailable to other organisms (Beneduzi et al. 2012). Although there are various reports indicating the use of microbe-mediated iron uptake to plants (Saha et al. 2015), commercially available bioformulations exclusively used in iron uptake are rare. In India Fe Sol B ®, a product of Agri Life Bio Solutions, is recently recognized as iron-mobilizing biofertilizer used for various crops.

1.6.2 Formulations for Biocontrol: Biopesticides

Worldwide, approximately 1400 biopesticide products are being sold (Marrone 2007), and the number of registered products is increasing day by day. Three types of microbes are always considered in developing a useful biopesticide formulation which are bacteria, fungi, and viruses. Although biopesticide formulations are dependent on the type of organism being used, the ultimate goal is that it must ensure that the agent is delivered in a form that is viable, virulent, and with sufficient inoculum potential to be effective in the field (Ash 2010). Many workers have discussed details of production methods of formulations (Burges and Jones 1998; Couch 2000; Ehlers and Shapiro-Ilan 2005).

1.6.2.1 Bacteria

Amongst all known microbe-based biopesticides, Bt and its subspecies enjoy a great reputation (Raddadi et al. 2008; Bravo et al. 2011). Great literature is available that confirms success of Bt in biopesticide market (Melnick et al. 2009; Sansinenea 2012). Rosas-Garcia (2009) classified Bt-based products: first-generation products contain mixture of spores and crystals from a native strain and govern mainstream of commercial products. By the advent of molecular biology techniques, it was possible to construct genetically engineered Bt strains (Cerda and Maurizio 2004) carrying numerous insecticidal crystal proteins (ICPs); these are second-generation products. Such formulation products have proved beneficiary because of their selective action on target pests. Recombinant *P. fluorescens* cells transformed with genes coding for Cry delta-endotoxins (Young et al. 2008) are classified as third-generation products. In such products engineered *P. fluorescens* expressing Bt Cry delta-endotoxin are cultivated and then chemically killed to fix the toxin within the cells, subsequently microencapsulates are prepared which not only stabilize the toxin but also reduce degradation when applied to plant leaves, so there is an improvement in the storage life of the product. Commercially Bt can be produced by (semi)solid state fermentation, whereas industrial production of Bt is performed by liquid state fermentation, but semisolid and solid state fermentation on small scale is also common in developing countries (Devi et al. 2005). Details of the production have been reviewed by Lisansky et al. (1993), Couch (2000), and El-Bandary (2006). Improvements have been suggested in experimental and commercial formulations of *Bacillus* spp. and other biocontrol agents (Schisler et al. 2004). In these days commercially available Bt products are proteins (ICP), viable spores, and enzyme systems (proteases, unknown virulent factors along with inerts/adjuvants (Brar et al. 2006).

Pseudomonas spp. strains are also extensively used in biocontrol of different phytopathogens (Ortet et al. 2011; Mishra and Arora 2012; Loper et al. 2012; Tewari and Arora 2014). For example, different strains of *Pseudomonas fluorescence* known to produce a variety of antibiotics or antifungal metabolites, are directly involved in suppression of diseases (Weller 2007). Bio-Save, BlightBan, Cedomon, Biocoat, and Victus are *Pseudomonas* spp.-based bioformulations and used in biocontrol of various plant diseases. Besides these *Agrobacterium radiobacter*, *Burkholderia cepacia*, and *Streptomyces griseoviridis* have been found in controlling soilborne seedling diseases and fruit and vegetable pathogens (Leonard and Julius 2000). Mostly entomopathogenic bacteria can be easily produced in vitro systems except *B. popilliae* and its close relatives which can only be produced in its natural host. Couch (2000) discussed detailed industrial fermentation and formulation of entomopathogenic bacteria.

Yan et al. (2007) reported the use of wastewater sludge to support growth of diverse *B. thuringiensis* serovars, yielding lower cell counts but higher entomotoxicity per spore compared to synthetic media. Similarly various other alternative raw materials have been also tested to minimize the cost of production (Ravensberg 2011).

1.6.2.2 Fungal

Since the discovery of *M. anisopliae* and *B. bassiana*, there have been plentiful attempts to develop commercial formulations based on fungi, but success is not as in the case of bacterial formulations (McCoy 1990). *B. bassiana* formulations alone contribute to 34 % of the commercial mycoinsecticides available in the market worldwide (de Faria and Wraight 2007). At least 750 species of fungi are known to be entomopathogenic, but very few have been established as control agents of insect pests (Copping 2009). Various reasons are responsible for these discrepancies, but mainly the shelf life of fungal formulations is short and mass production costs are high. One more reason is that preservation of active ingredients beyond a certain limit of time is low due to the fragile nature of the conidia and hyphae. Many of the registered formulations contain *Trichoderma harzianum*, *Trichoderma asperellum*, *Trichoderma gamsii*, *Coniothyrium minitans*, *Aspergillus flavus*, and *Chondrostereum purpureum* (Auld 2002). *Trichoderma* spp. are among the most studied fungal biocontrol agents and their commercialization is increasing day by day (Vinale et al. 2008), and new techniques are being developed for mass production of fungi. Genetic manipulation has also been used for fungi to increase their action on target pests. Techniques including Ca^{2+} polyethylene glycol (PEG)-mediated protoplast transformation, electroporation, and particle bombardment are best suited for fungal transformations (Bianca and Berg 2013). Mass production of entomopathogenic fungi by solid state fermentation (SSF), also called solid substrate fermentation (SSF), and by liquid state fermentation (LSF), also called submerged culture fermentation (SCF), is described by Jaronski (2014). A wide variety of organic materials have been used as substrates for mass production of entomopathogenic fungi. Among them broken rice, cassava chips, cotton and coconut cake, finger and kodo millet, wheat bran, and rice husk are more common (Jaronski 2014). Recently several inorganic substrates such as calcined diatomaceous earth (diatomite) (Jaronski and Jackson 2012) and open-pored clay granules have been also used (Jaronski 2014). As much of the work is done on Bt in bacterial formulations, development in case of fungal formulation *B. bassiana* is also done by solid-substrate production of aerial conidia (Feng et al. 1994). Mycotech Corp. of Montana has gained attention in *B. bassiana* production due to higher production of conidia (10^{13} conidia kg^{-1}) (Bradley et al. 1992). Production of *B. bassiana* and *Metarhizium* conidia by growing on a carrier in plastic autoclavable bags or in trays is common in developing countries (Mendonça 1992). Solid state fermentation by complex bioreactor is also used by some world famous companies: Koppert, Laverlam, and Prophyta (Bradley et al. 1992). Many fungi that are difficult to produce efficiently on solid substrates can be readily cultured in liquid media. In submerged cultures, fungi may produce blastospores or submerged conidia and/or mycelial parts or pellets. This depends on the species, the strain, and the production medium and parameters (McCoy et al. 1988). Industrial wastewaters and dewatered sludge have been also reported as rich nutrient sources for production and formulation of fungi (Verma et al. 2007). Jaronski and Jackson (2012) described a medium that works very well with a wide range of *Beauveria and Metarhizium* isolates.

1.6.2.3 Viral

Seven families of viruses, *Baculoviridae*, *Reoviridae*, *Iridoviridae*, *Poxviridae*, *Parvoviridae*, *Picornaviridae*, and *Rhabdoviridae*, cause diseases in insects. These viruses infect insects and form occlusion bodies which confirm their role in biocontrol (Kalawate 2014), but *Baculoviridae* is commonly used (Harrison and Hoover 2012). The family *Baculoviridae* have four genera: *Alphabaculovirus* (containing

nucleopolyhedroviruses (NPVs) that infect lepidopteran insects), *Betabaculovirus* (containing the granuloviruses (GVs) found in lepidopteran insects), *Gammabaculovirus* (NPVs infecting hymenopteran insects), and *Deltabaculovirus* (NPVs infecting insects in the order Diptera) (Reid et al. 2014). According to an estimate, more than 20 species and 30 different products of baculoviruses have been registered as commercially available insecticides (Rao et al. 2015). China is the world's largest viral insecticide producer (more than 32 registered products) (Sun 2015). Europe and the United States also have good market for viral insecticides. Here they are sold by the trade name Madex 3 (Andermatt Biocontrol), Granupom (AgrEvo), Carpovirusine (NPP-Calliope), Carposin (Agrichem), Virin-Gyap (NPO Vector), and CYD-X (Thermo Trilogy). Several recombinant baculoviruses have also been developed experimentally for insect control (Inceoglu et al. 2006), and some success was attained as the expression of insect-specific toxin in *Autographa californica* NPV (AcNPV) (Zlotkin et al. 1971) results in hyperactivity of the nervous system and musculature, cessation of feeding, and paralysis of the pest. Other recombinant systems involve insertion of juvenile hormone esterase (JHE) in AcNPV, gene of diuretic hormone of the tomato hornworm (*Manduca sexta* Joh), silkworm (*Bombyx mori* L.) virus, and straw itch mite (*Pyemotes tritici*) toxin TxP-1 into AcNPV (Copping and Menn 2000). The use of recombinant baculoviruses is also reviewed by Szewczyk et al. (2006). Viral biopesticides can be produced in vitro and in vivo. In in vivo production, original host is used and further descriptions of in vivo production methods in different insects are given by several workers (Tani et al. 2003; van Beek and Davis 2007). The main difficulty with in vivo production is contamination with microorganisms, mainly bacteria, and degeneration of the virus (Tani et al. 2003). Problem associated with in vivo production of viruses, specially control of process, resulted in recommendation of in vitro production which was found to be much better (Szewczyk et al. 2006; Nguyen et al. 2011), although requirements of productive insect cell lines and culture media are also a matter of consideration for in vitro production of baculovirus (Baines 2002; Verónica et al. 2006). Pawar and Thombre (1992) also gave detailed account on mass production of baculoviruses, whereas Rhodes (1996) provided all the economic aspects related to in vitro production. Commercially, viral insecticides are produced in the form of concentrated wettable powders apart from liquid or granular forms.

1.6.3 Consortia-Based Inoculants

Although most of the available bioformulations contain single strain, mixed cultures or co-inoculation with other microorganisms is proving to be better approach for overall plant growth and development. Practices such as use of rhizobial co-inoculation with mycorrhiza showed better results with legumes. This dual association not only improves nutritional status of nodulated plants but also reported to increase drought or osmotic tolerance in lucerne (Ardakani et al. 2009), soybean (Gao et al. 2012), broad bean (Jia et al. 2004), chickpea (Tavasolee et al. 2011), and pigeon pea (Bhattacharjee and Sharma 2012). Studies also confirmed that combined application of PSB and nodule-forming bacteria in legumes stimulated plant growth (Messele and Pant 2012). Phosphate and potassium rocks are a cheaper source of P and K, and integrated application of PSB with the co-inoculation of K-solubilizing bacteria may provide a faster and more continuous supply of these nutrients for optimal plant growth and best regarded for sustainable crop production (Xiufang et al. 2006). Recently consortia formulations have been developed by various workers and patents are also filed (Paikray and Malik 2010). In a study conducted by Maiyappan et al. (2010), a consortium bioformulation containing nine strains of the genera *Bacillus* spp., *Streptomyces* spp., *Azotobacter* spp., and *Frauteria* spp. was prepared as a wettable

powder and proved to be beneficial to black gram. In a similar study, consortium bioformulation of *Burkholderia* sp. MSSP with three other PGP bacteria were tested for growth enhancement of *Cajanus cajan* by using various carrier materials including sugarcane bagasse, sawdust, cocoa peat, rice husk, wheat bran, charcoal, rock phosphate, and paneer whey as liquid carrier, and their finding confirmed increase growth of pigeon pea plant when consortium was used in the formulation (Pandey and Maheshwari 2007). Tajini et al. (2012) showed that combined inoculation of AM fungi and rhizobia provides higher N and P accumulation in the shoots of common bean plants compared with single inoculum. Recently Zayadan et al. (2014) recommended that consortia of cyanobacteria, microalgae, and *Azotobacter* can be used as a biostimulator and biofertilizer for crops. BioGro™ is a consortium-based biofertilizer used in Vietnam, containing strains of *P. fluorescens*, two bacilli, and a soil yeast (Cong et al. 2009).

1.7 Conclusion and Future Steps

A bioformulation is not effective until it does not have an impact in field conditions, market existence and reliability and cost-effectiveness (Brar et al. 2006). Social and public interactions toward Bt-based biopesticides are given by Navon (2000). He concluded that the toxicity of protein as an oral insecticide and environmental conditions reduces the efficacy of the product. Production of bioformulation is not only dependent on the detailed knowledge of microbial as well as plant physiology, but a number of technological challenges are also involved such as fermentation process, formulation type, population of microbe, and delivery systems (Malusá et al. 2012). Barea (2015) suggested that prior to formulation development and application, it is necessary to understand the ecology of the PGPR–host–pest interaction. Fermentation depends on the medium used in the production process and concentration of constituents, oxygen transfer, incubation temperature, time of harvest, and postharvest treatments (Montazeri and Greaves 2002). Delivery system also decides usability of formulation and any bioformulation will not sustain until its delivery is not proper. For Bt water-miscible formulations and oil-miscible flowable (OF) are more preferable which are sprayed in ultralow volume (ULV). Himel et al. (1990) and Bateman (1993) described spray droplet size in detail and embodied all the mechanical aspects of droplet application. For foliar spray environmental factors, temperature, dew period, UV irradiation, and desiccation are involved (Bailey et al. 1996). In this direction technological advantage is preparation of bioformulation with very small particles or droplet size is now feasible and getting very good results when used as suspension or emulsion (Peng and Wolf 2011).

Impetus of Hynes and Boyetchko (2006) was to introduce research initiatives in the art and science of formulation development. They addressed various obstacles responsible in formulation technology development, including insufficient literature, registration process, and existing formulation technology. Current scenario toward bioformulation development and application is not satisfactory. The bioformulation policy including both for biofertilizers and biopesticides needs to be revived, and this is why Greaves and Grant (2010) have mentioned that the "biological control industry has the weakest policy network" and confirmed that the technical knowledge is not always matched by an understanding of political processes.

Albeit the research is ongoing, we have not succeeded in producing such an elite formulation which not only has broad spectrum activity but also fulfills economic challenges. Although many microbial inoculants have been developed, very few products are found to be promising. At present development of bioformulation involves collective effort of both microbiological and technological aspects, and vigorous research efforts are required for technological part (Arora 2015). Besides this there is urgent requirement in the field of bioformulation technology to reevaluate the whole process and plugging of loop holes. We

have to consider every step such as selection of organism, production method, delivery system, application technology, factors affecting development, persistence in the environment, and ultimately market availability of product.

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Formulation Technology of Biocontrol Agents: Present Status and Future Prospects

2

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

Contents

Abstract

Recent shift in trends of agricultural practices from application of synthetic fertilizers and pesticides to organic farming has brought into focus the use of microorganisms those carryout analogous functions. Formulations of rhizomicroorganisms available in global markets range from talc-based and liquid and secondary metabolite-based formulations. The ideal conditions required for development of high efficiency formulations of biopesticides include selection of potent strains, shelf life, storage, application technology, quality control, biosafety, and registration. In this chapter, we will discuss the constraints associated with development and commercialization of bioinoculants. Moreover, special emphasis will be on the next generation of antimicrobial secondary metabolite formulations which will not only have a much longer shelf life but also a higher efficiency against soilborne phytopathogens particularly against bacteria; also, a consortium of antimicrobial metabolites against individual pathogens could be formulated and used regardless of geographic location where the incidence of that particular disease is high. This approach would be unsurpassed by current technology, as the formulation would specifically target a particular pathogen while remaining soil microbiota would remain unaffected.

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

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_2

2.1 Introduction

Conventional farming around the world is primarily based on chemical fertilizers and pesticides for plant nutrition and disease management, a practice which pounded huge negative impacts on human and environment health. Globally, rising awareness of the hazardous effects of synthetic pesticides has increased the demand for safer alternatives. Various microorganisms are currently being explored and utilized as biological control agents (BCAs) or biopesticides. Popular BCAs include *Trichoderma* spp., *Pseudomonas fluorescence*, *Bacillus* spp., *Ampelomyces quisqualis*, *Agrobacterium radiobacter*, nonpathogenic *Fusarium*, *Coniothyrium*, and atoxigenic *Aspergillus niger* (Singh 2006; Keswani et al. 2014, 2015; Mishra et al. 2015). Approximately, there are 1400 biopesticide products being sold worldwide (NAAS 2013). The total market share of biopesticides was around 0.2 % of the total pesticides' market during the year 2000, and it amplified to 4.5 % by 2010. The market value is expected to reach around US$ 1 billion (Singh et al. 2012). In India, currently 34 microorganisms have been included in the schedule of Gazette of India for registration as biopesticide with Central Insecticide Board, Faridabad, under sections 9 (3B) and 9(3) of the Insecticides Act, 1968 (Table 2.1) (Keswani et al. 2013a).

Various classes of biocontrol agents have shown significant antagonism to a range of phytopathogens in vitro, but generally they have irregular performance in field conditions. Various factors are responsible for inconsistent performance which includes poor shelf life, susceptibility of microbial strain to various abiotic stresses, and low organic carbon content in the soil. Formulation technologies are used for stabilizing the microorganisms during production, storage, and

Table 2.1 Microbes listed in the Gazette of India for production of biopesticides and registration under sections 9 (3B) and 9(3) of the Insecticides Act, 1968

Bacterial	Fungal	Viral
Burkholderia cepacia	*Verticillium chlamydosporium*	Granulosis viruses
Agrobacterium radiobacter strain 84	*Streptomyces griseoviridis*	Nuclear polyhedrosis viruses (NPV)
Agrobacterium tumefaciens	*Streptomyces lydicus*	
Erwinia amylovora (hairpin protein)	*Candida oleophila*	
Alcaligenes spp.	*Fusarium oxysporum* (non pathogenic)	
Photorhabdus luminescences akhurustii strain K-1	*Penicillium islanidicum* (for groundnut)	
Photorhabdus luminescences	*Pythium oligandrum*	
Serratia marcescens GPS 5	*VAM* (*fungus*)	
Bacillus subtilis	*Trichoderma* spp.	
Pseudomonas fluorescens	*Aspergillus niger – strain AN27*	
	Gliocladium spp.	
	Beauveria bassiana	
	Verticillium lecanii	
	Metarhizium anisopliae	
	Nomuraea rileyi	
	Hirsutella sp.	
	Ampelomyces quisqualis	
	Phlebia gigantean	
	Coniothyrium minitans	
	Chaetomium globosum	
	Myrothecium verrucaria	
	Paecilomyces lilacinus	
	Piriformospora indica	

distribution, aiding in the application and handling, protecting the microorganism from damaging environmental factors, and enhancing the activity of the organism (Jones and Burges 1997). In a microbial formulation, the major focus is to preserve microorganism for enhancing their antagonism against target pathogens. Potency of microbial formulation is primarily dependant on the strain of microorganism used, though there may be crucial physical and nutritional requirements of the microorganism to remain active for longer time. Beneficial microorganisms are considered as eco-friendly, and it is mandatory that any additives in the formulation should be eco-friendly.

Commercial success of these formulations is based on the capability of a microorganism to survive and proliferate in the field condition, shelf life, and efficiency to control pest and disease, market price, ease of handling, and application (Lisansky 1985). Decision of selecting formulation depends fundamentally on the target organism to be managed, as well as on the ecology and biology of the biocontrol agents and host plant (Jacobsen and Backman 1993). Moreover, the best feature of this approach is that it can easily be integrated with different pest management modules. BCAs have been formulated in various ways such as wettable powders, liquid, and granules for application such as sprays, seed treatments, drenches, and dips and incorporation into soil and pot mix.

2.2 Types of Formulations

Although pesticides are formulated in various ways including dry formulations such as dusts (DP), granules (GR), and microgranules (MG); seed dressing formulations such as powders for seed dressing (DS); dry formulations for dilution in water including dispersible granules (WG) and wettable powders (WP); liquid formulations for dilution in water such as emulsions, suspension concentrates (SC), oil dispersions (OD), and capsule suspensions (CS); and ultralow volume formulations (Knowles 2005, 2006) however, globally biopesticides available in the market are in wettable powder, liquid, and granular formulations (Singh et al. 2012, 2014) (Table 2.2).

Dusts (DP) are formulated by adding an active ingredient on fine solid mineral powder such as clay and talc with particle size ranging from 50 to 100 μm. Dusts are applied directly to the target, either manually or mechanically. Inert ingredients used for dust formulation are anticaking agents, ultraviolet protectants, and adhesive materials to enhance adsorption. Dusts usually contain <10 % of microorganisms by weight.

Granule (GR) particles are heavier and larger compared to dust. Microgranules (100–600 μm) and coarse particles size (100–1000 μm) are made from mineral materials such as silica, kaolin, starch, attapulgite, polymers, ground plant residues, and dry fertilizers (Tadros 2005). Concentration of microorganisms in granules ranges from 5 to 20 %. Three types of granule formulations are currently available: (1) the microorganism is sprayed on a rotating granular carrier without a sticker, (2) the microorganism is attached to the outer surface of a granular carrier by a sticker, and (3) the microorganism is incorporated into a carrier paste or powder as a matrix.

2.2.1 Wettable Powders and Liquids

BCAs such as *Bacillus subtilis*, *Pseudomonas putida*, and *Trichoderma* spp. are used to control various diseases and are generally applied as dip or drenches and sprays to fruit after harvest (Tronsmo and Dennis 1983; Colyer and Mount 1984; Pusey and Wilson 1984; Wilson and Pusey 1985). Spraying of *Penicillium* sp. to pineapple fruit resulted in reduced postharvest diseases (Lim and Rohrbach 1980).

2.2.2 Granular Formulations

Lignite silage was applied to produce granules containing *Trichoderma harzianum* and *Gliocladium roseum* to control *Rhizoctonia solani* in soil causing damping-off of peanut (Jones et al. 1984). Lignite was grinded to produce granules of 425–2000 μm in diameter and

Table 2.2 Types of pesticide formulations (Modified from Patanjali and Raza 2013; Jones and Burges 1997)

Formulation	Abbreviation	Features
Formulations diluted in water		
Emulsifiable concentrate	EC	Emulsion formed when added to spray tank
Water-in-oil emulsion	EO	Preformed emulsion
Oil-in-water emulsion	EW	Preformed emulsion
Suspension concentrate	SC	Suspended insoluble AI
Capsule suspension	CS	AI contained in capsules
Soluble concentrate	SL	Used for water-soluble AI
Water-soluble powder	SP	Powder soluble, but may contain inert ingredients
Water-soluble granule	SG	Used for water-soluble AI
Tablet	TB	Used for portable water-soluble AI
Briquette	BR	Controlled-release formulation
Wettable powder	WP	Typically consist of AI, clay carrier, and surfactants
Water-dispersible granule	WG	AI dispersed, but not dissolved, in water
Formulations diluted with organic solvents		
Oil-miscible liquid	OL	AI dissolved in organic solvent
Oil-miscible flowable	OF	Suspension in organic liquid
Oil-dispersible powder	OP	Powder to be applied in oil
Formulations applied undiluted		
Dustable powder	DP	AI carried on free-flowing powder
Encapsulated granule	CG	Controlled-release granule
Microgranule	MG	Diameter below O.6 mm
Electro-chargeable liquid	ED	Used with electrostatic spray equipment
Spreading oil	SO	Applied to water surface
Ultralow volume liquid	UL	Applied through UL V sprayers
Ultralow volume suspension	SU	As above
Granule	GR	Applied to soil and water
Seed treatments		
Powder for dry seed treatment	DS	Liquid suspension
Flowable concentrate	FS	
Solution for seed treatment	LS	
Coated seed	PS	
Water-dispersible powder	SS	
Miscellaneous		
Bait concentrate	CB	Bait diluted before application
Bait	RB	

then amended with the product of sorghum fermentation. Isolates of *T. harzianum* and *G. roseum* were allowed growing on these granules for 7 days. These granules were allowed to air-dry followed by incubation before application in *R. solani*-infested soil. Trapping of antagonistic microorganism in calcium alginate granules, also known as prill, has been used widely used (Connick 1988). Though most of the commercially available alginates are derived from kelp, other organisms are also reported to produce alginates. Alginates produced by *Azotobacter vinelandii* can be used in place of kelp alginate for the production of microbial formulation to control plant pathogens (De Lucca et al. 1990). Further research may lead to less costly alginates. Sodium alginates showing great variation in viscosity and purity are available commercially. More granular sodium alginates like Kelgin HV, Kelgin, and sodium alginate IG-350 are easier to handle than the more powdery alginic acids.

Daigle and Cotty (1995) reported that 5 % gluten from wheat grains improved the

performance of a toxigenic *Aspergillus flavus* but that concentrations were very complex to process. The final formulated product contained 5 % gluten, 1 % sodium alginate, and 5 % corn cob grits. Likewise, in case of *Streptomyces* spp. formulation, clay was handy to keep in suspension, formulated in alginate with carrier polyamide.

2.3 Biocontrol Products Containing Fungi and Their Formulations

Plethora of antagonistic fungi and their products have been registered as commercial biopesticides globally (Table 2.3). Different types of formulations of these antagonists are currently available in markets and are successfully used against various phytopathogens. *Trichoderma* spp., *Gliocladium* spp., *Coniothyrium*, and nonpathogenic strains of fungal genus are available as different formulations.

Various biopesticide products (Binab-T, Bio-Fungus, Supresivit, RootShield, T-22HB, T-22G, Trichodex, Trichoseal, Trichopel, Trichodowels, BioMax, BioVam, Trichoject, *Trichoderma* 2000) contain *Trichoderma* spp. to control a variety of pathogens, including *Fusarium*, *Botrytis*, *Gaeumannomyces*, *Rhizoctonia*, *Pythium*, *Sclerotinia*, *Verticillium*, *Sclerotium*, and wood-rot fungi. *Trichoderma* product formulation varies considerably. Combination of *Trichoderma viride* and *T. harzianum* is formulated differently, for example, as a pellet for soil (Trichopel), as dowels for injecting in woods (Trichodowels), as a wettable powder for resuspension to apply on wounds with brush (Trichoseal), and as a wettable powder in syringe (Trichoject). Bio-Fungus is available as a granule, as crumbles for soil incorporation, and as wettable powder impregnated in sticks. *Gliocladium virens* formulation is available in alginate prill under the trade name SoilGard and effectively used against *R. solani* and *Pythium* spp. Nonpathogenic strain of *Fusarium oxysporum* has been used against pathogenic *F. oxysporum* and *Fusarium moniliforme* on carnation, tomato, basil, and cyclamen. Commercially available products of nonpathogenic *F. oxysporum*, namely, Biofox C, are formulated as alginate prill or dust, and Fusaclean is available as microgranule. Aspire containing yeast *Candida oleophila*, is formulated as wettable powder for postharvest application in citrus and pome fruit to control *Penicillium* spp. and *Botrytis*. *Ampelomyces quisqualis*, parasite of powdery mildew fungi, is commercially available as water-dispersible granule (AQ10) and applied to the leaves of apples, grapes, strawberries, tomatoes, cucurbits, and ornamentals. *Pythium oligandrum* has been formulated as granule or powder for use in seed treatment for management of pathogenic *Pythium* spp. (Jones and Burges 1997).

Lewis and Lumsden (2001) prepared a solid matrix formulation of *Gliocladium* and *Trichoderma* using wheat bran and vermiculite effective against *R. solani*. Formulated product was applied at a rate of 1.0 % (w/w). The product was tested, and a significant reduction in damping-off of pepper seedlings was observed. Efficacy of *Trichoderma* spp. against *Sclerotinia sclerotiorum* causing sunflower head rot was evaluated in the field. *Trichoderma* formulation contained viable hyphal fragments, *Trichoderma* conidia, milled corn kernels, and industrial talc. Sunflower heads were infected with *S. sclerotiorum* after 2 days of the first delivery of *Trichoderma* formulation by honeybees. Head rot incidence was reduced in sunflower when 100 g formulation was carried by honeybees in a 10 h per day period (Escande et al. 2002). An invert emulsion based on soybean and coconut oils offered the lowest viscosity (27 $\pm$ 0.81 cps) and most stable emulsion layer (93 % v/v) for formulating *T. harzianum* conidia (Batta 2004). In this formulation, 36 months of shelf life of conidia was recorded with 50 % declined viability at 20 $\pm$ 1 °C after 5 months. *Botrytis* sporulation on the fruit lesion surface was also inhibited after 10 days of inoculation. Biocontrol potential of *Trichoderma* isolated from rhizosphere of *Musa* sp. was evaluated against *F. oxysporum* in vitro. Among different isolates, *T. harzianum* Th-10 was found most significant

Table 2.3 Some commercially available fungal biocontrol products

Organism	Product name	Disease against used	Formulation	Application
Ampelomyces quisqualis	AQ10 Biofungicide	Powdery mildew	Water-dispersible granule	Spray
Candida oleophila	Aspire	*Penicillium* spp. *Botrytis* spp.	Wettable powder	Postharvest application to fruit as drench, drip, or spray
Trichoderma harzianum, *T. polysporll*	Binab-T	Pathogenic fungi causing wilt, take-all, root rot, internal decay of wood products, and decay in tree wounds	Wettable powder	Postharvest application to fruit as drench, drip, or spray
Fusarium oxysporum	BiofoxC	*F. oxysporum*, *F. moniliforme*	Dust or alginate granule	Seed treatment soil or incorporation
Trichoderma spp.	Bio-Fungus	*Sclerotinia*, *Rhizoctonia solani*, *Pythium* spp., *Fusarium*, *Verticillium*, *Phytophthora*	Granules, wettable powders, sticks, and crumbles	Applied after fumigation, incorporated in soil sprayed or injected
Coniothyrium minitans	Contans	*Sclerotinia sclerotiorum* and *S. minor*		Spray
Myrothecium verrucaria (killed cells)	DiTera	Root-knot, citrus cyst, stubby root, sting, lesion, and burrowing nematodes	Wettable powder, emulsifiable liquid or granule	
Fusarium oxysporum (nonpathogenic)	Fusaclean	*F. oxysporum*	Spores, microgranule	In drip to rock wool; incorporate in potting
Phlebia gigantean	Rotstop	*Heterobasidion annosum*	Spores in inert powder	Spray, chain saw oil
T. harzianum	Root Shield strainT-22	*Pythium* spp., *R. solani*, *Fusarium* spp.	Granules	Mix with soil or potting medium
Gliocladium virens	Soil Gard (formerly GlioGard)	*Rhizoctonia solani*, *Pythium* spp.	Granule (1×10^6 cfu g^{-1})	Granules are incorporated in soil or soilless growing media prior to seedlings
T. harzianum	Supresivit	Various fungi		
T. harzianum and *T. viride*	T-22G 19, T-22 HB	*Pythium* spp., *R. solani*, *Fusarium* spp., *Sclerotinia homeocarpa*	Granules or dry powder (both at 1×10^7cfu g^{-1})	Granules added in furrow with granular applicator, by broadcast application to turf, mixed with green house soil
T. harzianum	Trichodex	*Botrytis cinerea*, *Colletotrichum* spp., *Plasmopara viticola*,	Wettable powder	Spray
T. harzianum and *T. viride*	Trichopel, Trichoject, Trichodowel Trichoseal	*Armillaria*, *Botryosphaeria*, *Fusarium*, *Chondrostereum*		
Trichoderma sp.	*Trichoderma* 2000	*R. solani*, *S. rolfsii*, *Pythium* sp.		Incorporated into soil or potting medium
T. harzianum NBRI-1055	Sardar Eco Green Biofungicide	*Pythium* sp., *R. solani*, *S. rolfsii*	Wettable powder	Spray
T. harzianum NBRI-1055	Tricha	*Pythium* sp., *R. solani*, *S. rolfsii*	Wettable powder	Spray

in pathogen inhibition. Five organic substrates including rice chaffy grain, rice bran, banana pseudostem, farmyard manure, and dried banana leaf were tested for the mass production, and dried banana leaf was found the best carrier material for *T. harzianum* growth. Dried banana leaves were colonized within a few days by strain Th-10 and produced propagules of high density (4.6×10^{32} cfu g^{-1} of leaf). Furthermore, addition of jaggery (10 % w/v) to the dried leaves enhances the growth of *T. Harzianum*, and more than 6 months of survival was recorded (Thangavelu et al. 2004).

Trichoderma atroviride isolate C52 was observed on onion roots when inoculated in soil with various formulations (McLean et al. 2005). Pellet formulation product maintained the concentration of *Trichoderma* up to 10^5 cfu g^{-1}soil in comparison to 10^1 cfu and 10^4 g^{-1} soil concentrations that were maintained by seed coating and solid substrate formulations, respectively. *Trichoderma* isolate C52 was inoculated into *Sclerotium cepivorum*-infested soil as both solid substrate and pellet formulations, and no difference was observed in disease control, but more healthy plants were observed in the pellet treatment. Increased root and shoot lengths, plant height, and dry weight were recorded after treatment of plants with *T. viride* formulated in talc. Application of *T. viride* formulation also resulted in a significant reduction of sheath blight caused by *R. solani* (Mathivanan et al. 2005). New carrier formulation was developed by using *T. harzianum* M1, and resistance to carbendazim showed inhibitory effect against *Pythium aphanidermatum*. Different formulations including lignite, talc, wettable powder, lignite + fly ash-based powder formulation, bentonite paste, gelatin-glycerin gel, and polyethylene and glycol paste were developed for seed treatment. Shelf life of the microbial formulations was assessed at 24 °C for 9 months. Up to 74 % reduction in disease incidence was recorded when *Trichoderma* formulation was applied as seed treatment. Additionally, enhanced plant biomass under field and greenhouse conditions was also recorded (Jayaraj et al. 2006). Pelletized formulations of kaolin clay and wheat bran in an alginate gel containing conidia, fermentor biomass, or chlamydospores of *G. virens* and *Trichoderma* spp. were prepared (Lewis and Papavizas 2007). Higher population densities of *Trichoderma* and *Gliocladium* were observed when soil is incorporated with alginate pellets containing chlamydospores rather than condia and bran rather than kaolin as the carriers.

A new *Trichoderma asperellum* formulation was developed using soybean oil dispersion. Complete inhibition of *Phytophthora megakarya* causing cacao black pod disease was recorded when formulation was applied to the pods. Ninety percent prevention of infection in treated pods was recorded after 1 week, and 50 % reduction after 3 weeks was recorded when formulation was sprayed on cacao clones susceptible to *P. megakarya*. The formulations showed a significant effect in disease management (Mbarga et al. 2014). Talc-based formulation of a novel *T. viride*, BHU-2953, successfully controlled damping-off of chili caused by *P. aphanidermatum* and tomato wilt caused by *F. oxysporum*. Significant reduction in diseases was observed when *T. viride* formulated as 2 % wettable powder was applied to the seeds and furrow (Singh et al. 2014). Formulations of *Trichoderma* with mixture of 1 % w/v Sure-Jell, 1 % w/v PDB, and 0.3 ml L^{-1} of the surfactant BreakThru 100SL (BT) and an invert oil emulsion of 50 % v/v corn oil, 2.5 % w/v lecithin, and 0.5 % w/v PDB (COP) were evaluated against frosty pod rot pathogen *Moniliophthora roreri*. *T. harzianum* DIS 219f and *Trichoderma ovalisporum* DIS 70a were applied (180 ml $tree^{-1}$, 2.46×10^7 conidia ml^{-1}) in the field. COP/DIS-70a formulation resulted in a maximum increase in yield as compared to other treatments (Crozier et al. 2015).

2.4 Biocontrol Products Containing Bacteria and Their Formulation

Several bacterial species having biocontrol potential have been formulated and are commercially available (Table 2.4). Nonpathogenic

Table 2.4 Some commercially available bacterial biocontrol products

Biocontrol agent	Trade name	Target pathogen	Formulation	Application
Pseudomonas syringae ESC 10	Bio-Save10	*B. cinerea*, *Penicillium* spp., *Mucor piriformis*, *Geotrichum candidum*	Wettable powder	Postharvest application to fruit as drench, dip, or spray
P. syringae ESC 11	Bio-Save11	*B. cinerea*, *Penicillium* spp., *M. piriformis*, *G. candidum*	Wettable powder	Postharvest application to fruit as drench, dip, or spray
P. fluorescens A506	Blight Ban A 506	Frost, *Erwinia amylovora*	Wettable powder	Drench dip or spray
P. fluorescens NCIB	Conquer	*P. tolaasii*	Aqueous biomass suspension	Spray
B. subtilis	Epic II	*R. solani*, *Fusarium*, *Alternaria* spp., and *Aspergillus* spp.	Dry powder (5.5×10^{10} spores g^{-1})	Added to a slurry, mix with a chemical fungicide
Agrobacterium radiobacter	Galltrol-A13	Crown gall disease *A. tumefaciens*	Petri dishes with pure culture grown on agar (1.2×10^{11} cfu $plate^{-1}$)	Root dips, drench
Pseudomonas cepacia	Intercept 14	*R. solani*, *Fusarium* spp., *Pythium* sp.	Wettable powder	Drench dip or spray
A. radiobacter	Nogall, Diegall 16	*A. tumefaciens*	Washed plates, culture suspension	Root dips
P. solanacearum (nonpathogenic)	PSSOL 12	*P. solanacearum*		
P. fluorescens NCIB 12089	Victus	*R. solani*, *Fusarium* spp.	Aqueous suspension	Spray
B. subtilis	System3	*S. rolfsii*, *S. sclerotiorum*	Dust	Seed treatment in planter box
B. cepacia-type Wisconsin M36	Blue Circle	*Fusarium*, *Pythium*, spiral, lesion, lance, and sting nematode	Peat carrier or liquid	Seed treatment or drip irrigation

A. radiobacter is commercially available under various trade names such as Nogall, Galltrol-A, Diegall, and Norbac 84C. Bacteria are generally suspended in non-chlorinated water and applied as dips and sprays to cuttings and stems or as soil drench. *Pseudomonas syringae* formulated as wettable powders is commercially available under trade names Bio-Save 10 and Bio-Save 11 and used for postharvest application to citrus and pome fruit for management of *Botrytis*, *Mucor*, *Penicillium*, and *Geotrichum*.

P. fluorescens strains isolated from rhizosphere of various crops with antagonistic potential against *Fusarium* spp. were formulated as talc-based and peat-based products. In talc-based and peat-based formulations, *P. fluorescens* survived for a maximum of 240 days. When formulation was applied to the chick pea seed, the total shelf life of the bacteria was recorded at 180 days (Vidhyasekaran and Muthamilan 1995). Peat-based formulation of *P. fluorescens* strains PfALR2 was developed and assessed for root treatment, seed treatment, foliar spraying, and soil application. All four treatments in combination resulted in the significant control of sheath blight in greenhouse condition (Rabindran and Vidhyasekaran 1996). *P. fluorescens* strain PF-1, isolated from rhizosphere of maize roots, showed antagonistic potential against *R. solani* f. sp. *sasakii* causing banded leaf and sheath blight of maize. Among the different carriers, talc and peat maintained the population at 18.3×10^7 and 19.5×10^7 cfu g^{-1} of the bacterium, respectively, after 40 days. Significant control of disease was recorded after the seed treatment with peat-based

formulation (Sivakumar et al. 2000). Formulations of *B. subtilis* AF 1 showed both plant growth promotion and biocontrol potential when prepared in peat. The formulation was supplemented with *A. niger* mycelium, 0.5 % chitin, and organic compost from cultivation of *Agaricus bisporus*. Biocontrol potential of formulated products was evaluated against two pathogens on groundnut and pigeon pea. Chitin, *A. niger* mycelium, and *A. bisporus* compost were used as supplement for improving the growth rate of *B. subtilis* AF 1A. Peat formulation supplemented with chitin when used for seed treatment demonstrated better control of wilt in pigeon pea caused by *Fusarium udum* and *A. niger* responsible for crown rot disease in groundnut (Manjula and Podile 2001). *P. fluorescens* strains FP7 and PF1 and their consortium were formulated as talc-based product and mixed both with and without chitin were assessed individually against sheath blight of rice. Significant reduction in disease incidence was recorded after the application of formulated product through seed, soil, root, and foliar spray. In field as much as 62.1 % reduction in sheath blight incidence was observed in the consortium treatment containing chitin (Commare et al. 2002). 0.1 % calcium hydroxide significantly promoted the growth of *B. amyloliquefaciens* strain B190 used against *Botrytis elliptica* in lily. Spraying *B. amyloliquefaciens* B190 mixed with 0.05 % sodium carbonate, 0.025 % calcium hydroxide, or 0.025 % ammonium nitrate suppressed the gray mold on lily. Concentration of adjuvant was kept below 0.1 % (v/v); carboxymethyl cellulose (CMC) and Tween 80 were effective to *B. amyloliquefaciens* B190 formulation against lily gray mold (Chiou and Wu 2003). The efficacy of talc-based formulations of *T. viride* and *P. fluorescens* alone and in combination on sheath blight disease, crop growth, and yield in rice was studied in field experiments. Application of formulated product of *P. fluorescens* and *T. viride* either alone or in combination resulted in increase in root and shoot lengths and plant height when compared with control. Significant reduction in sheath blight incidence was also recorded after application of *P. fluorescens* and *T. viride* (Mathivanan et al. 2005).

Bacillus licheniformis strain N1 exhibiting the biocontrol activity against *Botrytis cinerea* was formulated using fermentation of the bacterial culture in Biji medium. Wettable powder formulation of antagonist based on olive oil corn and starch was selected for evaluation of the disease control. A dose of 100-fold-diluted *B. licheniformis* N1E was found to be the optimum spray formulation and significantly reduced the disease. 90.5 % reduction in disease by formulated product was recorded in comparison to the 77 % reduction by synthetic fungicide including carbendazim and diethofencarb. Results of this study indicated that the olive oil- and corn starch-based formulation of *B. licheniformis* using liquid fermentation will be effective against tomato gray mold (Lee et al. 2006). Nineteen isolates of antagonistic *Pseudomonas* and twelve isolates of yeast were screened for the biocontrol activity against *Colletotrichum musae* causing banana anthracnose. *P. fluorescens* strain FP7 showed a maximum inhibition of *C. musae* mycelial growth. Water-in-oil formulation of *P. fluorescens* FP7 was formulated by adding various oils such as rice bran (28.50 %), coconut (28.50 %), and castor (28.50 %) separately to the bacterial culture and bacterial populations were reported to survive for 210 days of storage. The application of water-in-oil formulation of bacterium significantly reduced the disease incidence (Peeran et al. 2014). *B. subtilis* strain BY-2 was unable to colonize the leaf surface and stem in oilseed rape when applied as pellet. Populations of BY-2 declined from 10^8 CFU seed^{-1} to 10^4 CFU g root^{-1} and $\leq 10^{23}$ CFU g stem^{-1} after 60 days. Significant reduction in disease was observed when compared to control (Hu et al. 2014). Efficacy of aqueous suspension (Serenade ASO or QRD 145) and foliar sprays of wettable powder formulation (Serenade MAX or QRD 141) of *B. subtilis* QST 713 alone and in combination with copper hydroxide was investigated against bacterial spot disease of tomato. The aqueous suspension of *B. subtilis* QST 713 alone significantly reduced bacterial spot on tomato foliage when compared to control. The wettable powder of *B. subtilis* QST

713 alone did not reduce bacterial spot, but in combination with copper hydroxide it reduced disease severity and enhanced the total fruit yield (Abbasi and Weselowski 2015).

2.5 Seed Treatment

The application of microbes to seed surface requires few technical considerations. Significant amount of the inoculums must survive the application procedure and must have the capacity to grow in vicinity of seed. Since seeds are at low moisture levels for most of the time during storage, microorganisms must have the ability to survive under low water activity. Microorganisms might likewise need to be mixed with other active ingredients, for example, insecticides and fungicides. These aspects raise issues of formulation stability and strain selection. Seed treatment with beneficial microorganisms has been a prime area of investigation for many years (Bisen et al. 2015). Microorganisms with various properties have been applied to seeds to perform various functions, including plant growth promotion, nitrogen fixation, phosphate solubilization, and biological control of plant pathogens (Keswani et al. 2013b). BCAs are applied to the seeds for protection of seed and seedlings from effects of various seed-borne and soilborne plant pathogens. For the successful biological control, applied microorganism must grow and colonize the rhizosphere in order to protect the plant, thus the release of microbes from the formulation and prerequisite condition for growth are of paramount importance.

The commercial wettable powder product Mycostop (Kemira AgroOy, Finland), containing *Streptomyces griseoviridis* strain K61, with 10^8 $cfug^{-1}$ is available for seed treatment. The powder is used at 5–8 g kg^{-1} seeds for control of seed-borne and soilborne fungal phytopathogens in herbs, vegetables, and ornamentals (Tomlin 1994). The shelf life of formulation is estimated to be 6 months at 8 °C or for 12 months at −12 °C and must be stored in airtight containers. Seed inoculation with bacteria to protect the seed from soilborne pathogens and promote plant has been well investigated (Merriman et al. 1974). Kodiak, a *B. subtilis*-based biopesticide, was used as seed inoculant for peanut, cotton, and beans to control the root diseases caused by *Fusarium* and *Rhizoctonia* (Mahaffee and Backman 1993). Quantum-4000, a *B. subtilis* strain A13-based product, is commercially available as seed inoculants for peanut, and another biopesticide based on strain GB 07 named Epic is available for cotton. Bacterial BCAs actively colonize the rhizosphere and compete with other microorganisms including pathogens (Bisen et al. 2015). Nonspore-forming bacterium *Enterobacter cloacae* showed antagonistic activity against *Pythium* spp. causing seed rot in cotton seeds. Simple seed inoculation technique with (CMC) as sticker in cotton seeds was carried out successfully (Nelson 1988). Liquid culture of *E. cloacae* was applied by solid matrix priming to tomato and cucumber seeds in combination with a fungicide (Harman and Taylor 1988).

Generally, in seed treatment, microbial formulation is applied to seed as powder or liquid. For example, *P. fluorescens* and *Burkholderia cepacia* were applied to pea seeds with or without captan for control of *Aphanomyces* root rot and *Pythium* damping-off (Parke et al. 1991). Potato tubers have been treated with ascospores of *Talaromyces flavus* in a pyrophyllite carrier (Fravel et al. 1985). In order to ensure the better adhesion of bioinoculants on seed surface, stickers are added to microbial formulations for seed treatment. To control *Pythium ultimum* attack in pea and soybean seeds, PelGel has been used to treat the seed with *P. putida* (Paulitz et al. 1992). In cucumber seed treatment, PelGel has also been used with *T. harzianum* (Harman 1991; Taylor et al. 1991). Another sticker Polynox-N-I0 was used for seed treatment with shale and then applied on bean seeds with conidia of *T. harzianum*, and efficient disease control was observed. Various concentrations of plant gum, methyl cellulose, and xanthane gum with talc have been used for treatment of potato tuber with PGPR (Kloepper and Schroth 1981). At 40 °C, population of rhizobacteria in talc and 20 % xanthane gum did not decline for 2 months and resulted in better increase in plant development in field potato. However, rhizobacteria did not last longer in formulation containing gum tragacanth or gum

karaya. CMC was applied with microbial agents to seeds to control *R. solani*. One percent CMC with clay carrier was applied to *Verticillium biguttatum* and other biocontrol agents to potato tubers (Jager and Velvis 1985). Various antagonists have been applied to treat the sugar beet seeds using either gum xanthan or methylcellulose in combination with a neutralized talc or peat carrier (Suslow and Schroth 1982). Similarly, to control take-all disease in wheat, surface-disinfested seeds were coated with *P. fluorescens* in combination with 1 % methylcellulose (Weller and Cook 1983). In seed treatment of Chinese aster with *T. flavus*, a polymer binder was used in quartz flour to pellet seeds (Nagtzaam and Bollen 1994), and antagonist *T. flavus* was isolated from seeds after 17 years. Strains of *E. cloacae* and *Trichoderma* were delivered to cucumber and tomato seeds through solid matrix priming (Harman and Taylor 1988). During priming seeds are brought to a certain moisture level just below the required level for germination and then mixed with moistened *Trichoderma* or *E. cloacae*, shale, sphagnum moss, or bituminous coal. The seeds and carriers are then mixed with water and incubated before planting.

2.6 Carriers and Adjuvant Used in Microbial Formulations

Variations in effectiveness of microbial formulations from beneficial microorganisms are credited to three main causes: (1) presence of the microorganism prior to inoculation in field, (2) poor survival of the organism in the environment, and (3) low quality of the microbial product itself. Success of biopesticides depends on the delivery of viable, active microorganisms in high numbers to the field which requires high-quality inoculants. The carrier substrate is the most critical part of the microbial formulation, and it must be capable of supporting high numbers of microbe. Carriers are inert ingredients, and they do not have biocontrol potential; however, they can affect the efficacy of the product and shelf life of microorganism (Table 2.5). Better survival of *Pseudomonas* spp. on minerals with small particle size, such as zeolite, montmorillonite, and vermiculite, than on minerals with larger particle size such as talc, pyrophyllite, and kaolinite was observed during storage at 20 °C (Dandurand et al. 1994). Backman and Rodriguez-Kabana (1975) compared diatomaceous earth and attapulgus clay for their various physical properties including water-holding capacity and strength after autoclaving. Attapulgus clay granules swelled in water and lost their integrity after autoclaving, whereas, diatomaceous earth granules did not swell in water and remained intact after autoclaving thus making it to absorb a molasses-based medium for delivery of *T. harzianum* to soil. Wheat bran-perlite mixture and poplar bark compost have been used as carrier for nonpathogenic *Fusarium* strains mixed with soil that induced resistance to *F. oxysporum* f. sp. *dianthi* (Garibaldi et al. 1987). Fine-ground tree barks have also

Table 2.5 Various adjuvants used in microbial formulations

Adjuvant	Type	Function
Oils	Mineral oils	Improve uptake, photostability
	Crop oil	
Surfactant	Wetting agents	Improve spreading, wetting, or dispersion
	Spreaders	
	Penetrants	
Stabilizing agents	Emulsifiers	Maintain stability during application
	Dispersants	
	Anti-flocculating agents	
Solvents	Cosolvents	Maintain AI in solution
	Coupling agents	
Hygroscopic agents	Humectants	Prevent premature drying of deposit

been used as a carrier (Stack et al. 1988). Peat is generally used as carrier for *Rhizobium* spp. and is also useful for soil applications and seed coating of biocontrol agents. Huber et al. (1989) used fine-ground peat with a methylcellulose sticker for wheat seed treatment with bacteria for control of *Gaeumannomyces graminis*. Alder bark has been used as a carrier to apply *T. flavus* to potato seed pieces (Keinath et al. 1990).

One of the mechanisms involved in biocontrol is the production of hydrolytic enzymes by the antagonistic microorganism. The amount and type of nutrients in the formulation must allow ample production of hydrolytic enzymes (Stack et al. 1988). Increased molar concentrations of carbon and nitrogen sources (0.02–0.18 M maltose and 0.006–0.024 M arginine) and increased carbon/nitrogen ratios (12:1–80:1) enhanced the proliferation of *Thielaviopsis basicola* and *Trichoderma* spp. on lignite granules (Stack et al. 1987). Likewise, *T. flavus* was formulated with eight different organic carriers and used against *Verticillium dahliae* on eggplant, and a maximum inhibition of pathogen was recorded in treatments with the highest carbon/nitrogen ratios (159:1 for pyrophyllite, 97:1 for corn cobs) (Fravel et al. 1985). As new information on mechanism of biocontrol revealed, it may be possible to express desirable biocontrol traits by manipulating nutrient composition of formulations. For example, biocontrol potential of *G. virens* GL-21 depends on the form of nitrogen in the formulation. Alginate prill of *G. virens* with wheat bran as a carrier resulted in significant control of *Sclerotium rolfsii* in comparison to vermiculite plus wheat bran (Ristaino et al. 1994).

2.7 Basic Information Required for Microbial Product Registration

Certain information about the various parameters is prerequisite for the biopesticide registration. These parameters include microorganism strain specifications, cfu count of microorganism used, target microorganism, moisture content of the product, type of formulation, and technical bulletin/product profile. Strain specifications include information on genus and species, rhizosphere competence, biological control potential, growth promotion potential, and growth parameters like pH and temperature.

2.7.1 Strain Specifications

Selection of potential antagonist strain under field and lab conditions ensures the effective and consistent performance of bioagent in field. Screening of effective strain can be done in different ways: selection of potential strain in relation to phytopathogens, screening of isolates with high biotechnological application, or search for economical viable substrates which are suitable for mass production of bioagent (Singh et al. 2003, 2006).

2.7.2 Shelf Life and Storage

For commercialization of a microbial product in the market, it is essential that it has long shelf life and can be stored at room temperature. It has been suggested that shelf life of a minimum 18 months is acceptable for commercial microorganism-based product. Storage at room temperature is an essential condition as the farmers cannot afford the equipment to keep the product at any temperature. According to the recent guideline of the Central Insecticide Board and Registration Committee, the data required for claiming 1-year shelf life of the product is for 15 months for talc-based formulation, i.e., the microbe should remain viable for 15 months.

2.7.3 CFU Count

According to the Central Insecticide Board and Registration Committee guideline, colony-forming unit (cfu) count should not be less than 2×10^6 spores ml^{-1} or g^{-1} on selective media (SM) for antagonistic fungi, and CFU count on selective medium should be a minimum of 1×10^8 ml^{-1} or g^{-1} for antagonistic bacteria (Singh 2012).

2.7.4 Pathogenic Contamination

The pathogenic contaminants such as *Salmonella*, *Shigella*, and *Vibrio* should not be present. Other microbial contaminants must not to exceed 1×10^4 counts ml^{-1}or g^{-1}.

2.7.5 Moisture Content

Maximum moisture content of the product should not exceed more than 8 % for dry formulation of fungi and 12 % for bacteria (www.cibrc.nic.in/2.1.22011.doc).

2.8 Constraints in the Production of Microorganism-Based Biopesticides

The main reasons for slow growth of microbial-based biopesticide industry include inconsistent performance of final product in field condition, short shelf life of microorganism in formulation, possibilities of contamination with other plant and human pathogens, lack of suitable application technology, small market size, and lack of proper knowledge about the biopesticides in farmers. Research should be focused on the development of superior formulation to protect the reliability of the product, because a single failure will jeopardize the whole trade's reputation. Production of biopesticides is a long process which includes selection of suitable strain for formulation, mass production of selected strain, screening of microorganism for suitable carrier for formulation, assessment of the shelf life of microorganism in selected formulation, and product efficacy in field condition. Several reports on contamination and low population of microorganism in biopesticides being sold in the market were registered (Singh et al. 2004a; Alam 2000; Arora et al. 2010). Due to low microbial count, it is obvious that their performance in the field is inconsistent and poor. The unpredictable seasonal nature of the existing demand needs capable storage for biopesticides. The storage requires sophisticated and special facilities which are generally not available to most producers, sellers, and farmers. Shelf life is a result of combining several factors including production technology, material used as carrier and packaging, and transport. The mass production of significant numbers of viable, efficient, and stable propagules of the microorganism is a prerequisite in biopesticide development (Singh et al. 2004b). Submerged fermentation system has been traditionally followed by the producers over the solid substrate fermentation because of its cost-effectiveness and easy technology (Churchill 1982; Stowell 1991). However, new large-scale production systems are required for bacteria and fungi that do not like to produce spores in liquid media. Unfortunately fermentation systems for mass production based on solid substrates are not frequently available (Connick et al. 1990). Submerged fermentation methods are generally well adopted for the mass production of secondary metabolites, antibiotics, organic acids, and bacteria; however, it is not suitable for the production of viable filamentous fungi. Therefore, selection of the cost-effective fermentation technology for the mass production viable and efficient propagules is a matter of concern. Choice of carriers and adjuvant used in the formulation is another technical problem in the development of stable and effective biopesticides. One of the major goals in formulating biopesticides is to maintain the viability and effectiveness of the active ingredient for a possible duration, preferably 2 years. After the production of microorganism, the main challenge faced by producers in formulation development is the shelf life of microorganism during the storage period. If the product carries less numbers of active ingredients due to shorter shelf life, the overall performance of the formulation will be affected.

2.9 Future Prospects

The necessity for more safe products for plant disease management prompts an inclination of microbial biopesticide formulations with efficient antagonism and good stability. Biopesticides give eco-friendly alternatives to synthetic pesticides, yet they confront various difficulties in their

production, formulation, and application. Since biopesticide generally contains live organism, maximum care is needed to maintain the microbial population and efficacy from beginning to the end use. Study of their formulation and production could enormously help in the commercialization of biopesticides. It appears to be that biopesticides will have a more extensive use in the future as their application techniques enhance as less expensive inert materials are recognized for different formulations. Introduction of new adjuvants showed significant increase in activity of microbes and proposed a new area of research. Selection of proper formulation may enhance the product stability, larger shelf life, and performance of microbes in field conditions. Biopesticides offer a more balanced plant protection product application, and in the future formulation products should have more balance between production cost and efficiency (El–Sayed 2005; Rao et al. 2007; Glare et al. 2012; Khater 2012). Development related to the formulation type would possibly shift from dusts to granules, from suspension concentrates and wettable powders to water-dispersible granules, and from single microorganism-based product to microbial consortium-based formulation. With the advances in nanotechnology science, different new microbial formulations such as nanosuspension, nanoemulsion, and nanocapsule suspension with superior efficiency will be released in the market (Rao et al. 2007; Ghormade et al. 2011; Glare et al. 2012). Significant advancement has been made in the production of new formulation products and application methods; however, there is still much work to be done. For further research to improve production and application techniques, scientist and researchers are likely to provide safe and effective products for plant disease management.

Eco-friendly and safer biopesticides play an important role in modern agriculture; however, their major drawbacks have led to the use of nanotechnology in agriculture (Mishra and Singh 2015). Silver nanoparticles (AgNPs) are the most frequently used metallic nanoparticles in various sector including agriculture (Jo et al. 2009; Kim et al. 2012; Mishra et al. 2014). Many workers have reported the antimicrobial activity of AgNPs against a vast range of phytopathogens. A nanosized silica-silver particle formulation was developed and showed significant antimicrobial activity against a wide range of phytopathogens including *Colletotrichum* sp., *Pythium* sp., *P. syringae*, *Xanthomonas compestris*, etc. (Park et al. 2006). Biosynthesized AgNPs of bacterium *Serratia* sp. BHU-S4 showed significant antifungal activity against *Bipolaris sorokiniana* (Mishra et al. 2014).

Recovering the real connections of AgNPs with agroecosystems including soil, soil biota, and plants and their poisonous quality level can be infer whether AgNP application could be helpful for agroecosystems. Considering the elements deciding destiny, transport, portability, and poisonous quality of AgNPs in soil, it is additionally accepted that the size of the poisonous quality of these particles could really be evaded by controlling them and henceforth requires more profound exploration. Specifically, research concentrating on this methodology utilizing biosynthesized AgNPs ought to get more thoughtfulness regarding an efficient comprehension of how incorporating biosynthesized AgNPs in agrarian applications contributes toward the farmer's benefits. One important point is that biosynthesized AgNPs have demonstrated their worth for agricultural applications by performing two noteworthy undertakings, viz., plant development upgrade and plant malady management. Hence, now it is high time when future studies must be coordinated toward upgrading the utility of biosynthesized AgNPs in regular environments keeping in mind the end goal to predict their future agricultural extension.

2.10 Conclusion

In recent past, disease management strategies have been inclined to much safer alternatives due to concerns over hazardous effects of chemical pesticides on human and plant health. Biological control of plant pathogens employing living microorganisms offers such safe

alternative to the chemicals. In order to improve the efficiency and shelf life of biocontrol agents, various formulations based on solid and liquid carriers have been developed. Maximum care is required in biopesticide production and formulation as it contains live organisms. It is quite a confirmation that biopesticides will have a more extensive use and share greater market space in the future. Specific procedures and technologies have been recently developed that would significantly affect biopesticide formulations. Selection of abiotic stress tolerance or rhizospheric competence could allow a wider range of applications (Keswani 2015). Moreover, fungicide tolerance screening would promote integrated management with reduced chemical inputs.

Acknowledgments HBS is grateful to the Department of Biotechnology (BT/PR5990/AGR/5/587/2012), New Delhi, for providing financial support. KB is grateful to BHU for financial assistance. BKS and CK are thankful to ICAR-AMAAS (Project No. P27/131) for financial support.

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Beneficial Microorganisms: Current Challenge to Increase Crop Performance

3

Márcia do Vale Barreto Figueiredo, Aurenivia Bonifacio, Artenisa Cerqueira Rodrigues, Fabio Fernando de Araujo, and Newton Pereira Stamford

Contents

M.d.V.B. Figueiredo (✉)
National Research and Technological Development, Agronomical Institute of Pernambuco (IPA/SEAGRI), 1371, Gen. San Martin Avenue, Recife, 50761-000 PE, Brazil
e-mail: mbarreto@elogica.com.br

A. Bonifacio • A.C. Rodrigues
Federal University of Piaui (UFPI), Teresina, PI, Brazil
e-mail: bonifacio.a@live.com; artenisacerqueira@hotmail.com

F.F. de Araujo
Faculty of Agrarian Sciences, University of West Paulista (UNOESTE), Presidente Prudente, SP, Brazil
e-mail: fabio@unoeste.br

N.P. Stamford
National Research and Technological Development, Department of Agronomy, Federal Agricultural University of Pernambuco (UFRPE), Recife, PE, Brazil
e-mail: newtonps@depa.ufrpe.br

Abstract

The major goal of agricultural microbiology is a comprehensive analysis of beneficial microorganisms. Fundamental knowledge of the ecology and evolution of interactions could enable the development of microbe-based sustainable agriculture. Plant growth-promoting bacteria (PGPB) have gained worldwide importance and acceptance for their agricultural benefits. This is due to the emerging demand to reduce dependence on synthetic chemical products within a holistic vision of developing and focalizing environmental protection. Beneficial microorganisms also help to solubilize mineral phosphates and other nutrients, enhance resistance to stress, stabilize soil aggregates, improve soil structure and organic matter content, and inhibit phytopathogens. Several efforts have been made in research to clarify definitions as well as develop commercial inoculants using these organisms, with a special emphasis on formulations that interact synergistically and are currently being devised. In addition, numerous recent studies indicate increased crop performance with the use of these commercial inoculants. In this chapter, the progress to date in the use of beneficial microbes for agricultural applications is summarized and discussed.

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_3

3.1 Introduction

Agricultural practices are the major factors underlying many environmental problems, and thus, contemporary agriculture faces enormous challenges (Hazell and Wood 2008). All around the world, agriculture is frequently produced using intensive production methods and indiscriminate agrochemicals that increase production costs and contribute to increased environmental pollution that significantly affects human and animal health (Javaid 2010). Therefore, alternatives to chemical-based conventional agriculture that increase a farmer's profit and that are environmentally safe are required (Singh et al. 2011).

Considering that agricultural productivity is strongly related to microbial activity in the soil system (Chaparro et al. 2014), the use of beneficial microorganisms makes a positive contribution to environmentally safe agriculture (Kloepper et al. 1980; Chanway 1998; Vessey 2003; Gray and Smith 2005; Figueiredo et al. 2012). In addition, the presence of these microorganisms in the soil is a known characteristic of pathogen-suppressive soils (Fliessbach et al. 2009). In biological studies, crustacean chitosan is frequently used to increase plant resistance against pathogens (Goy et al. 2009). On the other hand, fungal chitosan has antimicrobial properties and increases the availability of nutrients (Franco et al. 2011; Berger et al. 2013).

The modes of action of the microorganisms and their various benefits to plants, as described in the literature, range from the simple occupation of biological empty spaces to ecological relationships such as antibiosis, competition, predation, and symbiosis, among others (Kilian et al. 2000; Kloepper et al. 2004; Avis et al. 2008). In addition, activities related to the production of hormones and enzymes, that are important for plants, occur in the soil or phylloplane (Araujo et al. 2005; Raaijmakers et al. 2009). The use of selected microorganisms may represent an important biotechnological approach to decrease the deleterious effects of stress in crops (Egamberdieva et al. 2013; Nadeem et al. 2014).

Studies have also shown that the growth-promoting ability of some bacteria may be highly specific to a certain plant species, cultivar, or genotype (Bashan 1998; Figueiredo et al. 2010). Many of these microorganisms synthesize extracellular polysaccharides or exopolysaccharides (EPS) with a commercially significant applicability (Nwodo et al. 2012). The formulation step is a crucial aspect of producing microbial inoculants, and it determines the success of a biological agent (Brahmaprakash and Sahu 2012).

In recent years, the strong potential of biopolymers to be used as inoculants has been studied (Rodrigues 2012; Bashan et al. 2014). Another possibility for the development of new inoculants or biofertilizers is the use of biofilm (Seneviratne et al. 2009). Furthermore, the role of these compounds in stress adaptation may be an important criterion for the selection of inoculant strains to increase plant productivity through biological nitrogen fixation (BNF) under different soil and climatic conditions (Bomfeti et al. 2011; Sharmila et al. 2014).

3.2 Combinations of Beneficial Microorganisms: Keys for Effective Use in Agriculture

The rhizosphere microbiome consists of three types of microbe groups (Figueiredo et al. 2012; Chaparro et al. 2014): microorganisms that are beneficial to plant growth, plant pathogenic microorganisms, and human pathogenic bacteria. Beneficial microorganisms can fix atmospheric nitrogen, decompose organic wastes and residues, detoxify pesticides, suppress plant diseases and soilborne pathogens, improve nutrient cycling, and produce bioactive compounds that stimulate plant growth (Singh et al. 2011). Thus, the use of beneficial microorganisms present in the rhizosphere is crucial for the development and health of plants and is advantageous to soil fertility (Chaparro et al. 2014). The group of microbes that is beneficial to plants consists of a wide variety of microorganisms, such as

nitrogen-fixing bacteria, plant growth-promoting bacteria (PGPB), and endo- and ectomycorrhizal fungi (Mendes et al. 2013).

Nitrogen-fixing bacteria constitute a microorganism group that carries out BNF, an important process in ecosystems that supplies nitrogen to plants in a utilizable form (Peix et al. 2015). These microorganisms are also called diazotrophic bacteria and may act as free-living bacteria or form a symbiosis with legumes and establish root nodules where BNF occurs (Bonfante and Anca 2009; Chaparro et al. 2014; Peix et al. 2015). Rhizobia, a group of associative diazotrophic bacteria, are often used in combination with other microbes, either bacteria or fungi, to improve the growth and yield of many plant species (Figueiredo et al. 2008, 2010; Hungria et al. 2013; Rodrigues et al. 2013a).

Various bacteria distributed across different genera, such as *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Serratia*, and *Variovorax*, are collectively designated as PGPB due to their noticeable positive effects on plant metabolism (Nadeem et al. 2014). PGPB are commonly used as plant biostimulants (Bhattacharyya and Jha 2012) and increase proliferation and elongation of roots, facilitate nutrient acquisition, modulate plant hormone levels, and act as biocontrol bacteria (Ali et al. 2014; Bashan et al. 2014). Considering these positive effects, PGPB are commonly used to improve crop yields and in combination with rhizobia may constitute an alternative to increase crop plant performance under normal and stress conditions (Figueiredo et al. 2010; Bhattacharyya and Jha 2012; Rodrigues et al. 2013b; Bashan et al. 2014).

Under stress situations, plants trigger defense mechanisms that involve gene expression that is linked with hormonal balance between roots and shoots (Munns and Tester 2008). In this sense, PGPB may provide beneficial effects for plant drought tolerance due to modulation stimulated by hormones, mainly abscisic acid, ethylene, and cytokinins (Bhattacharyya and Jha 2012; Ali et al. 2014). The inoculation of wheat with the PGPB *Azospirillum lipoferum* alleviated plant drought stress and increased the growth and yield (Arzanesh et al. 2011). Drought situations may trigger oxidative stress in plants and induce a decline in nitrogen fixation rates (Arzanesh et al. 2011; Rodrigues et al. 2013b; Larrainzar et al. 2014). To cope with detrimental oxidative situations, a triple inoculation with rhizobia, *Paenibacillus graminis* and *P. durus*, was used in cowpea, which improved symbiotic performance and BNF as well as decreased the deleterious effects of the oxidative stress (Rodrigues et al. 2013a).

The use of selected PGPB may represent an important biotechnological approach to decrease the deleterious effects of salt stress in crops (Egamberdieva et al. 2013; Nadeem et al. 2014). Approximately 45 million hectares of irrigated land worldwide are affected by salinity, which represents a limiting factor to plant growth and reduces crop productivity (Munns and Tester 2008). Under salt stress, the PGPB *Bacillus amyloliquefaciens* promotes the growth of rice plants (Nautiyal et al. 2013). Moreover, positive effects on plant biomass were also observed in maize inoculated with the PGPB *Azotobacter chroococcum* when cultivated under saline stress (Rojas-Tapias et al. 2012). Additionally, the combined action of rhizobia and PGPB *Pseudomonas* spp. alleviated deleterious symptoms of salt stress in *Galega officinalis* and improved root and shoot growth (Egamberdieva et al. 2013).

Among PGPB, *Pseudomonas*, *Bacillus*, and *Azospirillum* are the most studied genera, and of these, *Azospirillum* is considered the most important due to the strong improvement in plant growth (Bashan et al. 2014). *Azospirillum* are free-living PGPB that are able to colonize root surfaces and often possess endophytic ability (Okon and Labandera-Gonzalez 1994). Moreover, these PGPB are capable of positively affecting the yield of many plants growing in different soils and regions, and co-inoculation with other microorganisms improves their beneficial effects (Mendes et al. 2013; Bashan et al. 2014). The co-inoculation of *Azospirillum* with phosphate-solubilizing bacteria allows an improvement in absorption of mineral nutrients

(Singh et al. 2011). Additionally, co-inoculation of *Azospirillum* and rhizobia induces positive responses in plant growth (Star et al. 2012; Hungria et al. 2013).

The rhizobia-PGPB symbiosis is often utilized to improve plant nutrition in terms of macro- and micronutrients. The presence of the microorganisms in the soil can efficiently affect the solubility and therefore the availability of several nutrients (Mendes et al. 2013); this is termed biofertilization (Adesemoye and Kloepper 2009). The use of a biofertilizer containing beneficial microbes, i.e., a mix of *A. chroococcum*, *Bacillus megaterium*, and *Bacillus mucilaginosus*, promoted the growth of maize and ameliorated the soil properties (Wu et al. 2005). In common bean (*Phaseolus vulgaris*), dual and triple inoculations with *Rhizobium*, *Bacillus subtilis*, and *B. megaterium* significantly increased the uptake of macro- and micronutrients (Elkoca et al. 2010). The co-inoculation of rhizobia with phosphate-solubilizing microbes, such as *Aspergillus*, *Pseudomonas*, and *Trichoderma*, increased the nodulation and availability of phosphorus to plants (Yadav et al. 2013).

Trichoderma are filamentous fungi that are widely used in agriculture as biocontrol agents due to their antagonistic abilities against phytopathogenic fungi, mainly *Fusarium* sp. and *Rhizoctonia* sp. (Brotman et al. 2013). Inoculation with *Trichoderma* induces systemic resistance to diseases in many crop plants that results in beneficial effects on plant growth (Hermosa et al. 2012). In Brazil, ten products containing *Trichoderma* are registered and commercially available to control various types of crop diseases. *Trichoderma* strains are frequently applied singly to many plants and induce resistance to biotic and abiotic stresses (Brotman et al. 2013; Colla et al. 2014). Nevertheless, the combination of *Trichoderma* with other microbes potentiates their beneficial effects, as shown by Colla et al. (2014) who studied many vegetable crops that were simultaneously co-inoculated with *T. atroviride* and *Glomus intraradices*, an arbuscular mycorrhizal fungi (AMF).

The term "mycorrhiza" (pl. mycorrhizae) is used to indicate nonpathogenic symbiotic associations between soil fungi and plant roots (Bonfante and Genre 2008). Mycorrhizal symbiosis plays important ecological roles that include the ability to increase nutrient uptake and protect plants from numerous stress situations (Figueiredo et al. 2012). Mycorrhizae are divided into ecto- and endomycorrhizae, and in the latter, the hyphae penetrate the root cells and establish an intracellular symbiosis (Bonfante and Anca 2009). Among the endomycorrhizae, AMF have great importance in tropical ecosystems and are the fungi that are most frequently found in the roots of crop plants (Bonfante and Genre 2008). AMF are a group of soil-dwelling fungi that are obligatory symbionts belonging to the *Glomeromycota* phylum; these fungi have a substantial influence on plant productivity (Redecker et al. 2013).

The positive effects of AMF colonization are commonly attributed to an improvement in nutrient uptake, mainly phosphorus, and to an increased pathogen resistance and herbivore tolerance in plants (Bonfante and Genre 2008; Smith and Smith 2012). Other advantageous effects of AMF-plant interactions are the increase in plant tolerance to stressful environments, phytoremediation promotion, and improvement of soil stability (Meier et al. 2012; Smith and Smith 2012; Estrada et al. 2013). Some rhizobacteria favor the formation of mycorrhizae on roots and are commonly referred to as "mycorrhiza helper bacteria" (MHB) (Frey-Klett et al. 2007). These MHB increase the spore germination and mycelial growth of AMF and are involved in the establishment of AMF-plant symbiosis. The MHB group includes nitrogen-fixing and PGPB (Table 3.1).

MHB aid AMF establishment in roots and the associations between AMF and MHB induce various positive aspects of plant development, mainly increases in the tolerance against drought and salt stress in plants (Gamalero et al. 2009; Lingua et al. 2013). For example, the co-inoculation with a mixture of AMF from the genera *Glomus*, *Gigaspora*, and *Acaulospora* and the rhizobia *Sinorhizobium terangae* resulted in a positive osmotic adjustment and an improved tolerance of *Acacia saligna* to saline soil

Table 3.1 Examples of the mycorrhiza helper bacteria (MHB) associated with arbuscular mycorrhizal fungi (AMF) identified in different host plants (Frey-Klett et al. 2007; Soliman et al. 2012)

Genres and groups of the MHB		Species of the AMF	Host plant
Gram-negative *Proteobacteria*	*Agrobacterium*, *Alcaligenes*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Bradyrhizobium*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rhizobium*, and *Sinorhizobium*	*Endogone* sp., *Acaulospora* sp., *Gigaspora margarita*, *Glomus caledonium*, *G. clarum*, *G. deserticola*, *G. fasciculatum*, *G. fistulosum*, *G. intraradices*, and *G. mosseae*	*Medicago sativa*, *Morus alba*, *Carica papaya*, *Zea mays*, *Solanum tuberosum*, *Allium cepa*, *Anthyllis cytisoides*, *Hordeum vulgare*, *Triticum aestivum*, *Trifolium* sp., *Acacia saligna, Cucumis sativum*, *Pennisetum americanum*, *Ipomea batatas*, *Uniola paniculata*, and *Licopersicum esculetum*
Gram-positive *Firmicutes*	*Bacillus*, *Brevibacillus*, and *Paenibacillus*	*G. etunicatum*, *G. fasciculatum*, *G. mosseae*, and *G. intraradices*	*Sorghum bicolor*
Gram-positive actinomycetes	*Streptomyces*	*Glomus intraradices*	*Sorghum* sp.

(Soliman et al. 2012). In cowpea co-inoculated with rhizobia, AMF *Glomus etunicatum* and the PGPB *Paenibacillus brasiliensis*, it was observed that AMF positively affected the nitrogen acquisition by rhizobia and increased the symbiotic efficiency, which enhanced plant growth (Lima et al. 2011). For all the above mentioned reasons, the use of beneficial microbes must be increased and disseminated considering that this is a sustainable and environmentally friendly agricultural practice.

3.3 Prospective Biocontrol Agents of Plant Diseases

Beneficial microorganisms have been widely used in agriculture to control diseases and pests of different plant species, in addition to other benefits they provide to plants. Within this group, we can highlight the bacteria of the genera *Bacillus*, *Azospirillum*, *Pseudomonas*, and *Rhizobium* and fungi of the genera *Beauveria*, *Gliocladium*, *Metarhizium*, and *Trichoderma*, as well as actinomycetes of the genus *Streptomyces*.

Within the microorganism groups in the soil, rhizobacteria can be highlighted, which develop a close relationship with plants and in most cases occupy the space known as the rhizosphere (Raaijmakers et al. 2009). In this environment, which is rich in sugars, amino acids, flavonoids, proteins, and fatty acids (Badri et al. 2009), there is a higher fungal and bacterial presence because these microorganisms are attracted by these compounds. However, there occurs a greater prevalence of rhizobacteria, which in most cases are able to form a biofilm that protects the root surface against pathogens (Bogino et al. 2013).

The maintenance of these beneficial microorganisms is supported by the host plant; the microorganisms drain carbon-rich compounds (exudates) produced by the roots that are essential for microbial nutrition in the rhizosphere (Vandenkoornhuyse et al. 2007). However, exudates may also act as antimicrobial molecules, inhibiting the growth of some species. This indicates that the host plant can select or attract microorganisms for their rhizosphere colonization (Chaparro et al. 2012), which has led to the occurrence of plant species that respond more to the presence of growth-promoting microorganisms in the soil.

Some practices, such as tillage, organic fertilization, crop rotation, and residue management, can also increase or decrease microbial activity in the rhizosphere (Raaijmakers et al. 2009). Chemical factors, such as pH and organic matter nutrient availability, are considered important

factors for maintaining microbial activity (Chaparro et al. 2012).

The presence of beneficial microorganisms in the soil is a known characteristic of pathogen-suppressive soils. These soils can maintain conditions favorable for diverse microfauna, preventing the prevalence of pathogens. It has also been found that soils rich in microbial biomass and diversity have been less responsive to microbial inoculant introduction, indicating that this introduction is more suitable for poor soils in this regard (Fliessbach et al. 2009).

Recent studies indicate that plants that interact with rhizobacteria are able to increase their photosynthetic capacity (Xie et al. 2009), soil salinity tolerance (Dimkpa et al. 2009), disease suppression, and iron absorption efficiency (Zhang et al. 2009). These results confirm the potential use of inoculants containing rhizobacteria in agriculture. Plants play a critical role in influencing the microbial community composition of the rhizosphere. Some species can attract larger amounts of microorganisms by releasing signaling substances in the rhizosphere. It has also been observed that depending on the plant growth stage, the signaling substances can influence the microbial community structure present in the rhizosphere (Chaparro et al. 2014).

The multiple beneficial effects related to rhizosphere microorganism activities are well explained by the diagram presented in Fig. 3.1. In this representation, it can be seen that microorganisms considered to possess biological control activity can play indirect roles in growth promotion, such as increasing the supply of macro- and micronutrients to plants. Moreover, it has been found that nitrogen-fixing species, such as *Rhizobium* sp., can play a role as biological control agents (Avis et al. 2008).

The main plant growth promotion forms are based on three important actions, i.e., phytostimulation, biofertilization, and pathogen control. Some microbial species exhibit good development of these three activities in the soil, indicating good potential for the market. Some of these species and the previously mentioned activities described in the literature are presented in Table 3.2.

In the case of *B. subtilis*, pathogen control is performed by different modes of action,

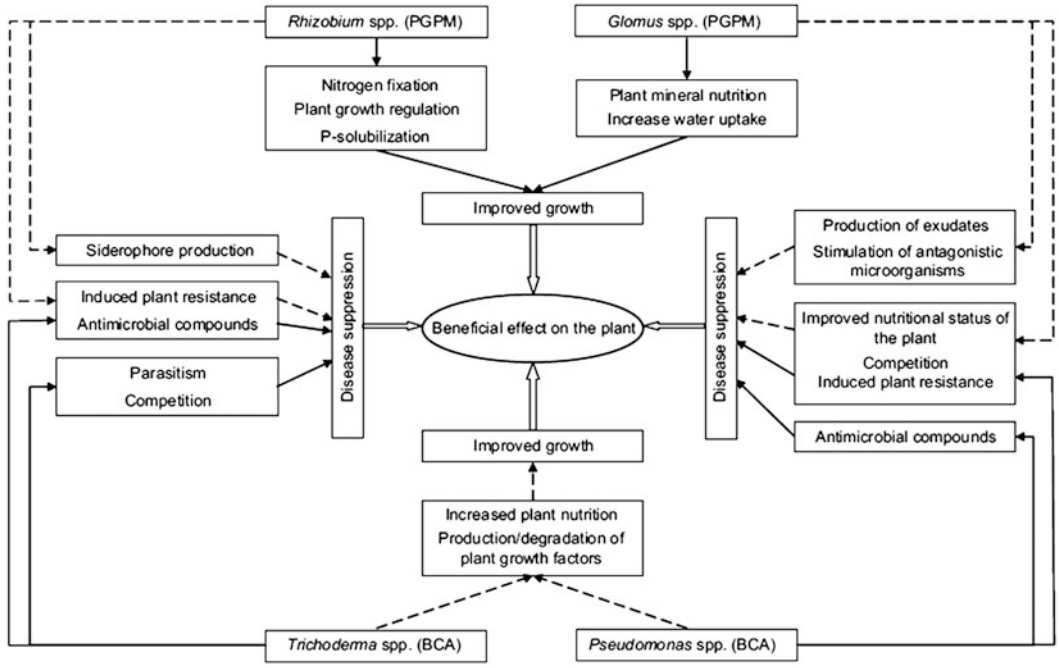

Fig. 3.1 Potential modes of action of plant growth-promoting and biological control agents (Avis et al. 2008)

Table 3.2 Simultaneous actions developed by different species of soil microorganisms within the beneficial activities for plants

Species	Biofertilization	Phytostimulation	Pathogen control
Azospirillum brasilense[a]	Nitrogen fixation	Auxins and gibberellins	Induced systemic resistance (ISR) and siderophores
B. subtilis[b]	Increase in phosphorus and nitrogen	Auxins	Antibiotics
Pseudomonas fluorescens[c]	Increase in phosphorus	Auxins	2,4-diacetyl-phloroglucinol
Streptomyces griseus[d]	Increase in phosphorus	Auxins	Chitinases
Trichoderma harzianum[e]	Increase in phosphorus and nitrogen	Auxins	Mycoparasitism and enzymes

[a]Dobbelare et al. (1999)
[b]Araujo et al. (2005) and Araujo (2008)
[c]Raaijmakers et al. (1997), Fernández et al. (2012), and Pallai et al. (2012)
[d]Hamdali et al. (2008)
[e]Contreras-Cornejo et al. (2009), Molla et al. (2012), and Vos et al. (2014)

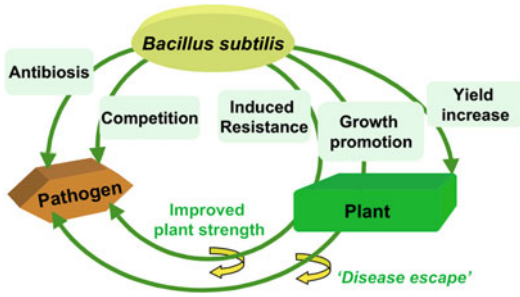

Fig. 3.2 Modes of action of *B. subtilis* and the interaction between *Bacillus*-plant pathogen (Kilian et al. 2000)

especially antibiosis, competition for ecological niches, and resistance induction in the host (Fig. 3.2). These main modes, in addition to some secondary modes, provide effective control of pathogens and reduce the possibility of the pathogens developing resistance. These secondary modes of action reinforce the immense potential that bioinoculants have in agriculture. The applications of different mixtures of rhizobacterial species to the soil can result in systemic resistance induction, which increases the efficiency against various pathogens (Ramamoorthy et al. 2001). Taking into account both the primary and secondary benefits related to the microorganisms mentioned above (Table 3.2), the addition of multiple microorganisms to agricultural production systems appears attractive (Avis et al. 2008). Many of the individual effects of co-inoculated microorganisms have been enhanced, resulting in synergistic effects in many cases (Kohler et al. 2007).

At present, the use of bacterial consortia in bioinoculant formulation has been highlighted with good prospects for the future, which may reflect multiple benefits in crop yield and the biological balance of the soil (Pindi 2012). This line of action of combining different microbial species with multiple functions into one product was recently used for the development of so-called biofilms (Triveni et al. 2012). In this case, there is the possibility of greater microorganism protection and survival with the use of the final product. The formulation of biofilms typically involves the combination of fungi and bacteria in a product developed in the laboratory. However, the analysis of this new technology is limited in recent studies that have examined the use and development of new inoculants.

Currently, more than 78 brands of products with a microbiological origin are being used in agriculture, covering more than 5 million ha. Some of these products are in the registration process or are marketed only to the organic agricultural market. It is also noted that most of these products are related to pest control. Worldwide, several commercial products developed using rhizobacteria are on the market and are mainly indicated for plant disease control (Table 3.3).

Table 3.3 Commercial products developed using different PGPR species

Species	Products	Intended crop
B. subtilis	Epic, Kodiak, Rhizo-Plus, Serenade, and Subtilex	Fruit, cereals, ornamentals, trees, and forage crops
P. fluorescens	BlightBan, Conquer, and Victus	Apple, cherry, peach, potato, strawberry, and tomato
T. harzianum	Custom and RootShield	Turf and ornamentals
A. brasilense	Azo Green	Forage crops
S. griseus	Mycostop	Ornamental and vegetable crops

The prospects for the future market are very promising due to a greater receptivity of organic products by farmers and also due to higher environmental and health restrictions on the registration and authorization of conventional chemicals used for disease control in plants. In this context, organic products are used more often in integrated control strategies.

3.4 Microbial Polysaccharides: Production and Application as a Vehicle for Inoculation

The term "microbial polysaccharides" includes polysaccharides produced by fungi and bacteria, which may be located inside the cell, on the cell wall or yet be released out of the cell (Donot et al. 2012). Polysaccharides are classified by the sugar composition in homopolysaccharides, which enclose a single type of monosaccharide and are usually neutral glucan, and heteropolysaccharides, which are composed of different sugar residues and usually display a regular backbone structure with a linear or branched repetitive unit (Delbarre-Ladrat et al. 2014). Heteropolysaccharides may also contain organic or inorganic substituents, such as phosphate, sulfate, acetate, and pyruvate, which confer polyanionic characteristics to these molecules (Schmid et al. 2011). Collectively, the sugar sequence in the molecule, the presence or absence of substituents, and how the chains intertwine influence the physicochemical features of polysaccharides (Rehm 2010).

Some bacteria and fungi secrete polysaccharides as an evolutionary adaptation to help them adhere to different surfaces or for adaptation to stressful environments (Sharmila et al. 2014). Polysaccharides protect against dehydration, serve as barriers to prevent viruses and antibodies from binding to specific sites on the cell wall, neutralize toxins, act as a carbon source, and also interact with plant cells in specific pathogenic or symbiotic relationships (Badel et al. 2011; Donot et al. 2012). In addition, microbial polysaccharides, especially those released to the external environment, have been reported to protect microbial cells against heavy metals or environmental stresses (Poli et al. 2011). For example, *Pseudomonas* strains survive under stress conditions due to polysaccharides released to the outside of the cell, which protect them from water stress and then regulate the diffusion of carbon sources in the microbial environment (Sandhya et al. 2009).

In addition to singular biological characteristics, polysaccharides exhibit chemical and physical properties, and these peculiar features represent advantages compared with plant polysaccharides for their use at large scales (Mahapatra and Banerjee 2013). Polysaccharides are generally nontoxic, biodegradable compounds (Rehm 2010) and are oftentimes named according to the biopolymers or gums. In contrast to traditional gum, microbial polysaccharide production is advantageous because it is more rapid and requires less space for manufacture (Badel et al. 2011). Among the polysaccharide types, the extracellular polysaccharides or EPS are the most interesting due the possibility of recovering these directly from the environment in which they are secreted, enabling higher productivity. Fungi and bacteria release EPS in extracellular media, and these EPS are useful to promote cell-cell recognition (Donot et al. 2012).

Table 3.4 Some extracellular polysaccharides (EPS) produced by fungi or bacteria with commercial and economic importance (Freitas et al. 2011; Delbarre-Ladrat et al. 2014)

EPS	Microorganism	Application
Homopolysaccharides		
Curdlan	*Agrobacterium*, *Rhizobium*, and *Alcaligenes*	Food additive, applied in pharmaceutical industries, bioremediation
Dextran	*Leuconostoc* and *Streptococcus*	Food industry, biomedical as plasma volume expander, biotechnological support for separation
Pullulan	*Aureobasidium pullulans*	Food, adhesive, and cosmetic additives; flocculant; thickener; viscosity stabilizer in preparation of nontoxic, biodegradable, edible plastic materials; health care
Scleroglucan	Fungi of the genus *Sclerotium*	Oil, ink, cosmetic, and pharmaceutical industries; animal feed; food additive
Heteropolysaccharides		
Alginate	*Pseudomonas aeruginosa* and *Azotobacter vinelandii*	Food hydrocolloid, wound care, drug encapsulating agent
Gellan	*Sphingomonas paucimobilis* and *Pseudomonas elodea*	Gelling in culture medium, food additive
Succinoglycan	*Alcaligenes, Agrobacterium*, and *Rhizobium*	Food and pharmaceutical industries, oil recovery
Xanthan	*Xanthomonas campestris*	Viscosifying and texturizing agent in various foods, used for oil recovery in petroleum industry, health care

Many microorganisms synthesize EPS with commercially significant applications (Nwodo et al. 2012). Biopolymers derived from natural resources, such as microbial EPS, have a competitive advantage due their sustainable production, biodegradability, and, often, biocompatibility (Rehm 2010). Due to their ability to alter the rheology of aqueous solutions and their potential use as a biomaterial, EPS possess great economic importance (Table 3.4) and wide applicability in various industry sectors with significant commercial value (Freitas et al. 2011; Finore et al. 2014). EPS show huge production in a short time as well as ease of isolation and purification in relation to intracellular and cell wall polysaccharides (Delbarre-Ladrat et al. 2014). They can function as thickeners, gelling, emulsifiers, stabilizers, lubricants, or as suspending agents and film formers (Freitas et al. 2011; Mahapatra and Banerjee 2013).

Although they exhibit a wide range of biological functions, the production of EPS by fungi remains poorly studied (Giavasis 2014). The EPS produced by fungi are frequently highly hygroscopic β-glucans (Mahapatra and Banerjee 2013; Sharmila et al. 2014) that possess numerous applications in the food and pharmaceutical industries. Basidiomycetes, filamentous fungi, and yeasts from different ecological niches are known as being capable of synthesizing EPS in different production systems (Delbarre-Ladrat et al. 2014). The production of fungal EPS depends on the fungal strain used and the physicochemical conditions applied inside the fermentative broth (Mahapatra and Banerjee 2013). Collectively, the oxygen levels, carbon and nitrogen sources, pH, and temperature influence the production and composition of fungal EPS (Badel et al. 2011; Mahapatra and Banerjee 2013; Zhang et al. 2013; Giavasis 2014).

Scleroglucan, schizophyllan, and pullulan are types of fungal EPS commonly produced at a large scale and that possess singular rheological properties. Scleroglucan is an extracellular glucan excreted by *Sclerotium glucanicum* or *S. rolfsii* that is used frequently in petroleum recovery and has great potential for use in the agricultural, food, and pharmaceutical industries (Ansari et al. 2012). Schizophyllan is an EPS secreted by the basidiomycete *Schizophyllum commune* that is used in many fields, such as the food industry and pharmaceuticals (Zhang et al. 2013). Pullulan is a water-soluble glucan gum commonly used as a food additive and is

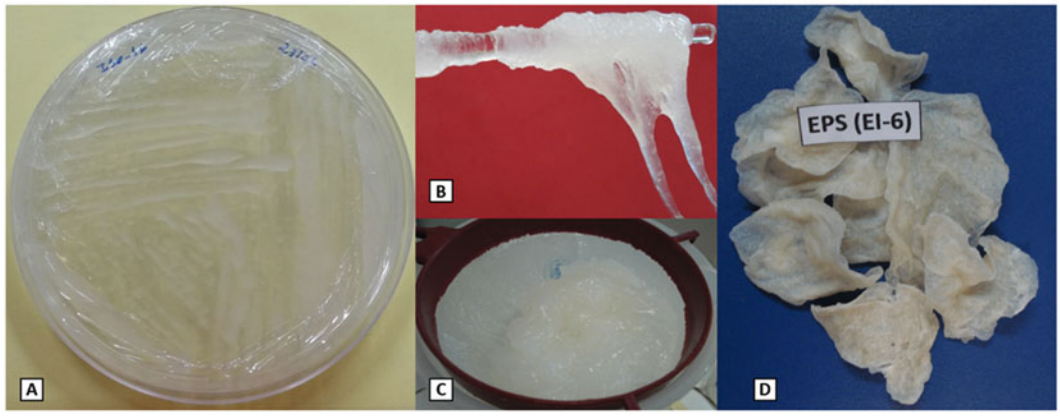

Fig. 3.3 Exopolysaccharides (EPS) produced by the EI-6 strain of *R. tropici*: (**a**) culture medium showing the EPS production, (**b**) EPS recovered from the fermentation broth, (**c**) EPS washed with 70 % alcohol, (**d**) dry EPS after 3 days in the stove at 30 °C (Photos courtesy of Artenisa Cerqueira Rodrigues)

produced by *Aureobasidium pullulans* (Singh et al. 2008). This compound has adhesive properties and the ability to form fibers and thin biodegradable films that are transparent and waterproof to oxygen because of their physical properties and distinctive linkage patterns (Cheng et al. 2011).

Researchers have extensively studied bacterial EPS due their structural variability that offers a large range of physicochemical and biological properties (Badel et al. 2011; Poli et al. 2011). Numerous bacterial EPS were reported in recent decades as biopolymers with industrial importance and significant commercial value, particularly xanthan, which occupies a prominent place in the market by presenting very different and unusual rheological properties (Freitas et al. 2011). Many soil bacteria from different habitats are recognized to produce complex and diverse EPS, such as *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Sinorhizobium* strains (Albareda et al. 2008; Staudt et al. 2012; Castellane et al. 2014). In a biological context, bacterial EPS are necessary to mediate *Rhizobium*-legume symbioses and are indispensable to promote the formation of efficient nodules on different hosts (Bomfeti et al. 2011; Castellane et al. 2014).

The viability of rhizobia in the field is maintained by their production of EPS (Staudt et al. 2012), and EPS can protect nitrogenase enzyme involved in the nitrogen-fixing process from high oxygen concentration in the nodules (Vu et al. 2009). Rhizobia are among the well-known EPS producers and can excrete large amounts of these polysaccharides in the rhizosphere (Albareda et al. 2008; Serrato et al. 2008; Bomfeti et al. 2011; Nwodo et al. 2012; Castellane et al. 2014) or culture medium (Fig. 3.3). Rhizobial inoculants have been used for many years to obtain greater yields in legumes and are often produced with peat, a material with a fossil origin (Rivera et al. 2014). Peat mines are situated in preserved environments where extraction is forbidden, and a lack of natural peat deposits exists in some countries (Albareda et al. 2008). The use of peat as a bacterial carrier, although established, has decreased, and alternative materials are being sought.

Alternative materials for bacterial inoculation can maintain the quality and efficiency of the inoculum and reduce production costs and negative environmental impacts compared with peat inoculants (Fernandes Júnior et al. 2009, 2012; Herrmann and Lesueur 2013; Rivera et al. 2014). For example, the combination of carboxymethylcellulose (CMC) and starch forms a polymeric carrier that adequately maintains rhizobial cell viability and has the same performance as peat inoculants (Fernandes Júnior et al. 2009, 2012). Rivera et al. (2014) tested eight polymers to use

Table 3.5 Number of nodules and amount of nitrogen accumulated in the shoots of cowpea plants inoculated with strain BR 3267 of *Bradyrhizobium* sp. using different inoculants (carriers): peat, a combination of the carboxymethylcellulose (CMC) and starch, and EPS produced by the EI-6 strain of *R. tropici*

Carrier	Number of nodules (nodule plant^{-1})	Nitrogen in shoot dry mater (mg N SDM^{-1})
Peat[a]	78.0	78.7
Combination of CMC and starch[a]	77.0	92.0
EPS from by *R. tropici* (EI-6)[b]	94.0	145.2

[a]Fernandes Júnior et al. (2009)
[b]Rodrigues (2012)

as vehicles for bacterial inoculation, and among them, sodium alginate and hydroxypropyl methylcellulose (HPMC) were able to provide a higher viability for strain G58 of *Rhizobium* sp. in comparison to the control (peat), without affecting the physiological process of nodulation in cowpea roots.

CMC and HPMC are competent bacterial inoculants; however, these compounds are semisynthetic polymers (Fernandes Júnior et al. 2012; Rivera et al. 2014). Because of their biological nature, EPS synthesized by rhizobia are currently considered as excellent carriers for inoculation (Table 3.5). EPS can encapsulate living bacterial cells and gradually release these to the soil in large quantities, which confirms that EPS are degraded by soil microorganisms (Albareda et al. 2008). *Rhizobium leguminosarum*, *R. meliloti*, and *R. tropici* (SEMIA 4080 and EI-6 strains) are known to produce significant amounts of rhizobial EPS (Rodrigues 2012; Rodrigues et al. 2013a, b; Castellane et al. 2014). The composition of the EPS produced by *R. tropici* SEMIA 4080 shows relatively higher contents of glucose and galactose combined with small amounts of mannose, rhamnose, and glucuronic and galacturonic acids (Castellane et al. 2014).

The EI-6 strain of *R. tropici* was first isolated from root nodules of cowpea (*Vigna unguiculata*) cultivated in an arid environment (Figueiredo et al. 1999; Oliveira et al. 2012) and is known to produce significant amounts of EPS with good quality (Xavier 2009; Santos 2010; Oliveira 2011; Rodrigues 2012). The EPS produced by the EI-6 strain of *R. tropici* (already shown in Fig. 3.3.) are a polysaccharide of glucose and galactose without the presence of uronic acids (Rodrigues 2012). Additionally, the EPS possess acetyl and pyruvate residues and a higher amount of sodium and potassium ions (Xavier 2009; Rodrigues 2012). This feature resulted in the EPS produced by *R. tropici* EI-6 being classified as polyanionic heteropolysaccharides (Rodrigues et al. 2013a). Currently, after intensive studies on *R. tropici*, it is possible to confirm that their EPS have high potential for use in agriculture.

3.5 Biofertilizers: Agricultural Innovations

Soluble fertilizers are of great importance for plant growth and production, but their use by low-income farmers is limited due to the high price. Furthermore, the fertilizers contain high amounts of soluble nutrients and may easily undergo lixiviation in the deeper soil layers and promote plant damage through soil and water contamination (Van Straaten 2007). In modern and sustainable agriculture, the application of slowly soluble fertilizers is required to economically increase food production and soil fertility while minimizing environmental damage (Stamford et al. 2008). Biofertilizers produced from phosphate and potash rocks mixed with elemental sulfur and inoculated with sulfur-oxidative bacteria (*Acidithiobacillus*) may be an alternative for effective and economic fertilization and have considerable influence on the availability of elements derived from rocks through the metabolic effect of sulfuric acid (Stamford et al. 2008), as shown in the equation below:

$$S + H_2O + 1.5\ O_2 \xrightarrow{Acidithiobacillus} H_2SO_4$$

Nitrogen (N) is one of the most important nutrients for plant growth and yield, and due to its role in some chemical compounds such as proteins, nucleic acids, and many other components, it is necessary for all types of life in the world (Berger et al. 2013). However,

phosphorus (P) and potassium (K) rock biofertilizers do not contain nitrogen for plants and microbial organisms in the soil, and the production of sulfuric acid may reduce soil pH, which damages and reduces plant growth. Therefore, rock biofertilizers can be mixed with organic matter (e.g., earthworm compound) that has a high pH (pH 7.9) and inoculated with selected free-living diazotrophic bacteria to promote N enrichment through the process of BNF (Lima et al. 2010).

In biological studies, crustacean chitosan is frequently used to increase plant resistance against pathogens. The chitosan displays better chelating properties compared with other polymers and can release nutrients into the environment (Boonlertnirun et al. 2008; Goy et al. 2009). The use of chitosan from fungal biomass has great advantages compared with crustacean chitosan, such as the independence of seasonal factors and the simultaneous extraction of chitin and chitosan (Franco et al. 2004). Mixed biofertilizers (NPKB) provide nutrients for plants, especially when inoculated with fungal chitosan such as *Cunninghamella elegans,* which also increases inorganic phosphate (Franco et al. 2004). Furthermore, the bioprotector (NPKP) has antimicrobial properties, promotes plant protection against pathogens, and increases the availability of nutrients (Berger et al. 2013; Franco et al. 2011).

3.5.1 Production of Biofertilizer (NPKB) and Bioprotector (NPKP)

The biofertilizer (NPKB) was produced from phosphate and potassium rock biofertilizers (PKB) mixed with organic matter (OM) in a 1:3 ratio and incubated for 30 days. The PK rock biofertilizers were produced at the Horticultural Experimental Station of the Federal Agricultural University of Pernambuco (UFRPE). Two furrows (10 m long, 1.0 m wide, 0.5 m deep) were used following the procedure described by Stamford et al. (2007).

The sulfur-oxidative bacteria were grown in Erlenmeyer flasks that contained 1000 mL of culture-specific medium (El Tarabily et al. 2006) and sterilized for 30 min at 120 °C. The Erlenmeyer flasks were shaken (150 rpm) for 5 days at 30 °C. The materials (phosphate and potash rocks plus elemental sulfur) were incubated for 60 days, and the humidity was maintained at a level close to field holding capacity. To avoid excessive humidity due to rain and to increase the efficiency of the sulfur-oxidative bacteria, the furrows were covered with black plastic (Fig. 3.4).

The biofertilizer (NPKB) was processed by mixing PK rock biofertilizers with organic biofertilizer (earthworm compound) enriched in N by inoculation with selected free-living diazotrophic bacteria (NFB 10001), as reported by Lima et al. (2010). The bioprotector (NPKP) represents the biofertilizer (NPKB) inoculated with *C. elegans* (UCP 542), which are fungi that contain chitosan in their cellular wall (Franco et al. 2004). The *C. elegans* fungus was purified in potato dextrose agar medium and grown for 10 days at 28 °C. A monosporic culture of *C. elegans* was obtained by growing the *Mucorales* fungus in potato dextrose medium using Erlenmeyer flasks (containing 1000 mL) maintained under shaking conditions (180 rpm, 96 h, 28 °C). The culture diluted in distilled water (20 L^{-1}) was applied using manual irrigation. The chemical differences between the P and K biofertilizers and the biofertilizer (NPKB) obtained following the procedure of Stamford et al. (2007) are presented in (Table 3.6).

The results of several experiments carried out in Brazilian soils cropped with different economic cultures such as cowpea, yam bean, grapes, melon, sugarcane, green pepper, and tomato showed that the biofertilizer (NPKB) and bioprotector (NPKP) increased crop performance and soil nutrients compared with the mineral soluble fertilizer. The NPKB and NPKP may be alternatives to replace conventional NPK soluble fertilizer and pesticides.

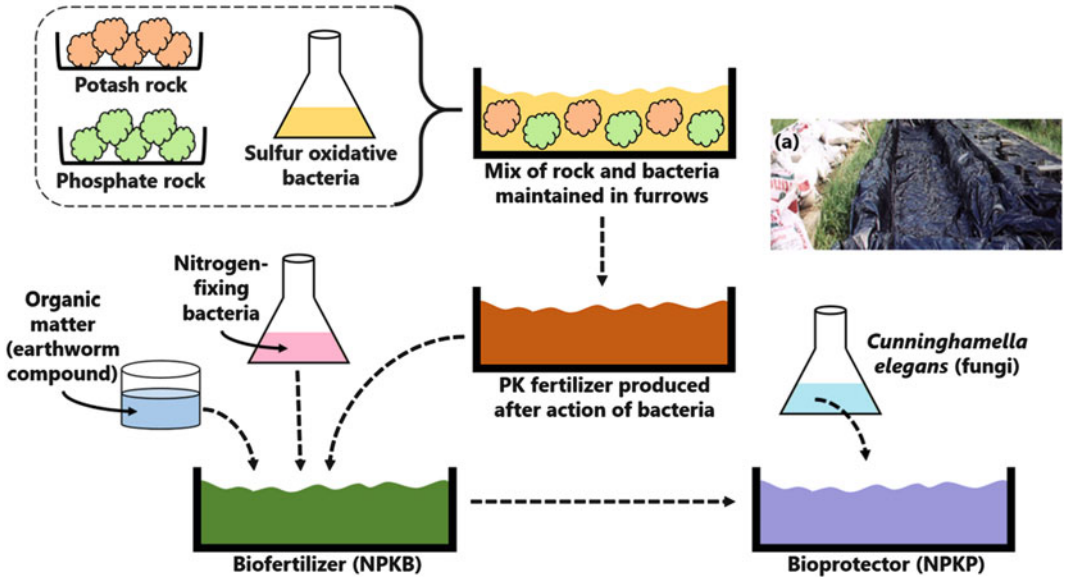

Fig. 3.4 Simplified schematic of the production of the biofertilizer (NPKB) and bioprotector (NPKP). The furrows covered with *black* plastic under field conditions are shown in (**a**)

Table 3.6 Chemical analyses of the P and K biofertilizers produced with sulfur-oxidative bacteria and of the biofertilizer (NPKB) after inoculation with selected free-living bacteria and mixed with organic matter as described in the text

Biofertilizer type	pH	Phosphorus (P)	Potassium (K)	Nitrogen (N)
P biofertilizer	3.8	60.0 g kg^{-1}	–	–
K biofertilizer	3.3	–	10.0 g kg^{-1}	–
PK rock after bacterial inoculation (NPKB)	6.4	21.0 g kg^{-1}	19.0 g kg^{-1}	20.0 g kg^{-1}

3.6 Conclusion

Beneficial microorganisms can be a potential tool for sustainable agriculture as well as a trend for the future. The beneficial effects include biological control of diseases, promotion of plant growth, increases in crop yield, and quality improvement. Knowledge of the complex environment of the rhizosphere, the mechanisms of action, and the practical aspects of inoculant formulation using these microorganisms is important in the search for new products to increase crop performance. New biotechnological methods for crop protection are based on the use of beneficial microorganisms applied as biofertilizers and bioprotectors. This approach represents an important tool for plant disease control and can lead to a substantial reduction of synthetic chemical products, which are important source of environmental pollution. Future studies are needed to identify management conditions that can contribute to the optimization of several mechanisms of the plant-microorganism interrelationship in real-life agricultural systems.

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The Production and Potential of Biofertilizers to Improve Crop Yields

4

Didier Lesueur, Rosalind Deaker, Laetitia Herrmann, Lambert Bräu, and Jan Jansa

Contents

Abstract

Extensive interactions of plant roots with soil microorganisms affect plant nutrition either directly by influencing mineral nutrient availability or indirectly through enhanced uptake efficiency via plant root growth promotion. Beneficial microbial interactions with roots may be either endophytic or associative and can be symbiotic, mutualistic, or incidental in nature. The increased understanding of the role of root – or rhizosphere –associated with microbes in the nutrition and/or yield of agricultural crops in particular has resulted in promotion of their use in agricultural production as alternatives or supplements to mineral or organic fertilizers. Despite this, there is an obvious lack of market penetration of microbial inoculants. This review specifically focuses on microbial inoculants, collectively termed biofertilizers, used to improve nutrition and yields of grain, legume, oil, tuber,

D. Lesueur (✉) • L. Herrmann
CIRAD, UMR Eco&Sols – Ecologie Fonctionnelle & Biogéochimique des Sols & Agroécosystèmes (SupAgro–CIRAD–INRA–IRD), Land Development Department, Office of Science for Land Development, Paholyothin Road, Chatuchak, Bangkok 10900, Thailand

School of Life and Environmental Sciences, Faculty of Science and Technology, Deakin University, 221 Burwood Hwy, Burwood, Victoria 3125, Australia
e-mail: didier.lesueur@cirad.fr; dllesueurdidier@gmail.com

R. Deaker
Faculty of Agriculture and Environment, University of Sydney, Level 4 Biomedical Building, 1 Central Ave, Eveleigh, NSW 2015, Australia

L. Bräu
School of Life and Environmental Sciences, Faculty of Science and Technology, Deakin University, 221 Burwood Hwy, Burwood, Victoria 3125, Australia

J. Jansa
Institute of Microbiology, Academy of Sciences of the Czech Republic, Vídeňská 1083, Praha 4 – Krč, Czech Republic

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_4

and other crops. A vast number of commercial biofertilizers are available worldwide; however, the quality and efficacy of many of them are not proven or tested. In the absence of efficacious biofertilizers of good and consistent quality, the dependence on the use of mineral fertilizers is not likely to decrease. Thus the availability of high-quality biofertilizers must be priority particularly in countries where crop plant production plays a key role in the economy and food security.

4.1 Introduction

4.1.1 Rhizosphere: Strong Interactions Between Plant Roots and Soil Microorganisms

The small volume of soil surrounding and under the direct influence of plant roots is called the "rhizosphere" and sustains great portion of life on Earth. Soil has the highest microbial diversity on Earth with thousands of species of prokaryotes and hundreds of species of eukaryotes in each gram. The rhizosphere probably represents the most dynamic habitat and the most important zone in terms of defining the quality and quantity of our terrestrial food resource (Hinsinger et al. 2009). Plant roots interact with the physical, chemical, and biological properties of soil and encounter an enormous variety of organisms in the rhizosphere (Roesch et al. 2007). Structural and functional characteristics of roots contribute to so-called rhizosphere processes that have a significant influence on the capacity of plants to acquire nutrients (Vacheron et al. 2013; Lamont et al. 2014). Extensive interactions of roots with soil microorganisms further affect plant nutrition either directly by influencing mineral nutrient availability and/or uptake or indirectly through plant (root) growth promotion enhancing uptake efficiency (Richardson et al. 2009). These "root–microorganism" interactions are ubiquitous across various trophic levels and are essential components of ecosystem functions. It has become increasingly evident that they are intricate and involve highly complex communities that function in very heterogeneous environments (Giri et al. 2005).

Microorganisms are a significant component in cycling/recycling of mineral nutrients and carbon in the soil and through their activities; nutrients can become more (through solubilization, mineralization) or less (through adsorption, immobilization) available to plants (Morgan et al. 2005; Leake et al. 2006). Through associations with a range of microorganisms, plants may also be protected from a variety of biotic and abiotic stresses including interplant competition (Morgan et al. 2005; Sanon et al. 2009; Smith et al. 2010). Beneficial microbial interactions with roots may be either endophytic or associative and can be symbiotic, mutualistic, or incidental in nature. Figure 4.1 shows the main interactions between plants and soil microorganisms. A few organisms, such as nodule-forming rhizobia and mycorrhizal fungi, have developed intimate symbiotic relationships with plants. Arbuscular mycorrhizal fungi (AMF) have become completely dependent on plant carbon and are unable to complete their life cycle without association with their host plant (van der Heijden et al. 2015). Other microorganisms are generally less dependent on the plant for carbon source, though in many instances, they derive a significant portion of their carbon from the plants (Drigo et al. 2010; Leake et al. 2006).

Plant-associated microorganisms trade plant carbon for mineral nutrients gathered in the soil. Examples include the mycorrhiza-assisted plant uptake of nutrients such as phosphorus (P) and zinc (Zn) that normally have low diffusion rates in the soil, plant sulfur (S) supply by both bacteria and mycorrhizal fungi, or nitrogen (N) transfer to plants after fixation from the atmospheric N_2 pool (Lendenmann et al. 2011; Gao et al. 2012; Smith and Smith 2012; Gahan and Schmalenberger 2014). Plant growth benefits are not only achieved through improved availability of limiting nutrients such as N and/or P but also through hormonal and enzyme-induced

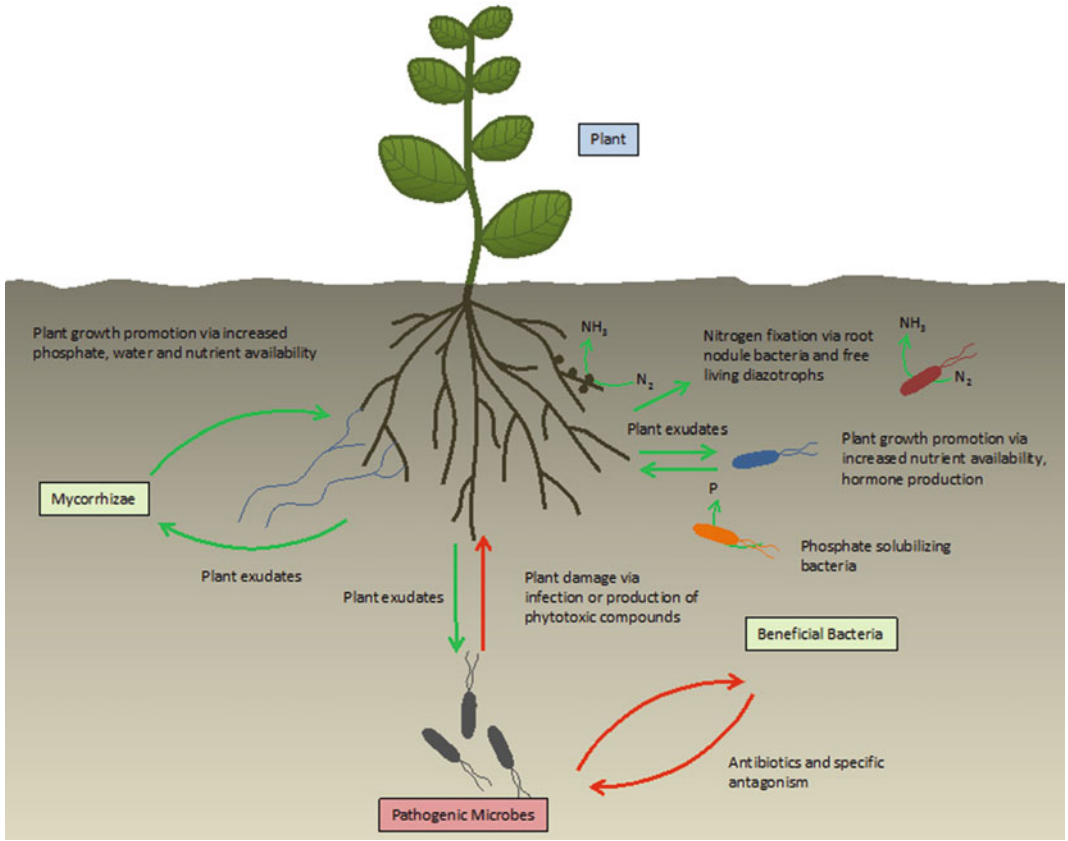

Fig. 4.1 Main beneficial interactions between plants and soil microorganisms (Figure modified from Richardson et al. 2009)

enhancement of root growth which improves nutrient uptake efficiency (Glick 2012; Hayat et al. 2010). Halpern et al. (2015) reviewed the mechanisms involved in the enhancement of plant nutrient uptake by various biostimulants. Biostimulants are defined as substances or materials except nutrients and pesticides which, when applied to seeds or plants, have the capacity to modify physiological processes in plants in a way that provides potential benefits to growth, development, or stress response. Included in this group are the plant growth-promoting rhizobacteria (PGPR) that facilitate atmospheric N_2 fixation and P and iron solubilization and can also induce changes in root morphology.

Microbes have also been shown to reduce the incidence of plant diseases by acting as antagonists of root pathogens or by inducing systemic resistance in plants (Babalola 2010; Choudhary et al. 2011; Singh et al. 2011). Berendsen et al. (2012) described that upon pathogen or insect attack, plants are able to recruit protective microorganisms and enhance microbial activity to suppress pathogens in the rhizosphere. As shown by Yang et al. (2009), PGPR can also elicit an "induced systemic tolerance" to abiotic stresses such as salinity and drought and provide protection to maintain plant health. Other mechanisms are likely to be involved in the PGPR–plants interactions but are still largely unexplored. Recently, Palacios et al. (2014) highlighted the potential role of vitamins in the interactions of plants with PGPR that would also require a deeper exploration.

This chapter will focus on microbial inoculants that influence plant nutrition and grain yields, collectively termed biofertilizers, and not on microbially mediated disease

suppression in plants that has been reviewed extensively elsewhere (Howell 2003; Harman et al. 2004; Compant et al. 2005; Avis et al. 2008; Berg 2009; Dutta and Podile 2010; Saharan and Nehra 2011).

4.1.2 The Potential of Soil Microorganisms to Improve Plant Nutrition

N and P are the most important and often the two most limiting mineral nutrients for living organisms. In terrestrial ecosystems, P is often considered as the most limiting nutrient, frequently more than N. Although most agricultural soils contain large amounts of inorganic and organic P, most of it is unavailable for plants. Even following the addition of P fertilizers to soils, plants may not access enough P as it is easily immobilized, becoming sparingly soluble and less accessible to plants. Hence, only a very small portion of soil P can be directly assimilated by plants and many soils are unable to supply adequate P to P-demanding crops (Bünemann et al. 2011; Dumas et al. 2011).

Economically mineable P deposits are finite and a better management of the P cycle is becoming increasingly important (Cordell et al. 2009). The world's main source of P is rock phosphate, a nonrenewable resource, and the mining and trading of rock phosphate contributes to global energy consumption, which is harmful to the environment and is extremely inefficient. The quality of rock phosphate, especially in terms of cadmium contamination, is continuously decreasing as cheap, high-quality reserves are becoming increasingly scarce and a major P shortage is predicted within few decades (Frossard et al. 2009). Currently, agricultural systems rely strongly on imported rock phosphate as well as various other rock phosphate derivatives from politically unstable regions such as Western Sahara. For example, while the European Union (EU) has for many decades been considered as a secure region in terms of food production, a report prepared by Schröder et al. (2010) demonstrates that the EU food system is actually highly vulnerable to future P scarcity, leading to decreased agricultural production (see Table 4.1 for world P demand).

Similar observations have been made concerning the use of N fertilizers. Global use of synthetic N fertilizers increased by 800 % between the years 1960 and 2000, and recently the anthropogenic N input was shown to be more than double the amount of N cycling through the biosphere (Canfield et al. 2010). Vast quantities of synthetic nitrogenous fertilizers are used worldwide and the Food and Agriculture Organization (FAO) estimates that nitrogenous fertilizer demand exceeds 130 million tones of N per year (Table 4.1). Our dependence on these resources is not only environmentally devastating (Rockström et al. 2009) but also economically shortsighted as synthetic N fertilizer production depends primarily on the use of fossil fuels (Canfield et al. 2010). While P is a limited natural resource, N occurs naturally in the atmosphere as molecular di-nitrogen (N_2) that can become a virtually unlimited resource after transformation into plant-available forms by a range of specific microorganisms (e.g., through biological nitrogen fixation (BNF); Fig. 4.2). BNF is a natural process of a prime importance in world agriculture (Werner and Newton 2005; Dakora et al. 2008; Canfield et al. 2010), and anything that can be done to increase BNF utilization will have global benefits. Herridge et al. (2008) reviewed and updated long-standing estimates

Table 4.1 World total demand for primary nutrients, 2011–2015 (thousand tons) – FAO current world fertilizer trends and outlook to 2015

Year	2011	2012	2013	2014	2015
Nitrogen (N)	130,844	133,951	136,846	139,517	141,682
Phosphate (P_2O_5)	48,119	49,256	50,323	51,180	51,940
Potash (K_2O)	32,190	33,361	34,359	35,482	36,367

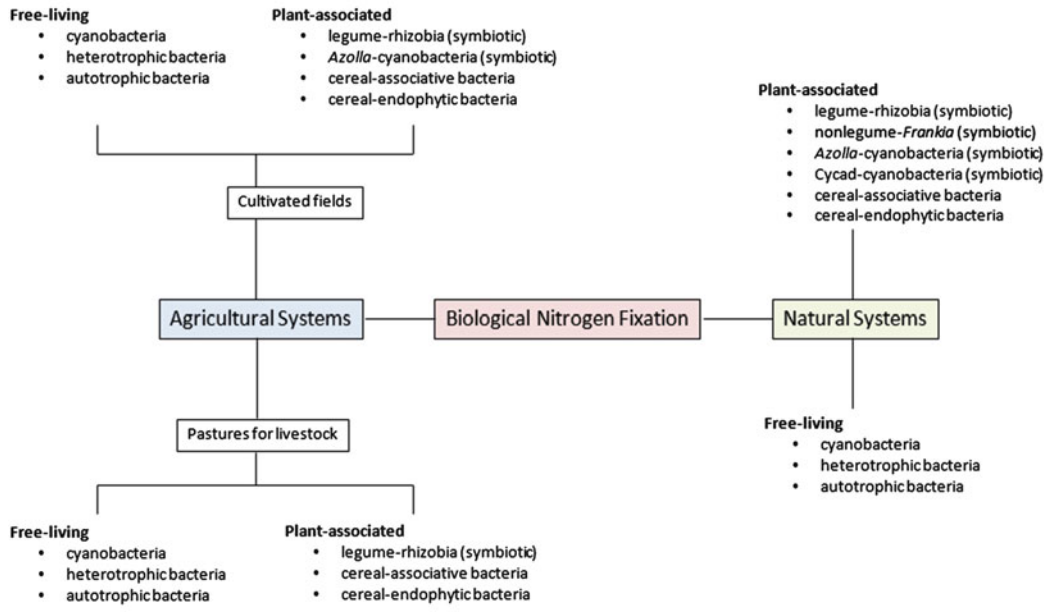

Fig. 4.2 Biological N-fixing agents in agricultural and terrestrial natural systems (Figure modified from Herridge et al. 2008)

of BNF in different agricultural systems, and the most important N_2-fixing agents are the symbiotic associations between legumes and rhizobia. Annual inputs of fixed N are calculated to be 2.95 Tg for pulses and 18.5 Tg for oilseed legumes. Soybean [*Glycine max* (L.) Merrill] is the dominant crop legume worldwide, representing 50 % of global area planted with crop legumes and 68 % of global legume crop production. It was estimated that soybean alone fixes up to 16.4 Tg N annually, representing 77 % of the N fixed by the crop legumes.

The increased understanding of the role of root- or rhizosphere-associated microbes in nutrition and/or growth, biomass accumulation and fitness of plants in general, and yield of agricultural crops in particular has resulted in their increased promotion for use in agricultural production as alternatives or supplements to mineral or organic fertilizers (Adesemoye and Kloepper 2009; Lugtenberg and Kamilova 2009; Compant et al. 2010; Miransari 2011; Tikhonovich and Provorov 2011; De–Bashan et al. 2012; Saikia et al. 2012). This aims to offset not only the increase in costs (while increasing the productivity of crops) but also the environmental and political issues related to the use of chemical fertilizer inputs. Inoculation of agricultural crops with different microorganisms such as rhizobia (for legumes), AMF, or other PGPR such as P-solubilizing microbes and associative diazotrophs has been practiced for many years with variable successes (Khan et al. 2007; Rooney et al. 2009; Sharma et al. 2012; Pérez–Montaño et al. 2014). The first patent for a biofertilizer (rhizobia inoculant) was registered more than a century ago (Nobbe and Hiltner 1896). In 2000, the area of legumes treated with commercial biofertilizers was more than 40 million hectares annually (Phillips 2004), with about half of this figure being represented by soybean production (Catroux et al. 2001). The commercial production of rhizobial inoculants has thus been in place for a number of decades, partially replacing the need for mineral N fertilizers on legume crops in many countries (Rodríguez–Navarro et al. 2011). In some regions and some crops (e.g., peas in North America), the proportion of inoculated acreage can reach almost 100 % of the total acreage for

the particular crop in that region, whereas for most crops, the figures are below 50 % of the planted acreage. In poor regions of Africa and elsewhere, application of rhizobia to leguminous crops still represents a negligible fraction of the overall legume production mostly due to lack of local research, information, infrastructure, and markets (Jansa et al. 2011). In contrast to rhizobial inoculants, P-solubilizing bacteria (e.g., *Bacillus* or *Pseudomonas*), root-associated diazotrophs (e.g., *Azospirillum*), and AMF have been used less frequently and on much lesser scale than the rhizobial inoculants. We estimate that no more than few thousand hectares are treated annually with non-rhizobial biofertilizers including AMF inoculants. This suggests that commercial non-rhizobial biofertilizer application does not affect global food production significantly, although specific inoculants (especially the *Azospirillum*-based products) may be important locally, e.g., in Cuba, Mexico, and Brazil (Saikia et al. 2010). The global agricultural crop production including legumes is estimated at some 1.6 billion hectares (www.fao.org/nr/solaw/solaw–home/en), but there is an obvious lack of market penetration by non-rhizobial biofertilizers in spite of decades of research. This indicates issues in the uptake and use of these products and is in contrast with the well-documented roles of microbes in specific aspects of plant nutrition (Douds et al. 2005; Cardoso and Kuyper 2006; Verbruggen et al. 2013) and with multiple research programs promoting large-scale application of such biofertilizers. Moreover, application of microbial inoculants does not always result in the desired effects as other factors such as abiotic and biotic soil conditions and climatic factors can play large and to a great extent unknown roles in the interplay between plants, microbes, and their shared environment (Wakelin et al. 2005). For example, Pereg and McMillan (2015) discussed the potential uses of beneficial microorganisms for increasing productivity in cotton cropping systems and pointed out that Australian cotton industry could greatly benefit from research into isolation of crop-specific beneficial microbes. While commercial biofertilizers are currently available for the cotton industry, they are not systematically applied because of inconsistent results. The authors also highlighted the need for taking into account the indigenous microbes because they often proved to be the most effective and most adapted to the environmental conditions in the cropping system for which they are intended. Some of these issues will be explored in this chapter, with a special focus on the biofertilizers that are used to improve nutrition and yields of grain, legume, oil, tuber, and other crops.

4.2 What Are the Biofertilizers?

Several definitions have been proposed over the years for the term "biofertilizer," and it generally refers to products containing one or more living microorganisms able to stimulate plant growth and development. Biofertilizers can increase the plant availability and/or uptake of mineral nutrients and are ideally formulated in an easy-to-use and economical carrier material (Arora et al. 2011; Malusa et al. 2012). According to Vessey (2003), a biofertilizer is a substance that contains living microorganisms which, when applied to seed, plant surfaces (leaves), roots or soil, colonize the rhizosphere or the interior of the plant and promote growth by several mechanisms that increase the supply or availability of primary nutrients to the host plant. Biofertilizers are also called microbial inoculants or bioformulations (Arora et al. 2011), but the term should not be used interchangeably with green manure, manure, intercrop, or organic-supplemented chemical fertilizer (Bhattacharyya and Jha 2012; Halpern et al. 2015). This term was first coined in 1978 for a group of microorganisms now known as PGPR (Kloepper et al. 1989) to define the free-living soil, rhizosphere, rhizoplane, and phyllosphere bacteria that, under some conditions, are beneficial for plants (Bashan and de Bashan 2005; Andrews et al. 2012). It has recently been proposed that the classification of PGPR should be confined to microbial strains that fulfill at least two of the three criteria such as aggressive colonization,

plant growth stimulation, and biocontrol (Khalid et al. 2006; Bhattacharyya and Jha 2012). This chapter focuses on the biofertilizers including rhizobia, AMF, and other nonsymbiotic microorganisms (PGPR) specifically involved in plant nutrition, but does not address the ectomycorrhizal symbiosis, though this may be relevant for mineral nutrition and growth of some energy crops (willows, poplars) but not food crops and production of edible mushrooms including truffles.

4.3 Current Contribution of Biofertilizers (Successes and Failures) to Agricultural Production

The most well-known success story of the use of microbial inoculants is clearly the inoculation of legumes with rhizobia. Inoculation of legumes with N_2-fixing rhizobia has been used in agriculture for more than 100 years (Deaker et al. 2004; Sindhu et al. 2010). It has largely transformed legume production systems so that high production rates are not totally dependent on N fertilizer. Theoretically, high N_2 fixation rates of 1–2 kg N ha^{-1} day^{-1} should be possible in all legumes and would satisfy most of the legume's needs. Alves et al. (2003), Hungria et al. (2006), and Melchiorre et al. (2011) have shown that the grain yields obtained in Brazil, Argentina, and the USA, respectively, can reach up to 4 t ha^{-1} per growing season through BNF and by the use of legume inoculants that contain both rhizobia and active compounds such as lipo-chito oligosaccharides (LCOs) which signal the symbiosis of rhizobia with legumes and the formation of nitrogen-fixing root nodules. There have been considerable efforts over many decades to enhance endophytic and associative nitrogen fixation in cereals and grasses through inoculation with free-living diazotrophs, most notably *Azotobacter*, *Azospirillum*, and more recently *Herbaspirillum*, *Gluconacetobacter*, and *Burkholderia* (Van Dommelen and Vanderleyden 2007; da Silva et al. 2012; Vargas et al. 2012). In Brazilian sugarcane plantations, nitrogen fixation rates of 40 kg N ha^{-1} $year^{-1}$ have been measured although the contribution of nonsymbiotically fixed N to plants remains small and difficult to establish (Unkovich and Baldock 2008).

Promotion of plant growth by bacteria from the genus *Azospirillum* is largely attributed to the production of phytohormones, predominantly indole-3-acetic acid (IAA); but gibberellins and cytokinins have also been implicated (reviewed by Baca and Elmerich (2007) and Halpern et al. (2015)). Plants inoculated with *Azospirillum* have been observed to increase root respiration rates and water and mineral uptake (reviewed by Dobbelaere et al. 2001). A selection of reviews on field application of *Azospirillum* covering 34 years and a range of sites worldwide indicated a high proportion (70 %) of increased plant growth and yield with reported averages typically just below 10 % (Table 4.2). However, publication of negative results may be suppressed and this may strongly affect the accuracy of the reported proportion of positive responses. The review by Dobbelaere et al. (2001) concluded that beneficial effects of *Azospirillum* are observed on lighter soils with low organic matter and intermediate levels of fertilizer and water. Early effects on plant growth do not necessarily result in increased yield, which is dependent on growing conditions during later stages of plant and grain development. Inoculants used in the described studies included liquid- and peat-based formulations of various species and strains of *Azospirillum* applied to both seeds, and soil and plant growth promotion was generally observed only after consideration was given to inoculum potential and appropriate formulation. Recent analyses of other field experiments indicate similar rates of increased yield (Díaz–Zorita and Fernández–Canigia 2009; Veresoglou and Menexes 2010).

Ferreira et al. (2013) evaluated maize response to *Azospirillum brasilense* inoculation and nutrient (macronutrients and micronutrients) applications under field conditions in clay and sandy soils of the Brazilian Cerrado. Maize growth was significantly affected when inoculated

Table 4.2 Summary of reviews on 34 years of field application of *Azospirillum* to cereal crops

Authors (year of publication)	Review period	Sites	Outcomes/conditions
Okon and Labanderra-Gonzalez (1994)	1974–1994	USA, India, Thailand, Israel, Egypt, Italy, France, Brazil, Mexico, Uruguay, Argentina	Significant yield increases 5–30 %
			Positive yield responses in 60–70 % of trials
Dobbelaere et al. (2001)	1994–2001	Belgium, Uruguay, Mexico, Israel	Positive responses dependent on conditions
			Lighter soils, intermediate levels of fertilizer and water
Díaz-Zoríta and Fernandez-Canígia (2009)	2002–2006	Trials at 297 sites in Pampas region of Argentina	Yield increased 8 % at 70 % of sites
			Response was independent of fertilization and other crop management practices
Veresoglou and Menexes (2010)	1981–2008	Meta-analysis of 59 articles	Yield increased 8–9 % effect greatest at low N responses varied with plant and microbial species

with *A. brasilense*, but the response was dependent on the soil type. The dual application of N fertilizer and *A. brasilense* increased grain yield by up to 29 % when compared to N fertilization alone. All these results on the field inoculation of crops with *Azospirillum* strains highlight the complexity of these plant–microbe interactions in the field and the number of factors playing a role on plant growth and grain yield. Marks et al. (2013) successfully tested the use of signaling molecules produced by rhizobia on both *Bradyrhizobium* spp. and *A. brasilense* field inoculation of soybean and maize, respectively. These results emphasize the potential of using secondary metabolites of rhizobia together with biofertilizers to improve the growth and yield of grain crops. According to Saikia et al. (2010), to gain the full potential of this association, the *Azospirillum* research should proceed toward more basic understanding of the underlying fundamental components of the system and less toward full-scale field experiments.

There is no doubt that bacterial biofertilizers can increase the yield of various crops significantly also through improved P acquisition (Hinsinger et al. 2015). However, field results are generally inconsistent despite some recent encouraging field inoculation studies. Krey et al. (2013) showed that maize plants inoculated with a P-solubilizing *Pseudomonas fluorescens* strain grew better and were significantly more infected by AMF than non-inoculated control plants. Kaur and Reddy (2014) identified two P-solubilizing bacterial strains playing an important role in plant growth promotion and improvement of soil fertility in different agroclimatic regions. Owen et al. (2015) reviewed the terminology, composition, and functions of P solubilizers and the many factors that impact their efficacy for increasing P availability in different soil and plant environments. These authors concluded that the effectiveness of the commercial biofertilizers is currently confounded by inadequate quality control standards and insufficient knowledge of the underlying mechanisms, which have led to contradicting reports on field performance.

AMF have been widely acknowledged as beneficial for plant P nutrition. Smith and Smith (2012) highlighted their capacity to very efficiently take up P from the soil to make it available for the associated plant. The outcome of this beneficial association will depend upon the soil characteristics and the environmental parameters such as temperature and soil moisture, as well as availability of other nutrients such as N (Johnson et al. 2015). For example, beneficial dual inoculation (AMF and rhizobia) has been successfully tested in the field to enhance woody legume growth (Lesueur et al. 2001; Lesueur and Duponnois 2005; Mortimer et al. 2013). Yet despite this, such kind of commercial biofertilizer remains rarely available on international markets, due mostly to production constraints and the lack of applied research.

Moreover, among the new biofertilizers that have recently appeared on various markets, many have not been subjected to scientific scrutiny. A 3-year Bill and Melinda Gates funded project (2009–2011) entitled "Evaluation and scaling up new chemical and biological commercial products for improving and sustaining crop yield in selected agro–ecological zones in sub Saharan Africa" (COMPRO) screened and evaluated several commercial biofertilizers from different countries of production (including rhizobia-, PGPR-, and AMF-based products) on major legume, cereal, and banana crops across diverse agroecological conditions in Kenya, Ethiopia, and Nigeria and under laboratory, greenhouse, and field conditions. Among the rhizobial inoculants, several were found very effective in increasing nodule biomass on soybean and increasing grain yield by up to 30 %, achieving a benefit–cost ratio of up to 5.0 (Thuita et al. 2012). The impact of inoculation was particularly significant in soil with low N content and when yields obtained without inoculation were low (0.5–1.0 t ha^{-1}). The efficiency seemed to be independent of the soybean variety and soil type. Results demonstrated economic returns of US$ 4 for every dollar invested for soybean production. However, when these effective rhizobial commercial inoculants were combined with biofertilizers containing AMF and/or P-solubilizing microorganisms, almost no additional effects were observed on crop yields (Jefwa et al. 2014). Commercial biofertilizers containing only PGPR bacteria were totally ineffective on major legumes, cereals, and banana crops not only under controlled conditions but also across diverse agroecological areas. Kavoo–Mwangi et al. (2013) demonstrated that tissue culture banana could benefit from application of AMF to improve survival and growth during the nursery phase as well as to enhance plant performance under field conditions. However, the effect of AMF was less evident in the field on both maize and soybean yields, particularly in the absence of mineral fertilizer application.

In the literature, a number of other studies have also looked at the effects of AMF inoculation, both under glasshouse and field conditions, to assess their impact on crop yields. The results were variable and often the direct impact of the AMF could not be quantified, as it is still difficult to (i) filter the contribution of the indigenous AMF out and (ii) measure the survival/function of the introduced genotypes under field conditions.

Other projects are currently ongoing in Africa aimed at improving the utilization of legumes and rhizobial inoculants to promote maize yields (SIMLESA project funded by ACIAR concerning East and Southern Africa http://aciar.gov.au/simlesa) and at promoting the utilization of Biological Nitrogen Fixation in African agricultural systems (N_2 Africa project funded by BMGF http://www.n2africa.org/). These projects should provide relevant results and technical protocols accessible to local African farmers for using appropriate and cheap biofertilizers to partially replace rare and expensive mineral fertilizers. Moreover, the information garnered from these projects should be transferable to other agricultural systems, particularly where a combination of high edaphic pressures and limited farmer access to fertilizer inputs results in poor crop yields.

4.4 Persistence of the Inoculated Microorganisms Contained Within Biofertilizers and Competition with Autochthonous Microbes

The soil is an extremely complex environment, inhabited by heterogeneous microbial communities interacting and communicating with each other via different chemical compounds secreted into the soil. Rhizobacteria constitute "common soil inhabitants" in all climate zones, from arctic to tropical areas, and are commonly found in all types of soils. After inoculation into soils, microorganisms contained in the biofertilizers must compete with the indigenous population for survival and are obviously disadvantaged as compared to the well-adapted native populations.

In the case of rhizobia, it has been shown that the presence, level, and diversity of native rhizobia in the soil are critical for inoculant performance. Successful inoculations were observed in soils with low native rhizobial populations and with a low N level. However, benefits are much less common in soils having strong established rhizobial populations, and inoculation may sometimes fail to produce the expected outcomes on nodule occupancy, BNF, and grain yield (McInnes and Haq 2007; Althabegoiti et al. 2008). Historically it was considered that only the presence of effective rhizobia in sufficient numbers in close proximity to the legume seed could ensure effective nodulation, BNF, and grain yield (Kaschuk et al. 2010; Torres et al. 2012). Depending on soil type, the required quantity of inoculated rhizobia in most cases was about 10^2–10^5 cells inoculated g^{-1} of soil (Andrade et al. 2002). There are numerous reports describing the effects of rhizobial inoculants in soils with a low level of soil N and few indigenous rhizobia. Skorupska et al. (2010) have made suggestions to explain why root nodules of legumes form a microenvironment not accessible to all rhizobia inhabiting rhizosphere: (i) the relatively high plant host–microsymbiont specificity as the nodules can be induced and consequently colonized only by rhizobia which recognize and exchange suitable molecular signals with the host and (ii) the variation in secreted Nod factors. Rhizobia recognized as "suitable microsymbionts" are subjected to a selection process during plant tissue invasion and colonization in two ways: they are exposed to intensive competition from other strains, and they are under some selective pressure from the host plant.

Soybean is one of the most important food crops in the world and provides an interesting example of whether or not to systematically employ rhizobial inoculation. High soybean grain yields require large amounts of N. To reach 1000 kg ha^{-1} of grain produced, the plant requires approximately 80 kg of N ha^{-1} although this varies between the different plant genotypes (Hungria et al. 2006). However, yields higher than 4000 kg ha^{-1} have been obtained in both Brazil and Argentina using BNF as the main source of N (Hungria et al. 2006; Rodríguez–Navarro et al. 2011). Soybean nodulation requires specific *Bradyrhizobium* species, and populations of these bradyrhizobia are seldom available in soils where soybean has not been grown previously (Abaidoo et al. 2007). In an attempt to find a solution to this problem, the soybean breeding program at the International Institute of Tropical Agriculture (IITA) in Nigeria developed soybean genotypes designated TGx (tropical *glycine* cross) able to nodulate effectively with indigenous *Bradyrhizobium* spp. populations (Kueneman et al. 1984). These TGx soybean genotypes (also called promiscuous varieties) have been tested in many western, eastern, and southern African countries without N fertilizer or *Bradyrhizobium* inoculation, and results indicate that indigenous strains do not always meet the N requirements of the plants despite good levels of nodulation (Mpepereki et al. 2000; Musiyiwa et al. 2005; Wasike et al. 2009; Thuita et al. 2012). The benefits of promiscuous soybean varieties are still being debated in the absence of efficient strains of rhizobia in soils. Previous studies carried out under greenhouse conditions with promiscuous soybean varieties showed that inoculation with selected commercial biofertilizers increased nodulation, N fixation, and biomass yield (Thuita et al. 2012). This illustrates the importance of evaluating the symbiotic performance of any new proposed strain in a field environment for suitability and adaptability before being recommended for use in an inoculant.

Mycorrhizal inoculants are also exposed to competition with autochthonous genotypes of AMF. In theory, these fungi already inhabit every soil on Earth (Jansa et al. 2006). However, solid information on competition introduced with native fungi is scarce due to the fact that, until recently, it was difficult to track the inoculant genotypes of AMF in non-sterile soil or the roots of field plants. The main reasons were the multi-genomic nature of these fungi, the lack of cultivability, and the slow growth of these microbes, limiting the amounts of pure DNA

for molecular genetic studies (Thonar et al. 2012). Some developments in sequencing AMF genomes and shifting focus from multi-allelic nuclear to more homogeneous mitochondrial genome (Lang and Hijri 2009) have enabled the design and use of specific molecular markers to identify individual genotypes (strains) of AMF, applicable both in the glasshouse and in the field (Boerstler et al. 2010; Kiers et al. 2011). However, not all results are yet available in a fully quantitative mode as some studies only apply specific genotype/strain markers using endpoint PCR (Sýkorová et al. 2012), whereas only few very recent papers employed specific markers in quantitative PCR (Kiers et al. 2011; Krak et al. 2012; Walker et al. 2012; Couillerot et al. 2013). Available data show that the inoculant strains sometimes survive for up to two seasons (Sýkorová et al. 2012). However their abundance generally rapidly decreases with time, and their beneficial effects established under model conditions (sterile soil) are masked due to intensive competition with native fungi (Biró et al. 2000), i.e., lack of long-term establishment of inoculants in the field. Yet another study substantiated that the identity of fungi applied on non-AMF seedlings greatly affected the community composition of AMF communities after transplantation into the field soils (Mummey et al. 2009), which may indicate that even a transient presence of the AMF inoculant may have long-lasting effects on the rhizosphere composition and functioning. Verbruggen et al. (2013) identified three factors determining inoculation success and AMF persistence in soils after inoculation: (i) species compatibilities, (ii) - field-carrying capacity, and (iii) priority effects. These authors explored how these factors can be employed for the establishment and persistence of AMF. Inoculant choice, plant choice, management practices, and timing of inoculation came up as being very important for the successful manipulation of the resulting AMF community. Lehmann et al. (2012) realized a meta-analysis study (from 1981 to 2010, 39 publications working on 320 different crop plant genotypes) about mycorrhizal responsiveness trends in annual crop plants and their wild relatives. They concluded that new cultivars were more mycorrhiza responsive (and possibly dependent) compared to ancestral genotypes. It means that no evidence was found indicating that new plant genotypes lost their ability to respond to AMF inoculation due to agricultural and breeding practices. The controversy about the low impact of AMF inoculation on crop yields thus cannot be explained by the utilization of these new plant genotypes.

4.5 Production of the Biofertilizers

The aim of inoculation is to supply a high number of viable, effective microorganisms to the rhizosphere soon after seed germination to optimize competition by introduced microorganisms with less effective resident strains. Development of adequate inoculants technologies to support the broad needs of current and potential biofertilizer microorganisms is challenging. Inoculant formulations must be designed to provide a suitable temporary microenvironment to support high numbers of viable cells and prevent the rapid decline of these populations during delivery under field conditions to maximize their effects on plants. A good inoculant must be nontoxic for plants, animals, and humans, permit optimal growth and survival of selected organisms during storage and inoculation (optimal water capacity, pH, composition), be made of a uniform and readily available carrier, and meet a low production cost. Different dispersal agents have been formulated depending on the microorganisms they contain, the target crop, the environmental conditions in which the products are used, the production cost, and the market availability and market demand. Four main dispersal products are distinguished currently in the market according to the kind of carrier materials used: powders, slurries, granules, and liquids. For each of them, a wide range of carriers are available. Most of the literature relating to commercial biofertilizers refers to bacterial inoculants, mainly due to the success story of rhizobial inoculants. However, there is a rapidly increasing interest in other types of inoculants, particularly incorporating AMF and other

microorganisms showing root growth promotion properties. The number of companies producing inoculum of mycorrhizal fungi has increased around the world over the last decade. Currently, in Europe, approximately 20 companies actively produce and distribute mycorrhizal biofertilizers, meaning that there are real market opportunities for their application (Vosátka et al. 2008). Companies have taken different market approaches, ranging from products with a single AMF strain for specific targets to mixed products for more general markets. AMF inoculants are generally provided as granular substrates made of peat, compost, vermiculite, perlite, sand, and/or expanded clay and contain spores, colonized roots, hyphal segments, or mixture of the three (Dalpé and Monreal 2004). Baar (2008) also described liquid formulations but these often contained only spores. The most frequently used AMF species for commercial inoculum are *Rhizophagus* sp. (formerly *Glomus intraradices*) and other *Glomus* species. Interest in the *Gigaspora*, *Scutellospora*, and *Acaulospora* genera is gradually increasing for commercial inoculum production (Dalpé and Monreal 2004; Baar 2008). Due to their obligate symbiotic status, AMF need to be associated with plants for growth and proliferation (Dalpé and Monreal 2004), and this remains a major obstacle for their commercialization since their high-scale production requires more specific technical skills and substantial infrastructures (IJdo et al. 2011). AMF production is generally performed using pot cultures, but other soilless techniques such as NFT (nutrient film technique), aeroponic, and monoxenic cultures are sometimes used (Dalpé and Monreal 2004; IJdo et al. 2011; Malusa et al. 2012). An in vitro method using split-plate cultures and Ri T-DNA transformed roots of carrots was also developed and resulted in very high concentrations of spores and hyphae in a very limited space (Dalpé and Monreal 2004).

In addition of the technical manual published by Deaker et al. (2011) entitled "Practical methods for the quality control of inoculant biofertilizers," two recent review papers have described in details the challenges of formulation and quality of biofertilizers for successful inoculation (Herrmann and Lesueur 2013) and the advances in biofertilizer technology (Bashan et al. 2014). Bashan et al. (2014) made a critical point on the practical aspects of bacterial inoculants for both agriculture and environmental restoration. They suggested that new developments should use renewable, non-contaminated natural resources opening up new avenues for research. Herrmann and Lesueur (2013) emphasized the importance of a suitable carrier to protect the microorganisms from the often–harsh conditions during storage and to ensure their survival and establishment after introduction into soils as well as the need for adequate quality controls, both during production and storage.

Despite 100 years of experience, 90 % of all rhizobial inoculants commercially produced worldwide are recognized to have little or no demonstrated impacts whatsoever on the productivity of the legumes for which they are used (Brockwell and Bottomley 1995; Catroux et al. 2001). Similar numbers are true for other kinds of inoculants (Gianinazzi and Vosátka 2004), although the knowledge is not assembled in as clear way as for the rhizobial products. As stated by Herridge et al. (2002), assessment of inoculant efficacy should start with their quality as assessment of inoculant efficacy is meaningless if it is of poor quality, if the product is contaminated or there is a low count of the focal microorganisms. Inoculant manufacturers increasingly commercialize new biofertilizers around the world. However, most of the manufacturers are not willing to improve the quality of their products, as the resultant increase in cost to the manufacturer and ultimately the farmer is a major disincentive. In addition, farmers are unlikely to judge the quality of an inoculant before they buy it (Lupwayi et al. 2000). As a consequence, farmers may purchase low-quality inoculants, obtain no or little and inconsistent effect on crops (thus

limited profit), and eventually lose confidence in the benefits of inoculation (Husen et al. 2007). The importance of the wider introduction of improved regulation and controls is widely acknowledged (Jenkins and Grzywacz 2000; Husen et al. 2007; Herridge 2008) to obtain better and more consistent results in the field and remove low-quality inoculants from the markets (Bhattacharyya and Jha 2012). These efforts have mainly focused on the bacterial biofertilizers in the past, but more recent establishment of the Federation of European Mycorrhizal Fungi Producers (www.femfip.com) aims at improving quality of mycorrhizal products at least at the European level.

However, two recent publications suggest why so far biofertilizers have not performed well under field conditions. Faye et al. (2013) assessed 12 commercial AMF inoculants sourced globally in a two-step experiment under greenhouse conditions. In this study, only three inoculants significantly increased root colonization levels compared to no sterile soil. Instead of the 12 declared AMF species, 13 fungal strains were extracted from the pot culture survey, including five undeclared species, while four declared species did not produce spores. Only three inoculants increased maize growth (shoot biomass) under controlled conditions. These authors highlighted the need to pre-evaluate commercial AMF inoculants on selected crop and soils before launching large-scale field use. Herrmann et al. (2015) characterized the microbial content of 65 commercial biofertilizers manufactured in the USA, the UK, Australia, South Africa, Thailand, Kenya, and Argentina. The results showed that 64 % of the products contained one or several strains of contaminants, while only 36 % of the products could be considered as pure. Rhizobial inoculants were generally of better quality than the other PGPR-based products, and 40 % of the tested biofertilizers did not contain any of the claimed strains but only contaminants, among which human pathogen such as *Comamonas testosteroni* or *Serratia marcescens* was found. These results highlight the need for better quality control systems, to ensure that efficacious inoculants reach the end users.

Inoculant quality is driven by several factors, primarily the number and viability of the selected strain(s) and the level of contaminants that may inhibit growth of selected strains as well as provide a potential risk to the health of humans, animals, and plants. A good formulation must also provide an extended shelf life under less than optimal storage conditions (Lupwayi et al. 2000; Catroux et al. 2001), and proper labeling must give sufficient information about the nature of the product, the shelf life, and instructions for its use (Hungria et al. 2003; Husen et al. 2007). However, shelf life, expiry date, and storage information are generally not clearly stated and difficult to obtain (Gemell et al. 2005; Husen et al. 2007), and this clearly makes the establishment of a functional and reliable quality control system difficult.

To achieve the goal of setting up a quality control system worldwide, standards must be defined. As raised by Herrmann and Lesueur (2013), to date, no international agreement of standards for inoculant quality is in place. Existing regulations generally concern rhizobial inoculants only, vary from country to country, and may or may not be enforced. Where specified, standards only concern the minimum number of viable rhizobia to be inoculated per seed or per unit of weight of inoculant and the maximum level of contaminants tolerated in the final products (Lupwayi et al. 2000). The minimum standards for rhizobia required to sustain adequate nodulation have generally been presented as a number to be applied per seed and vary depending on seed size. While there are no global standards, there is general agreement in the published literature that numbers should be of 500 cfu seed^{-1} for legumes with very small seeds (e.g., white clover), 10^3 cfu seed^{-1} for small seeds (e.g., subterranean clover and lucerne), 10^4 cfu seed^{-1} for medium seeds (e.g., lentil), and 10^5 cfu seed^{-1} for large seeds (e.g., soybean and faba bean) (Lupwayi et al. 2000;

Table 4.3 Summary of the main commercial legume inoculant formulations and their properties in relation to application and efficacy

Product	Properties relating to survival and efficacy
Moist peat: still major inoculant carrier for legumes in Australia and Canada	Long shelf life – cells undergo physiological and morphological changes that improve survival on seed
Liquids most common in the USA and South America (soybean) – applied to seed, large market for pre-inoculated soybean seed (the USA)	Shelf life and survival on seed of liquid formulations vary according to formulation and strain
Other formulations developed to provide flexibility in application: granules and freeze-dried products	Clay and peat based granules can be applied directly to the seedbed. Use of granules can be an advantage where survival on seed is a limiting factor (Brockwell et al. 1980)
	Water injection using freeze-dried inoculants eliminates issues of blockages in filters and spray nozzles
Formulations developed to improve survival on seed – dry clay powder	Dry clay powder (the USA) developed for pre-inoculated seed production (Dormal®) – claims of better survival on seed

Herridge et al. 2002). There is evidence that increasing numbers above these levels can further increase nodulation, N fixation, and yield in both competitive and noncompetitive soil environments (Denton et al. 2013). It is interesting to point out that legume yield is not decreased when rhizobia are applied in excess, and therefore there are no conceivable maximum doses for rhizobial inoculants. On the other hand, the mode of application of the rhizobial inoculants can significantly affect the response of the plant even when the amount of rhizobia applied remains constant (Odee et al. 2002; Diouf et al. 2003).

There are no available standards for PGPR and it may explain the results described by Herrmann et al. (2015) on the quality of this category of biofertilizers. The authors of reviews on field experiments with *Azospirillum* (listed in Table 4.3) agreed that high numbers (i.e., 10^7–10^8 cfu seed^{-1}) of *Azospirillum* are required to produce positive yield responses in cereals (Okon and Labandera–Gonzalez 1994; Dobbelaere et al. 2001). However, positive responses were observed in Argentina after application of liquid inoculants at a rate of 10^5 cfu seed^{-1} (Díaz–Zorita and Fernández–Canigia 2009). Inoculants prepared using dried alginate microbeads can provide a cell concentration of up to 10^9 cfu seed^{-1} and deliver an inoculum potential of 10^5 cfu seed^{-1} (Bashan et al. 2002, 2014). Peat cultures of *Azospirillum* have been produced commercially (i.e., Azogreen, Liphatech, France) and applied in field experiments, but survival of *Azospirillum* in peat is relatively poor as viable cell numbers can drop from 10^9 to 10^7 cfu seed^{-1} in a matter of months (Okon and Itzigsohn 1995). The relationships between minimum cfu seed^{-1}, selected strain, plant genotype, inoculant formulation, and positive plant growth responses are still unclear from the published information on field application of *Azospirillum*.

Likewise, there are no broadly established standards for application of mycorrhizal inoculants. As mycorrhizal fungi are present in virtually all soils, introduced strains of mycorrhizal fungi must always compete with indigenous communities unless these have previously been removed by soil sterilization such as in intensive glasshouse production (Jansa et al. 2006). However, results described by Faye et al. (2013) strongly suggest a change in the current production system in order to significantly improve the quality of the biofertilizers and to deliver better performing AMF biofertilizers. However, the control of the purity and the load of infective propagules of AMF biofertilizers are technically more difficult than the control of bacterial biofertilizers. Some fungal isolates abundant (mainly) in tropical soils are also known to establish extensive mycelium networks, but hardly

any dormant structures such as vesicles or spores, which makes them very susceptible to substrate disturbance or inoculant storage (Boddington and Dodd 2000; Jansa et al. 2003). A recent study (Ehinger et al. 2012) showed that different variants of the same AMF species with novel phenotypes could be selected among clonal spore lines generated from one initial single AMF spore. This highlights the complicated dynamic nature of AMF genetics, which needs to be better understood and exploited for the improvement of the AMF inoculant efficiency and to guarantee long-term stability of the product quality.

4.6 Future Research Needs

It is reasonable to expect that the need for BNF in crop production will at least double by 2050 if we are to feed a growing global population, and the expanded use of leguminous species has the potential to ameliorate a suite of problems facing agriculture around the world. BNF has the potential to reduce the application of manufactured nitrogenous fertilizer by approximately 160 million tons per annum, equating to a reduction of 270 million tons of coal or equivalent energy consumed in the production process. Given current concerns about climate change caused by greenhouse gas emissions, this represents a valuable improvement. In addition, N is likely to become increasingly expensive to produce as the cost of fossil fuels used in manufacturing and transportation processes escalates. Improvements in symbiotic BNF therefore promise significant economic benefits to farmers and consumers. Reduced reliance on external inputs such as synthetic N can improve ecosystem health and enhance the sustainability of agricultural systems ranging from industrial to subsistence farming.

Although field experiments with nonsymbiotic biofertilizers show real potential, it is still unclear as to whether these products will reliably substitute chemical fertilizer inputs to any great extent. Little is understood of the conditions under which these inoculants may work, and the lack of information about their quality, application practices, and potential in published studies makes it difficult to determine if these are optimized for any given conditions. What is very much needed, especially in the area of AMF inoculants, is the assessment of inoculum potential required for a given farming system, soil, and climatic conditions. This has been acknowledged for some time and algorithms to solve these issues were developed decades ago as illustrated in Fig. 4.3. There is also a pressing need for a more universal quality control system, providing the same regulations and standards to be used by manufacturers during formulation and production. The availability of good quality biofertilizers for farmers in countries where crops play a key role in the economy and food security must be a priority. In the absence of high-quality and efficacious biofertilizers, the dependence on the use of mineral fertilizers will not decrease. Moreover, the confidence of the end users in biofertilizers may be completely undermined if an inoculant does not clearly result in enhanced yield and/or reduced input requirements.

A real effort is required to develop new carriers and new formulations, providing "easy-to-use" products, more adapted to the farmers' requirements, together with improved quality and shelf life under less than optimal conditions. These new products are likely to contain additives that promote the survival of microorganisms during distribution and application as well as enhancing plant growth-promoting functional characteristics (Herrmann and Lesueur 2013; Bashan et al. 2014). One important aspect that is often overlooked is the fact that commercial inoculants, because they contain living microorganisms, are often dispersing potentially invasive and/or pathogenic organisms in their nonnative range (Schwartz et al. 2006). This practice has an inherent risk of environmental damage, but very little effort (at least to our knowledge) has so far been undertaken to quantify these risks and compare them rigorously with the economic and social benefits of inoculant application.

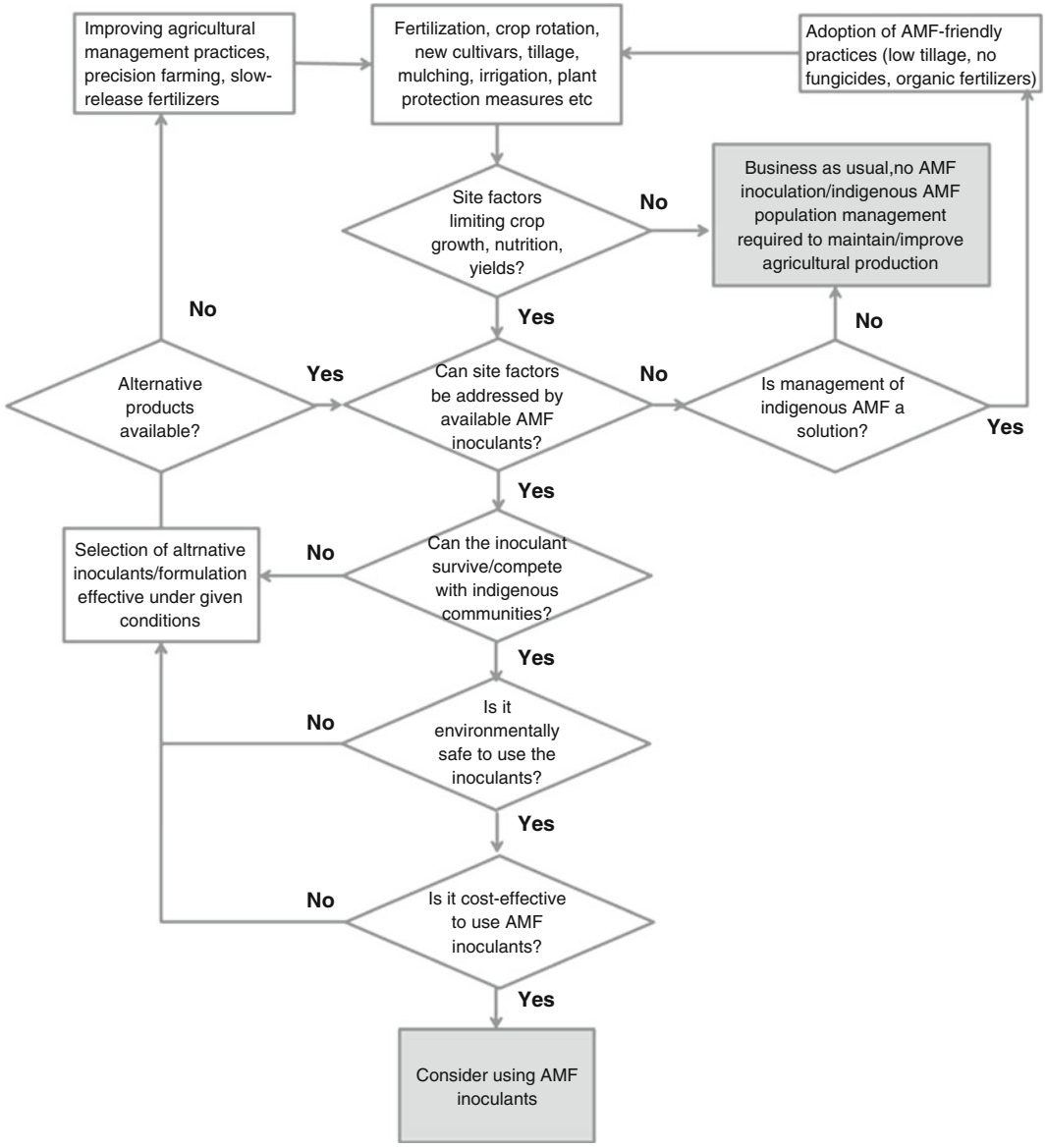

Fig. 4.3 Scheme of a site-specific decision strategy on the use of arbuscular mycorrhizal fungal (AMF) inoculants in agricultural production. Combined and modified from Dodd and Thomson (1994), Findlay and Kendle (2001) and Schwartz et al. (2006)

4.7 Conclusion

Biofertilizers can significantly improve plant growth. However, in the field, beneficial impacts of biofertilizers are unfortunately rare. The way forward for changing that is to strengthen collaboration of scientists with biofertilizer manufacturers because it would be of great benefit at expediting the development of new and efficacious biofertilizers. While scientists are mainly looking at the development of new products at the laboratory scale, the private sector can assist in addressing the issue of industrial scale-up and economic feasibility of the newly developed biofertilizers. That way, all the required parameters (such as strain selection, culture conditions, specifications for different

crops and target markets, as well as logistical considerations that could influence the final product) would be considered in the development of efficacious commercially viable new inoculants. Clearly, alongside the development and production aspects of biofertilizers, one of the most promising ways to increase biofertilizer efficacy is to introduce robust quality control systems. End users may then buy these products with confidence and compare their usefulness and cost–benefit ratios with traditional mineral fertilizer inputs and/or other management interventions in their specific farming system, edaphic, and environmental conditions.

Acknowledgments JJ was supported by the Fellowship of J E Purkyně, Czech Science Foundation (P504121665), Ministry of Education, Youth and Sports (LK11224), and the long-term development program RVO 61388971. LH and DL were partially supported by the "COMPRO" project funded by Bill and Melinda Gates Foundation (2009–2011).

The authors are indebted to Prof David Herridge for his useful review of the Ms and his important comments and suggestions to improve it.

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Effect of Bioinoculants on the Quality of Crops

5

Elisa Bona, Guido Lingua, and Valeria Todeschini

Contents

Abstract

Soil microorganisms like arbuscular mycorrhizal fungi (AMF) and plant growth-promoting rhizobacteria (PGPR) are known to positively affect plant health, growth, and nutrition. Their use was often suggested in order to reduce the input of chemicals in agriculture. Recently, it has been observed that AMF and PGPR can also affect some quality features of various crops. In this chapter we review the literature concerning the effects of soil microbes, focusing on AMF and PGPR as the most common plant-associated microorganisms, on the quality of crops. Such effects were considered according to commercial and agronomic plant categories. Current limitations of the available information and some possible future developments have been indicated.

5.1 Introduction

Several soil microorganisms, belonging to very different taxa, have shown the ability to improve plant growth and health, especially under conditions of low nutrient availability. The involved microbes include phosphate-solubilizing, indole acetic acid (IAA)-releasing, 1-aminocyclopropane 1-carboxylate (ACC) deaminase-producing bacteria, actinomycetes, and a number of fungal species, mycorrhizal or

E. Bona • G. Lingua (✉) • V. Todeschini
Dipartimento di Scienze e Innovazione Tecnologica, Università del Piemonte Orientale "A. Avogadro", viale T. Michel 11, 15121 Alessandria, Italy
e-mail: guido.lingua@uniupo.it

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_5

not. The establishment of relationships between such microorganisms and plants results in improved plant nutrition and better tolerance of biotic and abiotic stresses. Hence, the use of inocula based on these microbes was proposed to reduce the impact of chemicals and to support sustainable agriculture.

In recent years, some of the above mentioned microorganisms have also shown the ability to affect the quality of plant products, including some fruits, vegetables, and secondary metabolites (e.g., essential oils) (Kapoor et al. 2002a; Copetta et al. 2006; Khaosaad et al. 2006; Zubek et al. 2012; Bona et al. 2015). Providing a definition for the "quality" of crops is a difficult task. It can depend on area-specific criteria, and it is often connected to economical and commercial factors or transformation processes. Anything concerning the improvement of desirable traits (like taste, smell, and look) and the healthiness of the products (e.g., increased concentration of healthy molecules, as antioxidants) can however gain a quite large consensus. The purpose of this work is, therefore, the revision of the existing literature concerning the improvement of some traits of various crops, related to the quality of products, in plants inoculated with AMF or PGPR. Other organisms, like *Trichoderma* species, can exert positive effects on plants; however, we limited this study to AMF and PGPR because they are the most widespread plant-associated microorganisms. AMF are obligate biotrophs classified into the *Glomeromycota*; they form a symbiosis with the roots of most land plants, improving plant nutrition and growth (Smith and Read 2008). PGPR are a very heterogeneous group of bacteria that can exert their beneficial role directly or indirectly; direct promotion of plant growth is usually accomplished via increased uptake of nutrients (especially nitrogen, phosphate, and iron) or interfering with the plant hormone balance, modulating the concentration of substances like auxins, cytokinin, and ethylene. Indirect beneficial effects are mostly observed when PGPR inhibit or circumscribe the noxious effects of plant pathogens (Glick 2012).

Although the above definition of "quality" is anthropocentric, the topic is of interest because it can help to:

- Broaden the possible applications for bioinoculants, providing suggestions and directions about their utility and use in agriculture, with the perspective of further economical return and lower environmental impact.
- Support reliable enterprises that commercialize inocula.
- Provide new perspectives for the definition of typical products, relying on specific interactions with microbes characteristic or dominant under certain conditions or in certain areas.
- Better understand the specificity of some interactions.
- Recognize what biological and agronomic aspects require further investigation.

The subjects of increased yield, improved P nutrition, better stress tolerance, and reduced fertilization have extensively been treated elsewhere (Gianinazzi et al. 2002; Smith and Read 2008) and will not be included in this essay. When appropriate, however, reference to the above mentioned topics will be introduced.

For the purposes of the present work, the various types of crops have been organized into categories which reflect similarities in the way of growing, in the used part of the plant, in the kind of metabolic pathway involved, and so on. The idea is that plants with similar features might benefit from the same kind of inocula. Such a criterion is of course arbitrary, but we expect that this would help in the formulation of new inoculants specifically addressed to some kind of cultivation and product.

Before starting the examination of the various crop categories, a disclaimer is necessary: we cannot make any claim of completeness. "Product quality" is almost never included in the keywords of a paper; therefore, the bibliographic search for this study went through the manual screening and sorting of (literally) thousands of papers resulting from searches based on the name of the plant

species and microbial group. We apologize for any paper that has skipped our attention. In addition, for reasons of simplicity, AMF have been indicated with the names used in the original articles, in spite of the relatively recent taxonomic revision (Schüßler and Walker 2010).

As described before, we chose eight main categories on the basis of the commercial use: (1) fresh fruits, (2) nuts and oilseeds, (3) root and bulb vegetables, (4) fruiting vegetables, (5) cabbages and leaf and stem vegetables, (6) fresh and grain legumes, (7) cereals, and (8) aromatic plants, spices, tea, coffee, herbal infusion, and cocoa.

5.2 Fresh Fruits (Table 5.1)

Fresh fruits include citrus, Maloideae, Prunoideae, berries, grape wine, small and large fruits with inedible peel, and fruits with edible peel. They are a nutritional source of sugars, vitamins (mainly C and E), carotenoids, flavonoids, and phenol compounds collectively called phytochemicals, with antioxidant activity. The consumption of fresh fruits and vegetables in the diet is important for the prevention of several diseases (chronic, cancer, and cardiovascular diseases) (Giovannetti et al. 2012; Sbrana et al. 2014). Both abiotic (soil chemical-physical properties, growth conditions, postharvest storage) and biotic factors (plant species or cultivar and interaction with soil microorganisms, mainly AMF and PGPR) can modulate the content of antioxidant compounds. Different studies highlighted the beneficial effects of these symbiotic microorganisms on plant physiology, but only a small part of them included the assessment of the quality of plant products (leaves, roots, fruits, or vegetables). Concerning the "citrus fruit" category, Nautiyal et al. (2008) demonstrated that the rhizospheric bacterium *Bacillus lentimorbus* induces the modulation of phenolic compounds and dietary antioxidants in *Citrus sinensis*.

"Berry fruits" (blackberry, blueberry, mulberry, cranberry, strawberry, etc.), mostly belonging to the family Rosaceae, are consumed as fresh fruits and are used to produce fruit juice or jam. They are a good source of vitamins C (ascorbic acid) and B9 (folic acid), fibers, minerals, and antioxidant compounds (anthocyanins, phenolic acids, and flavonols). Many epidemiological studies reported that anthocyanin consumption decreases the risk of obesity, coronary heart disease, and various types of cancer (Karlsen et al. 2007; Krikorian et al. 2010; Kaume et al. 2012).

Blackberry (*Rubus* sp.) is an aggregate fruit, composed of small drupelets, especially rich in polyphenols included flavonoids (anthocyanins) with medicinal properties (Kaume et al. 2012; Ramos-Solano et al. 2014). In a field experiment, Ramos-Solano and coworkers demonstrated that blackberry inoculation with *Pseudomonas fluorescens* N21.4 (CECT7620) affected secondary metabolism increasing fruit production and quality in terms of flavonoids and other phenol compounds (Ramos-Solano et al. 2014). Similar results were reported for fruits of *Fragaria x ananassa* Duch (strawberry), by Castellanos-Morales et al. (2010) in plants inoculated with the AM fungus *Glomus intraradices* (Castellanos-Morales et al. 2010), by Palencia et al. (2013) in plant inoculated with *Glomus mosseae* and *G. intraradices*, and by Lingua et al. (2013) and Bona et al. (2015) in plants of the same species inoculated with *P. fluorescens* Pf4 and *Pseudomonas* sp. 5Vm1K alone or in combination with a commercial inoculum consisting of a mix of AMF (Mybasol s.r.l.). Moreover, it is noteworthy that in this last study, bioinoculants affect sugar metabolism and vitamin concentration (vitamin C and B9). Sweeter fruits are produced in PGPR-inoculated plants than in controls (Pešaković et al. 2013), and this increase of the sweetness index is especially due to sucrose concentration, as shown by Bona et al. (2015). Strawberry flavor is due to the balance of organic acids and soluble sugars (Kallio et al. 2000). Citric and malic acid are the most important acids in strawberry fruits, while glucose, fructose, and sucrose are the principal sugars, representing about the 99 % of the total carbohydrate content (Perez et al. 1997;

Table 5.1 Fresh fruits

Reference	Plant family	Plant species	Rhizosphere microbe	n. fruits	Maturation time	Fruit size	Acidity	Total solids	Sugars	Organic acids	Vitamins	Minerals	Flavonoid	Sweetness index	Phenol compounds
Attia et al. (2009)	Musaceae	*Maghrabi banana*	PGPR (*P. fluorescens*; *B. megaterium*)				✔	✔				✔			
Bona et al. (2015)	Rosaceae	*Fragaria x ananassa*	PGPR (*P. fluorescens* (Pf4) and *Pseudomonas* sp. (5Vm1K)); AM fungi (commercial inoculum Mybasol s.r.l. (*R. intraradices*, *G. aggregatum*, *G. viscosum*, *C. etunicatum*, *C. claroideum*))	✔	✔	✔	✔		✔	✔	✔			✔	
Castellanos-Morales et al. (2010)	Rosaceae	*Fragaria x ananassa* Duch	AM fungi (*G. intraradices*)									✔	✔		✔
Esitken et al. (2006)	Rosaceae	*Prunus avium* L. (sweet cherry)	PGPR (*Pseudomonas* sp., *Bacillus* sp.)									✔			
Lingua et al. (2013)	Rosaceae	*Fragaria x ananassa*	PGPR (*P. fluorescens* (Pf4) and *Pseudomonas* sp. (5Vm1K)); AM fungi (commercial inoculum Mybasol s.r.l. (*R. intraradices*, *G. aggregatum*, *G. viscosum*, *C. etunicatum*, *C. claroideum*))										✔		
Mamatha et al. (2002)	Moraceae, Caricaceae	Mulberry (*Morus* sp.); papaya var. Solo (*Carica papaya* L.)	PGPR (*B. coagulans*); AM fungi (*G. fasciculatum*); mixed culture of *G. mosseae* and *G. caledonium*	✔		✔									
Nautiyal et al. (2008)	Rutaceae	*C. sinensis*	PGPR (*B. lentimorbus* B-30488)												✔

Orhan et al. (2006)	Rosaceae	*Rubus idaeus* L. (raspberry)	PGPR (two *Bacillus* strains)	✔			✔						✔		
Palencia et al. (2013)	Rosaceae	*Fragaria* × *ananassa* Duch. "Splendor"	AM fungi (*G. mosseae* and *G. intraradices*)				✔	✔							
Pešaković et al. (2013)	Rosaceae	*Fragaria x ananassa*	PGPR (*K. planticola)*; liquid inoculum (*A. chroococcum*, *A. vinelandii*, *Derxia* sp., *B. megaterium*, *B. licheniformis*, and *B. subtilis*)		✔	✔	✔	✔	✔						
Ramos-Solano et al. (2014)	Rosaceae	*Rubus* sp. var. Lochness (blackberries)	PGPR (*P. fluorescens* N21.4 (Spanish Type Culture Collection accession number CECT 7620))					✔					✔		✔
Sinclair et al. (2014)	Rosaceae	*Fragaria* × *ananassa* Duch. cultivars Albion, Charlotte, and Seascape	AM fungi (*F. mosseae*; *F. caledonius*; *R. irregularis*)				✔								
Verginer et al. (2010)	Vitaceae	*V. vinifera* L.	PGPR (*Paenibacillus* sp.).							✔			✔		

Summary of the main parameters influenced by the interaction between plants, belonging to the "fresh fruits" category, and microorganisms (PGPR or AMF). The term "fruit size" includes weight, size, and biomass of the crop fruit. References are listed in alphabetical order

Castellanos-Morales et al. 2010). Besides their impact on flavor, acids can affect the gelling properties of pectin (Castellanos-Morales et al. 2010; Sinclair et al. 2014). Vitamin C is associated with healthy properties of fruits, so this is an attractive feature for consumers. Citrus fruits have the greatest reputation for a high content of vitamin C, but there are some other fruits, such as strawberries, that have a higher content of ascorbic acid (Perez et al. 1997; Bona et al. 2015).

Finally, as far as berries are concerned, Orhan et al. (2006) reported an increase of berry number in raspberry plants inoculated with *Bacillus* M3 alone or in combination with *Bacillus* OSU-142.

AMF and PGPR are able to modulate fruit quality also in woody plants such as mulberry and papaya. Mamatha et al. (2002) reported the results obtained from the inoculation of 10-year-old mulberry plants with *Glomus fasciculatum* and 1.5-year-old papaya plants (var. Solo) with a mixed culture of *G. mosseae* and *Glomus caledonium*, with or without *Bacillus coagulans*. Increased yield and P uptake are the most relevant results. Sweet cherry is one of the most important fruit crops grown in Anatolia, and Turkey is the biggest sweet cherry-producing country in the world (Esitken et al. 2006). Floral and foliar applications of *Pseudomonas* BA-8 and *Bacillus* OSU-142 in sweet cherry plants enhance fruit size, compared with the control (Esitken et al. 2006).

In some crops, volatile compounds are important for the flavor of the final products, as in the case of grapevine (*Vitis vinifera* L.). In fact, in this plant, compounds such as 1-propanol, 2-butanone have a strong impact on the aroma of the produced wines and give them their characteristic flavor. Verginer et al. (2010) reported the isolation and identification of different rhizospheric microorganisms (bacteria and fungi) that influenced the production of volatile compounds in fruits.

Finally, Attia and coworkers reported that banana plants inoculated with *P. fluorescens* and *Bacillus megaterium* show enhanced fruit size, acidity, total soluble solids, and micro- and macronutrient content of fruits (Attia et al. 2009).

5.3 Nuts and Oilseeds (Table 5.2)

In this section we have included plants with dry seeds for the production of oils and olive trees. Some cereals, like maize, might have been considered as well, but we decided to discuss each species only in one section.

Overall the amount of available information is rather limited. For instance, there are thousands of papers concerning the interaction between sunflower and AMF or PGPR, but, to the best of our knowledge, none reports information concerning the quality of seeds or oil.

In the case of peanuts (*Arachis hypogaea*), *G. mosseae* and *Rhizobium* sp. can increase the shoot and root content of phenolic compounds; the effect is highest with dual inoculation, and it is associated to the appearance of some new molecules, absent in the uninoculated plants (Devi and Reddy 2002). These results suggest that it might be worth searching for some effects on the fruits.

Flax (*Linum usitatissimum*) can be grown in different varieties, for the production of fiber or oil. Inoculation with *G. mosseae* or *G. intraradices*, or their mixture, results in increased fiber production. In oil flax, the same two AMF slightly decrease the amount of non-saturated fatty acids and increase that of saturated fatty acids. These two AMF also show competition against *Sinorhizobium* sp., decreasing its abundance when co-inoculated. As a consequence, in this condition, the effect of co-inoculation is not synergistic (Rydlová et al. 2011).

AMF can also colonize various woody (shrubs or trees) species, including hazelnut (*Corylus avellana*), walnut (*Juglans regia*), macadamia (*Macadamia tetraphylla*), and olive (*Olea europaea*), whose fruits or seeds are used for the production of oil. So far, the effects of colonization on fruit quality have not been investigated, but it was shown that *G. mosseae* or *G. intraradices* BEG 72 can dramatically improve the survival of micropropagated walnut plants if inoculated in the post-acclimatization

Table 5.2 Nuts and oilseeds

Reference	Plant family	Plant species	Rhizosphere microbe	n. fruits	Fruit size	Minerals	Phenol compounds	Oil content
Devi and Reddy (2002)	Fabaceae	*Arachis hypogaea* L.	PGPR (*Rhizobium* sp.); AM fungi (*G. mosseae*)				✓	
Estaun et al. (2003)	Oleaceae	*O. europaea* L.	AM fungi (*G. intraradices*, *G. mosseae*)	✓	✓			
Rydlova et al. (2011)	Linaceae	*L. usitatissimum* L. (oil and fiber flax)	PGPR (*Sinorhizobium* sp.); AM fungi (*G. mosseae*, *G. intraradices*, *G. mosseae* + *G. intraradices*)					✓

Summary of the main parameters influenced by the interaction between plants, belonging to the "nuts and oilseeds" category, and microorganisms (PGPR or AMF). The term "fruit size" includes weight, size, and biomass of the crop fruit. References are listed in alphabetical order

phase (Dolcet-Sanjuan et al. 1996). In the case of micropropagated hazelnut plants, colonization with AMF improves growth rather than survival (Mirabelli et al. 2009). Drought tolerance of Macadamias improves when mycorrhized (Yooyongwech et al. 2013). Similarly, we could not find any information concerning the effect of PGPR on the quality of *O. europaea* fruits; actually, the only report concerns the rooting induction of some auxin-producing bacteria on olive cuttings (Montero-Calasanz et al. 2013). Olive trees can form arbuscular mycorrhiza (Citernesi et al. 1998) although species-specific responses have been reported: three different cultivars of olive plants have been found to be colonized by a *Gigaspora* species, but only one of the three by a *Glomus* species (Chatzistathis et al. 2013). The use of inocula has been evaluated in order to increase the success of transplantation (Palenzuela et al. 2002), to improve water-deficit tolerance (Caravaca et al. 2003), and to protect plants against parasites (Castillo et al. 2006). Inoculation with *G. mosseae* or *G. intraradices* improves plant growth and fruit yield compared to non-inoculated plants, with more pronounced effects for the latter fungus; however, under field conditions, the inocula effects tend to diminish with time, due to the presence of naturally occurring propagules which trigger the colonization of uninoculated plants (Estaun et al. 2003). The phospholipid and glycolipid content of leaves increases in olive plants inoculated with *G. intraradices* DAOM 197198 (Mechri et al. 2014); unfortunately, no data are yet available on the lipid content of fruits.

5.4 Root and Bulb Vegetables (Table 5.3)

Potatoes, sweet potatoes, carrots, radishes, turnips, onions, green onions, garlics, and shallots belong to this category. Data present in the literature on the microorganism-plant interaction in food quality mostly concern the bulb vegetables, with particular attention to the family Alliaceae.

Table 5.3 Root and bulb vegetables

Reference	Plant family	Plant species	Rhizosphere microbe	n. bulbs/ roots	Bulb size	Firmness	Total solids	Organic acids	Vitamins	Minerals	Carotenoids	Flavonoid	Phenol compounds	Alliin
Abdel-Razzak and El-Sharkawy (2013)	Alliaceae	*A. sativum*	PGPR (commercial inoculum Halex-2 (*Azospirillum sp.*, *Azotobacter sp.*, *Klebsiella sp.*))	✔	✔									
Albrechtova et al. (2012)	Alliaceae	*Allium cepa* L. cv. Alice	AM fungi (commercial inoculum Symbivit (Symbiom s.r.o) (*G. intraradices* BEG 140, *G. mosseae* BEG 95, *G. etunicatum* BEG 92, *G. claroideum* BEG 96, *G. microaggregatum* BEG 56, *G. geosporum* BEG 199. Single inoculation *G. intraradices* BEG 140))		✔					✔				
Borde et al. (2009)	Alliaceae	*A. sativum*	AM fungi (*G. fasciculatum*)		✔									✔
Charron et al. (2001)	Alliaceae	*Allium cepa* L.	AM fungi (*G. intraradices*, *G. versiforme*)			✔								
Mogren et al. (2008)	Alliaceae	*A. cepa* L.	AM fungi (*G. intraradices* Schenck and Smith (BEG 87))									✔		
Nautiyal et al. (2008)	Apiaceae	*Daucus carota*	PGPR (*B. lentimorbus* B-30488)										✔	
Perner et al. (2008)	Alliaceae	*A. cepa* L.	AM fungi (TerraVital Hortimix comprising *G. mosseae*, *G. intraradices*, *G. claroideum*, and *G. microaggregatum*)									✔		✔
Wang et al. (2011)	Apiaceae Alliaceae	*Daucus carota*, *Allium fistulosum* L.	AM fungi (*G. intraradices* BEG 141, *G. mosseae* BEG 167)		✔									

Summary of the main parameters influenced by the interaction between plants, belonging to the "root and bulb vegetables" category, and microorganisms (PGPR or AMF). The term "fruit size" includes weight, size, and biomass of the crop fruit. References are listed in alphabetical order

For instance, Abdel-Razzak and El-Sharkawy (2013) found an increase of bulb and clove number in *Allium sativum* plants treated with a commercial inoculum Halex-2 (consisting of growth-promoting nonsymbiotic N-fixing bacteria *Azospirillum* sp., *Azotobacter* sp., and *Klebsiella* sp.) (Abdel-Razzak and El-Sharkawy 2013) and with AMF inoculation (Borde et al. 2009). With onion, garlic is one of the most cultivated species of bulb vegetables. It is used as a spice and condiment and has high medicinal (e.g., it contrasts high blood pressure and arteriosclerosis), antioxidant (Chung 2006), antimicrobial, and antiviral properties (Harris et al. 2001) due to the presence of allicin (Borde et al. 2009). This organo-sulfur compound is produced from alliin by the enzyme alliinase (Corzo-Martinez et al. 2007), and it is responsible of the bulb flavor and aroma. A significant increase of alliin and alliinase content was shown in garlic plants, grown in field and inoculated with the AMF *G. fasciculatum* (Borde et al. 2009).

Allium cepa production is common worldwide and according to estimates it is over 250,000 tonnes (FAOSTAT 2010). The beneficial effects derived from onion consumption depend on two groups of chemical compounds: the flavor precursors alkenyl sulfoxides and flavonoids (Griffiths et al. 2002; Mogren et al. 2008). Yellow onions contain high concentration of flavonols, and the main flavonol glycosides have been shown to be quercetin 4-′-monoglucoside and quercetin 3,4′-diglucoside (Price and Rhodes 1997). It was reported that these compounds are modulated by AMF as shown by Albrechtova et al. (2012) and Perner et al. (2008). However, it should be underlined that different fungi induce different response in the same plants. In fact, a mix of *Glomus* sp. induces the best total antioxidant capacity in *A. cepa* cv. Alice bulbs if compared with *G. intraradices* BEG 140 alone that, on the contrary, is most effective in increasing mineral (Mg and K) accumulation (Albrechtova et al. 2012). Similarly, Charron et al. (2001) demonstrated that *Glomus versiforme* induces the production of firmer bulbs than *G. intraradices* (Charron et al. 2001).

Inoculation with *G. intraradices* BEG 141 leads to increased biomass either in carrot or in green onion (Wang et al. 2011). To the best of our knowledge, no data concerning PGPR effects on bulb quality are available in the literature. Within "root and bulb vegetables" category, the only paper about carrot quality reported an increase of phenol compounds in plants inoculated with *B. lentimorbus* B-30488 (Nautiyal et al. 2008).

5.5 Fruiting Vegetables (Table 5.4)

Solanaceous species, like tomato and pepper, establish root symbiosis with different AMF. Tomato, in particular, has become a model species for studies on plant interactions with AMF and PGPR, and effects of the microorganisms on the quality of tomatoes have been demonstrated. In particular, Zouari et al. (2014) demonstrated that AM symbiosis with *Funneliformis mosseae* induces the upregulation of genes involved in photosynthesis, stress response, transport, amino acid synthesis, and carbohydrate metabolism and the downregulation of ethylene response genes in tomatoes. Also Salvioli et al. (2012) previously demonstrated the modulation of gene transcription and of amino acid composition in fruits of plants inoculated with AMF. Nzanza et al. (2012) reported increase in the number of marketable fruits and of the concentration of nutrients such as minerals (P, K, Ca, Mg), vitamin C, and total flavonoids in tomatoes of plants inoculated with *Trichoderma harzianum* and *G. mosseae*. Similar results were reported by Giovannetti et al. (2012) (enhancement of lycopene concentration in tomatoes of plants inoculated with *G. intraradices*), by García-Fraile et al. (2012) (enhancement of fruits size and mineral concentrations in plant inoculated with *Rhizobium* sp.), by Sirichaiwetchakul et al. (2011) who showed that AMF (*Glomus* sp., *G. mosseae*, *Acaulospora* sp., *Entrophospora schenckii*, *Scutellospora fulgida*) raise the amount of total soluble solid and ascorbic acid in tomatoes, by Lenin

Table 5.4 Fruiting vegetables

Reference	Plant family	Plant species	Rhizosphere microbe	Allergens and metabolism	Flowers	n. fruits	Maturation time	Fruit size	Acidity	Total solids	Sugars	Organic acids	Minerals	Carotenoids	Flavonoid	Chlorophylls	Xanthophylls
Castillo et al. (2009)	Solanaceae	*C. annuum* L. "Cacho de cabra"	AM fungi (*G. intraradices* (commercial; IC); *G. claroideum* (native; IN))				✔		✔	✔		✔					
Del Amor et al. (2008)	Solanaceae	*C. annuum* L. (sweet pepper)	PGPR (Biopron (*A. brasilense*, *P. dispersa*))									✔					
Garcia-Fraile et al. (2012)	Solanaceae	*Solanum lycopersicum*, *C. annuum*	PGPR (*R. leguminosarum*)		✔	✔		✔					✔				
Giovannetti et al. (2012)	Solanaceae	*S. lycopersicum* L. var. Moneymaker	AM fungi (*G. intraradices* (IMA6))										✔	✔			
Kaya et al. (2009)	Solanaceae	*C. annuum* L.	AM fungi (*G. clarum*)										✔				
Lee et al. (2011)	Solanaceae	*C. annuum* L.	PGPR (*M. oryzae*)					✔									
Lenin et al. (2010)	Solanaceae	*S. lycopersicum* L., *S. melongena* L., *C. annuum* L.	AM fungi (*G. fasciculatum*)										✔			✔	
Lu et al. (2006)	Cucurbitaceae	*C. sativus*	AM fungi (*G. versiforme*, *G. mosseae*, *G. intraradices*, and their mixtures)			✔					✔						
Mandyal et al. (2012)	Solanaceae	*C. annuum* L.	PGPR (*Bacillus* sp.)			✔							✔				
Mena-Violante et al. (2006)	Solanaceae	*C. annuum* L. cv. San Luis	AM fungi (*G. fasciculatum*, Los Tuxtla (AMFT), AMFD)					✔						✔			✔
Nzanza et al. (2012)	Solanaceae	*Solanum lycopersicum* L.	AM fungi (commercial inoculum *T. harzianum* DB 103; Biocult© *G. mosseae*)			✔		✔					✔	✔	✔		
Ortas (2010)	Cucurbitaceae	*C. sativus*	AM fungi (*G. mosseae*, *G. etunicatum*, *G. clarum*, *G. caledonium*)			✔							✔				
Salvioli et al. (2012)	Solanaceae	*S. lycopersicum* L.	AM fungi (*G. mosseae*)	✔	✔	✔											
Schwarz et al. (2011)	Solanaceae	*S. lycopersicum* L.	AM fungi (*G. mosseae*)	✔													

Sirichawetchakul et al. (2011)	Solanaceae	*S. lycopersicum* L. (cherry tomatoes)	AM fungi (*Glomus* sp., *G. mosseae*, *Acaulospora* sp., *E. schenckii*, *S. fulgida*)			✓	✓		✓		✓		✓					
Ulrichs et al. (2008)	Solanaceae	*S. lycopersicum* L.	AM fungi (*Glomus* sp.)											✓				
Zouari et al. (2014)	Solanaceae	*S. lycopersicum* L.	AM fungi (*F. mosseae*)	✓														

Summary of the main parameters influenced by the interaction between plants, belonging to the "fruiting vegetables" category, and microorganisms (PGPR or AMF)

et al. (2010) (increase of chlorophylls, proteins, and minerals and reduction of starch and sugars in tomatoes of plants inoculated with *G. fasciculatum*), and by Ulrichs et al. (2008) (increment of lycopene, β-carotene, and phenolic contents in fruits of AM plants).

Finally, Schwarz et al. (2011) reported a modulation of different proteins potentially allergenics in tomato fruits, but they explain that this modulation is not sufficient to increase allergenic skin reactivity in a group of patients being allergic to tomato.

The eggplant or aubergine (*Solanum melongena* L.), belonging to the family Solanaceae, is cultivated worldwide for its fruits. Only one work reports the use of AMF (*G. fasciculatum*) to improve eggplant quality (Lenin et al. 2010), describing the accumulation of minerals, proteins, and chlorophylls in inoculated plants.

Capsicum annuum, with cultivated varieties including bell, sweet chili, and paprika peppers, is a perennial herbaceous plant belonging to the family Solanaceae, which originated in Central and South America and the Caribbean.

Different papers reported data concerning rhizospheric microorganisms (mostly PGPR) producing effects on fruit quality of *C. annuum*. In particular, Mena-Violante et al. (2006) and Kaya et al. (2009) reported results of AMF inoculation on fruits growth of chile ancho (*C. annuum* L. cv. San Luis) (Mena-Violante et al. 2006) and of pepper fruits (Kaya et al. 2009). Moreover, Castillo et al. (2009) reported that inoculation of *C. annuum* plants with *G. intraradices* and *Glomus claroideum* induces a precocity in fruit production (49 days earlier) and a 177 % higher fresh weight than fruits of uninoculated plants. Concerning PGPR effects on pepper fruits, del Amor et al. (2008) investigated the influence of a commercial product (Biopron), consisting of the bacteria *Azospirillum brasilense* and *Pantoea dispersa* on sweet pepper fruits, and reported an increased concentration of citric, ascorbic, and succinic acids in green fruits compared to the fruits of uninoculated plants. Significant effects on fruit growth were reported by Lee et al. (2011) in red

pepper inoculated with *Methylobacterium oryzae* CBMB20. The same result was reported by García-Fraile et al. (2012) in pepper var. "verde italiano" inoculated with *Rhizobium* strains and by Mandyal et al. (2012) in sweet pepper inoculated with *Bacillus* sp.

Cucumis sativus, the garden cucumber, is a widely cultivated plant in the gourd family (Cucurbitaceae), which includes squash, and in the same genus are muskmelon and cantaloupe. Cucumber originated in India, where it appears to have been cultivated for more than 3000 years, and then spread to China. Hundreds of cultivars of varying size and color are now grown in warm areas worldwide, commercially and in home gardens. Scanty data are available about the use of PGPR and AMF in cucumber cultivation. The effects of different AMF on the quality of cucumber fruits have been reported in a couple of papers. They included the increase of P and Zn concentration (after inoculation with *G. mosseae*, *G. etunicatum*, *G. clarum*, *G. caledonium*, and a mixture of them, Ortas 2010) and the enhancement of soluble sugar, amino acid, and soluble protein concentrations in fruits of inoculated (with *G. versiforme*, *G. mosseae*, and *G. intraradices*) plants of *C. sativus* (Lu et al. 2006).

5.6 Cabbages and Leaf and Stem Vegetables (Table 5.5)

Cabbages are a popular, nutritious vegetable crop, belonging to the family Brassicaceae, which also includes brussels sprouts, cauliflower, broccoli, turnips, etc. They are very rich in vitamin C, vitamin A, and vitamin E which, together with cysteine, glucosinolate, and sulforaphane, are antioxidant components and whose ingestion protects against free radicals and the diseases that they cause, such as different types of cancer (Rosen et al. 2005). To our knowledge, there are few data concerning the use of bioinoculants (only PGPR because most Brassicaceae do not form mycorrhizae) in cabbage cultivation and the effects that these microorganisms could have on crop quality. Verma and Maurya (2013) reported the effects of *P. fluorescens* on cabbage cv. Golden and Yildirim (2011) who investigated the influence of *Bacillus cereus* (N_2 fixing), *Brevibacillus reuszeri* (P solubilizing), and *Rhizobium rubi* (both N_2 fixing and P solubilizing) on broccoli. In both studies, size and mineral concentration are enhanced following the inoculation with PGPR. Moreover, Yildirim (2011) demonstrated that heads of cabbages inoculated with the tree strains tested have higher chlorophyll content than controls (14.7 %, 14.0 %, and 13.7 %, respectively, in plants inoculated with *B. cereus*, *R. rubi*, or *B. reuszeri*).

Lettuce (*Lactuca sativa* L.) is one of the most used vegetables in the human diet. So, the total production of lettuce and chicory in the European Union in 2010 achieved 3,000,000 t (FAO Statistic Division), with Italy and Spain as main producers. This vegetable exhibits healthy properties mainly due to anthocyanins, carotenoids, and phenolics, with high fiber content and useful amounts of some minerals in its tissues (Azcón et al. 2003; Baslam et al. 2011a). These characteristics are modulated by both the use of fertilizers and the inoculation with AMF. In particular, the association of lettuce with AMF (a commercial inoculum consisting in a mixture of *G. intraradices* and *G. mosseae*) results in a greater quantity of anthocyanins in plants grown with different fertilization (Hewitt solution and/or water-insoluble fraction of a "rhizosphere-controlled fertilizer") (Baslam et al. 2011a, b, 2013). To our knowledge, no results are reported in the literature about the effects of PGPR on lettuce.

Fennel (*Foeniculum vulgare* Mill.) belongs to the family Apiaceae. Besides its use as a vegetable, the pharmacopoeial use is focused on the fruits and the important ingredient is the oil (Mahmoud and Hassan 2013). Fennel is composed of a white or pale green bulb from which closely superimposed stalks are arranged. The stalks are topped with feathery green leaves near which flowers grow and produce fennel seeds. The bulb, stalk, leaves, and seeds are edible. Fennel is also considered as a spice due to

Table 5.5 Cabbages, leaf, and stem vegetables

Reference	Plant family	Plant species	Rhizosphere microbe	Fruit size	Sugars	Organic acids	Minerals	Carotenoids	Flavonoid	Chlorophylls	Phenol compounds
Azcón et al. (2003)	Compositae	*L. sativa* L.	AM fungi (*G. mosseae*)				✔				
Baslam et al. (2011a)	Compositae	*L. sativa* L. var. capitata, *L. sativa* var. longifolia	AM fungi (*G. fasciculatum*, AEGIS endo granulo)				✔	✔	✔		✔
Baslam et al. (2011b)	Compositae	*L. sativa* L. var. Capitata	AM fungi (AEGIS endo granulo: commercial inoculum containing *G. intraradices* and *G. mosseae*)		✔	✔	✔	✔	✔	✔	✔
Baslam et al. (2013)	Compositae	*L. sativa* Cogollos de Tudela (CT) (var. longifolia); Batavia Rubia Munguía (BRM) (var. Capitata); Maravilla de Verano (MV) (var. Capitata)	AM fungi (*G. fasciculatum*; commercial inoculum AEGIS Endo Gránulo (*R. intraradices F. mosseae*))	✔			✔	✔	✔	✔	
Ceccarelli et al. (2010)	Asteraceae	*C. cardunculus* L. var. *scolymus*	AM fungi (*G. intraradices*, *G. mosseae*)								✔
Mahmoud and Hassan (2013)	Apiaceae	*F. vulgare*	Biofertilizer + compost	✔			✔			✔	
Verma and Maurya (2013)	Brassicaceae	*Brassica oleracea* var. capitata cv. Golden acre	PGPR (*P. fluorescens*)	✔			✔				
Yildirim (2011)	Brassicaceae	*B. oleracea* var. italica	PGPR (*B. cereus*, *B. reuszeri*, *R. rubi*)	✔			✔			✔	

Summary of the main parameters influenced by the interaction between plants, belonging to the "cabbage, leaf, and stem vegetables" category, and microorganisms (PGPR or AMF). The term "fruit size" includes weight, size, and biomass of the crop fruit. References are listed in alphabetical order

terpenic compounds isolated from its fruits. It has been demonstrated that the inoculation with biofertilizers in association with compost enhances production of essential oil and chlorophylls (Mahmoud and Hassan 2013).

A special case is that of artichoke (*Cynara cardunculus* L. var. *scolymus*) because flower heads constitute the edible part. An increase in total phenolic content and antioxidant activity was reported in the leaves and flower heads after inoculation with *G. intraradices*, either alone or in mixture with *G. mosseae*. The effect was observed both in greenhouse and in field experiments (Ceccarelli et al. 2010).

5.7 Fresh and Grain Legumes (Table 5.6)

Common beans represent the most important source of dietary proteins from plants, especially in developing countries, where its cultivation has often been relegated to marginal areas, characterized by poor soils (Ballesteros-Almanza et al. 2010). So, the use of biofertilizers can improve soil production and restoration of soil fertility (P and N availability). Yadegari and Rahmani (2010) demonstrated that the inoculation with *P. fluorescens* P93, *Azospirillum lipoferum* S21, and *Rhizobium* strains 133 and 136 increases fruit number, fruit size, and protein concentration in bean.

Chickpea (*Cicer arietinum*) is the second most widely grown legume crop after soybean, accounting for a substantial proportion of human dietary nitrogen intake and playing a crucial role in food security in developing countries. The inoculation with PGPR *A. brasilense*, as reported in Hamaoui et al. (2001), enhances chickpea fruit number and yield.

Faba bean is a legume which is used as a green vegetable or dried, fresh, or canned in the Middle East, Mediterranean region, China, and Ethiopia. Also, it is considered as a cash crop in Egypt and Sudan (El Wakeil and El Sebai 2007). Two papers report the effects of PGPR inoculation on faba bean quality: in the first, Hewedy

Table 5.6 Fresh and grain legumes

Reference	Plant family	Plant species	Rhizosphere microbe	n. fruits	Fruit size	Protein	Total solids	Sugar	Vitamins	Minerals
Abbas (2013)	Fabaceae	*Vicia faba* cv. Giza 3	PGPR (*B. licheniformis*)		↘			↘	↘	
Hamaoui et al. (2001)	Fabaceae	*C. arietinum*	PGPR (*A. brasilense*)	↘	↘					
Hewedy (2011)	Fabaceae	*Vicia faba* cv. Giza 843	PGPR (*S. chibaensis*)	↘	↘				↘	
Kumari et al. (2010)	Fabaceae	*Pisum sativum* L.	PGPR (*Rhizobium* sp.)			↘				↘
Yadegari and Rahmani (2010)	Fabaceae	*Phaseolus vulgaris*	PGPR (*P. fluorescens* P93, *A. lipoferum* S21, *Rhizobium strain* 133 and 136)	↘	↘	↘				

Summary of the main parameters influenced by the interaction between plants, belonging to the "fresh and grain legumes" category, and microorganisms (PGPR or AMF). The term "fruit size" includes weight, size, and biomass of the crop fruit. References are listed in alphabetical order

(2011) showed that inoculation of faba bean with *Streptomyces chibaensis* induces an increased accumulation of minerals (N, P, K), proteins, and vitamins (ascorbic acid, riboflavin, and thiamin) and a yield enhancement (Hewedy 2011). In the second, Abbas (2013) described the influence of biostimulants (bacteria and yeast) on the growth and biochemical composition of faba bean. In particular, this work demonstrated that, besides increased biomass production, biostimulants (*Bacillus licheniformis* and, interestingly, also yeast) significantly increase pigment, carotenoid, and total carbohydrate content (Abbas 2013).

Finally, only one work reported the effects of rhizobial inoculation on the quality of pea: Kumari et al. (2010) reported that seed inoculation with biofertilizer (*Rhizobium* and other PGPR) induces an increase in the number and size of peas.

To our knowledge, no data are available on the effects of AMF on bean quality. This is an important point because AMF could be very relevant for the exploitation of marginal areas.

5.8 Cereals (Table 5.7)

According to FAO, cereals are defined as a group of species generally, but not exclusively, belonging to the gramineous family (i.e., Poaceae) that produce dry seeds rich in starch. Therefore, this word does not define a botanical taxon. This definition excludes Poaceae used as fodder, but includes plants of other families that produce grain seeds with high starch content. The Poaceae cereals (as wheat, rice, maize, sorghum, oats, barley, rye, millet, etc.) are sometimes considered "true cereals," while species belonging to other families, like buckwheat, quinoa, and amaranthus, are referred to as "pseudo-cereals." Because of their high content of starch, cereals are the staple food for mankind. Rice is the main cereal in Asia, wheat in Europe, and maize in South America; however, the most largely cultivated species is maize, because of its multiple use, as a food and as a source of bioplastic and biofuel. However, several cereal seeds also contain proteins and oils. The concentration of these two groups of substances can be important for their features, for instance, durum wheat is considered of better quality when richer in proteins, and oils can be extracted for use as well (as it happens for maize and rice). Concentration of proteins and oils is often regarded as a measure of cereals quality.

Cereals have been selected for disease resistance and high yield traits and not for their ability to form mycorrhizae. However, colonization has been reported, either in field or controlled conditions, for most species of this group (Raju et al. 1990; Braunberger et al. 1991; Secilia and Bagyaraj 1994; Hawkins and George 1997; Zhu et al. 2003; Likar et al. 2008; Criado et al. 2015), with the exception of quinoa (*Chenopodium quinoa*), which shows negligible AM root colonization and no arbuscule formation at all (Urcelay et al. 2011). This is not strange, since it belongs to the family Chenopodiaceae, known to include mostly non-mycorrhizal species.

Cereals inoculated with soil microorganisms (AMF, bacteria or their combination) have been tested for resistance to drought (Sajedi et al. 2010), for response to various levels of P availability (Braunberger et al. 1991; Al-Karaki and Clark 1999), to high temperatures, salinity, or pathogens. Results often showed better P nutrition, higher biomass and grain yield (also related to a higher number of tillers – Khan and Zaidi 2007; Midrarullah and Mirza 2014), increased concentration of Zn in grains (Sharif et al. 2011; Subramanian et al. 2013; Berta et al. 2014; Abaid-Ullah et al. 2015), and lower concentrations of some potentially phytotoxic elements, like Mn or Al (Karagiannidis and Hadjisavva-Zinoviadi 1998; Cornejo et al. 2008), in comparison to uninoculated plants.

Al-Karaki and Clark (1999) were the first to investigate parameters related with quality in mycorrhizal plants of durum wheat grown at different P concentrations. The grain protein and lipid concentration are not increased by AM colonization, but their total content per plant is increased due to enhanced yield (via

Table 5.7 Cereals

Reference	Plant family	Plant species	Rhizosphere microbe	n. fruits	Fruit size	Starch	Minerals	Lipid	Protein	Carotenoids
Abaid-Ullah et al. (2015)	Poaceae	*T. aestivum* L.	PGPR (*S. liquefaciens*, *S. marcescens*, *B. thuringiensis*)		✔		✔			
Al-Karaki and Clark (1999)	Poaceae	*T. durum* L.	AM fungi (*G. mosseae*)				✔	✔	✔	
Behara and Rautaray (2010)	Poaceae	*T. turgidum* var. durum	PGPR (*Azotobacter* sp., *A. brasilense*, *P. vulgaris*, *Kurthia* sp., *K. planticola*, *B. subtilis*); AM fungi (*G. fasciculatum*)							✔
Behn (2008)	Poaceae	*T. aestivum* L.	PGPR (*P. fluorescens* RA56); AM fungi (*G. mosseae*)	✔	✔					
Berta et al. (2014)	Poaceae	*Zea mays*	PGPR (*P. fluorescens* Pf4); AM fungi (commercial inoculum Mybasol s.r.l. (*R. intraradices*, *G. aggregatum*, *G. viscosum*, *G. etunicatum*, *G. claroideum*))		✔	✔	✔		✔	
Cornejo et al. (2008)	Poaceae	*T. aestivum* L.	AM fungi (*G. etunicatum* CH110)				✔			
Criado et al. (2015)	Poaceae	*Hordeum vulgare*	AM fungi (*R. intraradices*)		✔		✔			
Karagiannidis and Hadjisavva-Zinoviadi (1998)	Poaceae	*Triticum turgidum* var. durum	AM fungi (*G. mosseae*)				✔			
Khan and Zaidi (2007)	Poaceae	*T. aestivum* L.	PGPR (*A. chroococcum*, *Bacillus* sp.); AM fungi (*G. fasciculatum*)		✔					
Kumar et al. (2011)	Poaceae	*T. aestivum* L.	AM fungi (*G. mosseae*)							
Midrarullah and Mirza (2014)	Poaceae	*Oryza sativa*	PGPR (*A. brasilense* R1, *A. lipoferum* RSWT1, and *Pseudomonas* Ky1)	✔						
Sajedi et al. (2010)	Poaceae	*Z. mays* L.	AM fungi (*G. intraradices*)	✔	✔					

Sharif et al. (2011)	Poaceae	*Z. mays*, *Sorghum bicolor L.*, *Pennisetum glaucum L.*, *Vigna mungo L.*, *Vigna radiata*	AM fungi (*G. fasciculatum*, *G. intraradices*, *G. mosseae*, *G. aggregatum*, *A. mellea*)	✓	✓			✓			
Subramanian et al. (2013)	Poaceae	*Z. mays* L.	AM fungi (*G. intraradices*)								

Summary of the main parameters influenced by the interaction between plants, belonging to the "cereals" category, and microorganisms (PGPR or AMF). The term "fruit size" includes weight, size, and biomass of the spike or grain. References are listed in alphabetical order

increased number of tillers). Plants grown at high P concentration show a similar effect, and thus the mycorrhizal effect might be explained as nutritional.

More recently, three further studies have explored the consequences of inoculation with combinations of plant beneficial microorganisms on the quality of grains in cereals. Khan and Zaidi (2007) have investigated the effect of several combinations of plant growth-promoting (PGP) microorganisms, with or without the AMF *G. fasciculatum*, on the growth, yield, and quality of *Triticum aestivum*. The different PGP microorganisms had been selected because of their ability to fix nitrogen (*Azotobacter chroococcum*) or solubilize phosphate (the bacterium *Bacillus* sp. 8 and the fungus *Penicillium variabile*). While not all the possible combinations were tested, it is most interesting and valuable that the experiment was run as a field test. In terms of productivity, the various inoculants are generally able to increase, at different degrees, the plant biomass (often because of the increased number of tillers), straw production, and grain yield. For what concerns quality, single inoculations are generally not able to increase the grain protein content compared to the control, with the remarkable exception of *A. chroococcum* which leads to a grain protein content that is 2.4 times higher than that of the uninoculated controls. Overall, the multiple combinations (inocula consisting of three or four microbes) were consistently resulting in better performance for grain protein content (about twice the control). While the positive effect of a N-fixing bacterium such as *A. chroococcum* on protein accumulation is not surprising, the effect of this microbe is also related to its ability to produce various growth-promoting molecules, affecting wheat growth and development. The effects observed for combined microorganisms are often larger than the sum of the single increases, suggesting a synergistic effect. It must also be observed that *P. variabile* mostly exerts a negative effect on the various parameters considered (in terms of growth, yield, and grain protein content), alone or in combination with other microbes.

The previous study concerned one AMF and three other PGP microorganisms; on the contrary, the effects of a mycorrhizal inoculum containing five fungal species (*R. intraradices*, *G. aggregatum*, *G. viscosum, G. etunicatum*, and *G. claroideum*) and/or of a single bacterial strain (*P. fluorescens* strain Pf4, a PGPB able to produce siderophores and IAA and to solubilize phosphate) were assessed in maize by Berta et al. (2014). Beyond improving plant yield in several ways (increasing seed size, number of seeds per spike, and spike length and width), they are also able to differently influence the storage compounds in the grains. Bacteria increase starch content (and decrease the protein content), while AMF induce a higher accumulation of proteins (either soluble or unsoluble) and reduce the amount of digestible starch. Each inoculum (AM mix, Pf4, or their co-inoculation) also results in higher concentration of iron and zinc in the seeds. Results concerning proteins are of special interest, since the main maize allergens are found to the water-soluble protein group (Pastorello et al. 2009).

The third study concerns *Hordeum vulgare*. The commercial quality of malting barley after inoculation with *R. intraradices* was evaluated by Criado et al. (2015).

For malting barley, grain size and N concentration (ideally between 1.6 % and 1.8 %) are some of the most important features. The AM symbiosis does not affect the grain size in a significant way, but it increases N concentration in seeds, while in non-mycorrhizal plants the grain N concentration does not meet the standards required by industry.

In addition, a short note by Kumar et al. (2011) describes the effects of wheat inoculation with *G. mosseae* and seed priming (soaking overnight before sawing) on yield and grain quality. The authors claim that "The fungal inoculation improved the quality of the grain, increasing protein content and wet gluten. The study revealed that the agricultural practices followed in the region are adversely affecting the colonization of indigenous mycorrhizae" but the data included in the paper suggest for a possible effect by seed priming and not by inoculation.

Rice is a special case among cereals, because it is generally grown on (temporarily) flooded soils. Early studies described many wetland plants as non-mycorrhizal (e.g., Khan 1974; Anderson et al. 1984). However, the AM colonization of several hydrophytic species has been reported (Brown and Bledsoe 1996; Turner et al. 2000; Bohrer et al. 2004), and rice plants form arbuscular mycorrhiza; yield of AM rice plants improves even when they are flooded (Solaiman and Hirata 1997). Arsenic accumulation is a major problem in rice cultivation because of the toxic and carcinogenic effects of this element. Since arsenate can use phosphate transporters, the role of AMF is of special interest in this context. The concentration of As, in mycorrhizal plants, seems to vary according to the different plant cultivars and fungal species (Li et al. 2011) or their combinations (Chan et al. 2013). This fact opens the possibility to select the best formulations of fungal specie (singly or in a mixture) to be inoculated in chosen rice varieties and, therefore, improve rice quality in terms of healthiness, lowering As content.

Some studies describe the lack of effects on quality. Behn (2008) reports the testing of *G. mosseae* and *P. fluorescens* strain RA56, alone or in combination, for protective effects against *Gaeumannomyces graminis* in *T. aestivum*. While both the microorganisms (alone or in combination) are able to protect the plant from the pathogen, they fail to affect the grain quality, measured as protein content; this is true with and without the pathogen. In another paper, the effect of various microbes (including AMF and phosphate-solubilizing and N-fixing bacteria) was tested on *Triticum turgidum* at 50 % fertilization for N, P, and K and compared with 100 % fertilization and unfertilized controls. The bacteria (alone or in combination) was unable to improve any of the quality parameters (β-carotene concentration, yellow berry, protein content, hectoliter weight) when compared with uncolonized plants at 100 % or 50 % fertilization; AM plants were the worst performers, but it must be noted that the fungal species composition of the inoculums is not indicated and the level of colonization was not assessed (Behara and Rautaray 2010).

Overall, the above mentioned results clearly indicate a role for microorganisms in determining the seed quality of cereals, with possible positive effects on the grain concentration of mineral nutrients (like Cu, Fe, or Zn), lipids, and proteins. In addition, inoculated plants often show a significantly higher yield and larger seeds. Inoculation with more than one microorganism frequently results in better results than single inoculations.

5.9 Aromatic Plants, Spices, Tea, Coffee, Herbal Infusion, and Cocoa (Table 5.8)

Several plants mainly belonging to the families Lamiaceae, Asteraceae, and Apiaceae are included in this category. Some of them are known as aromatic plants, while others as spices or drugs. They are cultivated worldwide for pharmaceutical, herbal, cosmetic, and culinary uses. A number of studies demonstrate that the associations of PGPR and AMF with aromatic and officinal plants positively affect the quantitative and/or qualitative profile of the secondary metabolites (e.g., essential oils and alkaloids), in addition to improved growth parameters, biomass and yield (Khaosaad et al. 2006; Copetta et al. 2007; Banchio et al. 2008; Arango et al. 2012; Roshanpour et al. 2014; Silva et al. 2014).

The genus *Ocimum* comprises more than 150 species and is one of the most representative genera of the family Lamiaceae (Evans 1996). *Ocimum basilicum* L. (sweet basil) is cultivated in several regions all over the world. It is an annual herb used as food flavoring, with antiviral/antimicrobial activities and good medicinal properties, mostly due to its essential oils, stored in leaf glandular hairs (in particular eugenol and linalool) (Simon et al. 1990; Jirovetz et al. 2003; Toussaint et al. 2008; Roshanpour et al. 2014). Because of all the above mentioned qualities, its cultivation is of great economic interest.

Copetta and coworkers (2007) tested the effects of three species of AMF (*G. mosseae*, *Gigaspora margarita*, and *G. rosea*) on sweet basil plants (*O. basilicum* var. Genovese) showing that these fungi differently affect the essential oil production: *G. mosseae* does not alter the concentration of the analyzed compounds relative to control plants, while *G. rosea* and *G. margarita* significantly increase bornyl acetate and methyl eugenol, respectively; both the *Gigaspora* species enhance δ-cadinene content. A modulation of the essential oil profile due to the AMF inoculation was also observed by Toussaint et al. (2008) in plants of the same variety in the presence of *G. mosseae* and by Zolfaghari et al. (2012) in *O. basilicum* L. inoculated with *G. mosseae*, *G. intraradices*, or *G. fasciculatum* (with the best results by *G. mosseae* and *G. fasciculatum*). Not only AMF but also PGPR influence the synthesis of basil essential oils. In fact, Banchio et al. (2009) found an increase of R-terpineol and eugenol in leaves of plants exposed either to airborne volatiles from the beneficial soil bacterium *Bacillus subtilis* (GB03) or to root inoculation with the same microorganism. Integrated application of three different bacteria, *A. chroococcum*, *A. lipoferum*, and *Bacillus circulans*, improves the essential oil yield and shoot biomass of basil plants (Roshanpour et al. 2014). Similar results on shoot parameters were reported by Maleki et al. (2013) in plants co-inoculated with AMF (*G. intraradices*) and PGPR (*A. lipoferum* and *A. chroococcum*).

Genus *Salvia* is another important and representative genus of the family Lamiaceae. *Salvia officinalis* L. is used either in food industry (as herb or spice) or in the pharmaceutical sector (against inflammations and infections of the mouth and throat). As basil, sage antioxidant activity is due in great part to the presence in leaves of essential oils (Deans and Simpson 2000) and of two polyphenol compounds, rosmarinic and carnosic acid (Cuvelier et al. 1996). It was shown that besides the AM symbiosis, the nutrient supply, especially P, affects secondary metabolism in sage (Nell et al. 2009; Geneva et al. 2010). Nell

Table 5.8 Aromatic plants, species, tea, coffee, herbal infusion, and cocoa

Reference	Plant family	Plant species	Rhizosphere microbe	Biomass	Minerals	Organic acids	Carotenoids	Flavonoid	Chlorophylls	Xanthophylls	Phenol compounds	Terpenoids	Alkaloids	Essential oil
Al-Fraihat et al. (2011)	Lamiaceae	*Majorana hortensis*, L. (or *Origanum majorana* L.)	PGPR (The nitrobein: *Azotobacter*, a nitrogen-fixing bacteria; Halex-2, a mixture of N-fixing bacteria of the genera *Azotobacter*, *Azospirillum*, and *Klebsiella*)											✔
Arango et al. (2012)	Lamiaceae	*Mentha piperita*	AM fungi (*G. mosseae* isolate SB1 (Spegazzini Institute Collection, UNLP); *G. intraradices*, isolate A4; *G. intraradices*, isolate B1 Glomeromycota (In Vitro Bank, BGI, Buenos Aires, Argentina))		✔									✔
Awasthi et al. (2011)	Asteraceae	*Artemisia annua*	PGPR (*B. subtilis*, *Stenotrophomonas* sp.); AM fungi (*G. mosseae*, *G. aggregatum*, *G. fasciculatum*, *G. intraradices*)	✔									✔	
Bagyalakshmi et al. (2012)	Theaceae	*Camellia sinensis*	PGPR (*P. putida* strain KSB (potassium-solubilizing bacteria))		✔		✔		✔		✔		✔	
Banchio et al. (2008)	Lamiaceae	*Origanum majorana* L.	PGPR (*P. fluorescens* WCS417r; *B. subtilis* 09; *S. meliloti* Rm1021; *Bradyrhizobium* sp. USDA 4438)	✔										
Banchio et al. (2009)	Lamiaceae	*Ocimum basilicum*	PGPR (*B. subtilis* strain GB03)											✔
Bharti et al. (2013)	Lamiaceae	*Mentha arvensis* cv. Kosi	AM fungi (*G. mosseae*, *G. aggregatum*, *G. fasciculatum*, *G. intraradices*)	✔			✔		✔					✔
Binet et al. (2011)	Asteraceae	*Artemisia umbelliformis*	AM fungi (*G. tenue*, *G. intraradices*, *G. claroideum*/*etunicatum*, *Acaulospora* sp. (isolated from soil))											✔
Chaudhary et al. (2008)	Asteraceae	*Artemisia annua* L.	AM fungi (*G. macrocarpum*, *G. fasciculatum*)		✔									
Chulan (1991)	Sterculiaceae	*Theobroma cacao* L.	AM fungi (*S. calospora*)											

Copetta et al. (2006, 2007)	Lamiaceae	*Ocimum basilicum*	AM fungi (*G. mosseae*, *G. margarita*, *G. rosea*)											✔
Darzi et al. (2012)	Apiaceae	*Anethum graveolens*	PGPR (mixture of *A. chroococcum* and *A. lipoferum*)								✔			✔
Farahani et al. (2008)	Apiaceae	*Coriandrum sativum*	AM fungi (*G. hoi*)	✔										✔
Freitas et al. (2004)	Lamiaceae	*Mentha arvensis* L.	AM fungi (*G. clarum*, *G. etunicatum*, *G. margarita*, *A. scrobiculata*)	✔										
Geneva et al. (2010)	Lamiaceae	*Salvia officinalis* L.	AM fungi (*G. intraradices*)			✔		✔			✔			✔
Gupta et al. (2002)	Lamiaceae	*Mentha arvensis* L. (cv. Kalka, Shivalik, and Gomati)	AM fungi (*G. fasciculatum*)	✔	✔									✔
Jurkiewicz et al. (2010)	Asteraceae	*Arnica montana*	AM fungi (*G. intraradices* or inoculum composed of several *Glomus* strains)								✔			
Kapoor et al. (2002a)	Apiaceae	*C. sativum*	AM fungi (*G. macrocarpum*, *G. fasciculatum*)		✔									✔
Kapoor et al. (2002b)	Apiaceae	*Anethum graveolens* L.	AM fungi (*G. macrocarpum*, G. *fasciculatum*)											✔
Kapoor et al. (2004)	Apiaceae	*F. vulgare* Mill	AM fungi (*G. macrocarpum*, *G. fasciculatum*)		✔									✔
Kapoor et al. (2007)	Asteraceae	*Artemisia annua* L.	AM fungi (*G. macrocarpum*, *G. fasciculatum*)											✔
Karagiannidis et al. (2011)		Oregano and mint (collected from Greek mountain)	AM fungi (two strains *G. etunicatum* and one *G. lamellosum* (collected from field))		✔									✔
Khaosaad et al. (2006)	Lamiaceae	*Origanum vulgare* ssp.	AM fungi (*G. mosseae*)	✔										✔
Maleki et al. (2013)	Lamiaceae	*Ocimum basilicum* L.	PGPR (*A. chroococcum*, *A. lipoferum*); AM fungi (*G. intraradices*)	✔										
Morone-Fortunato and Avato (2008)	Lamiaceae	*Origanum vulgare*	AM fungi (*G. viscosum*)	✔										✔

(continued)

Table 5.8 (continued)

Reference	Plant family	Plant species	Rhizosphere microbe	Biomass	Minerals	Organic acids	Carotenoids	Flavonoid	Chlorophylls	Xanthophylls	Phenol compounds	Terpenoids	Alkaloids	Essential oil
Nell et al. (2009)	Lamiaceae	*Salvia officinalis* L.	AM fungi (*G. mosseae* (BEG 12) and *G. intraradice* (BB-E)-BIORIZE/AGRAUXINE; commercial inoculum "Symbivit®" consisting of *G. mosseae, G. intraradices, G. cladoideum, G. microagregatum, G. caledonium* and *G. etunicatum*)		✔									✔
Nell et al. (2010)	Valerianaceae	*Valeriana officinalis* L.	AM fungi (*G. mosseae* and *G. intraradices* (BB-E), BIORIZE/AGRAUXINE; commercial inoculum "Symbivit®" consisting of *G. mosseae*, *G. intraradices*, *G. cladoideum*, *G. microagregatum*, *G. caledonium*, and *G. etunicatum*)	✔								✔		
Oliveira et al. (2014)	Passifloraceae	*Passiflora alata* Curtis	AM fungi (*G. albida*)					✔			✔			
Roshanpour et al. (2014)	Lamiaceae	*Ocimum basilicum*	PGPR (*A. chroococcum*, *A. lipoferum*, *B. acillus circulans*)	✔										✔
Silva et al. (2014)	Lamiaceae	*Mentha x piperita* L. var. citrata	AM fungi (*A. morrowiae*, *R. clarus*, *S. calospora*)	✔	✔									✔
Singh et al. (2010)	Theaceae	*Camellia sinensis* (L.) O. Kuntze	AM fungi (consortia containing: *A. spinosa*, A. sp. 1, *G. aggregatum*, *G. ambisporum*, *G. clavisporum*, *G. geosporum*, *G. mosseae*, *G. pustulatum*, and *Glomus* sp. 7.; consortia containing *A. foveta*, *A. scrobiculata*, *G. ambisporum/heterosporum*, *G. clarum*, *G. fasciculatum*, *G. heterosporum*, *G. intraradices*, *Glomus* sp. 6, *Glomus* sp. 8, *Glomus* sp. 9, and *Glomus* sp. 10)	✔							✔		✔	

Toussaint et al. (2008)	Lamiaceae cv. Genovese	*Ocimum basilicum*	AM fungi (*G. mosseae*)						✔			✔		
Zitterl-Eglseer et al. (2015)	Apiaceae	*Angelica archangelica* L.	AM fungi (*G. mosseae* and *G. intraradices* (BB-E), BIORIZE/AGRAUXINE; commercial inoculum Symbivit containing *G. mosseae* (BEG 99), *G. intraradices* (BEG 98), *G. claroideum* (BEG 93), *G. microaggregatum* (BEG 56), *G. caledonium* (BEG 97), and *G. etunicatum* (BEG 92))	✔	✔							✔		
Zolfaghari et al. (2012)	Lamiaceae	*Ocimum basilicum*	AM fungi (*G. mosseae*, *G. fasciculatum*)	✔										✔
Zubek et al. (2012)	Clusiaceae	*Hypericum perforatum*	AM fungi (*R. intraradices* (BEG 140); *F. mosseae*; mix: *F. constrictum* (262-5 Walker), *F. geosporum* (UNIJAG.PL.12-2), *F. mosseae*, *R. intraradices* (BEG 140))	✔									✔	

Summary of the main parameters influenced by the interaction between plants, belonging to the "aromatic plants, spices, tea, coffee, herbal infusion, and cocoa" category, and microorganisms (PGPR or AMF). The term "biomass" includes weight, size, and biomass of the shoots. References are listed in alphabetical order

et al. (2009), testing three different fungal inocula and two levels of phosphate, observed that P supply, but not AM inoculation, increases total phenolic and rosmarinic acid concentrations in leaves of *S. officinalis*. However, Geneva et al. (2010) observed that AM colonization combined with foliar fertilization (N, P, K) improves dry biomass production and enhances the content of antioxidant metabolites, such as ascorbate and reduced glutathione. Plants inoculated with *G. intraradices* also show an increase of essential oil content, in particular bornyl acetate, 1,8-cineole, and α- and β-thujones, while foliar fertilization increased bornyl acetate and camphor. Similar results were obtained by Karagiannidis et al. (2011) in AM-oregano plants grown at different P levels.

However, in some cases the improvement of essential oil production is not directly dependent on mineral nutrition (especially P) as observed by Khaosaad et al. (2006) on three cultivars of *Origanum vulgare* L. inoculated with *G. mosseae*. This is in accordance with results obtained by Copetta et al. (2006) in sweet basil plants. In such cases other factors as plant genotype, fungal species, and their interaction should be considered. An analysis of the components of essential oils was conducted in micropropagated oregano plants, inoculated or not with *G. viscosum*, at various times after transplanting, showing a combined effect of time and inoculation on the molecular composition of the oil (Morone-Fortunato and Avato 2008).

Majorana hortensis Moench., also known as *Origanum majorana* L., is a perennial herbaceous plant from the Mediterranean area. It has a peculiar, spicy smell and taste due to its isoprenoid compounds of which pinene, limonene, linalool, terpinene, and eugenol are the most abundant (Al-Fraihat et al. 2011). The symbiotic interaction between *O. majorana* plants and four different PGPR results in the increase of essential oil content, depending on the rhizobacterial species, without alteration of oil composition (Banchio et al. 2008). The best results were observed in presence of *Bradyrhizobium* sp. and *P. fluorescens*. According to these findings, Al-Fraihat and coworkers (2011) reported an enhancement of essential oil content and yield per plant and per hectare, a larger biomass production, and an improvement of mineral accumulation in marjoram plants treated with two commercial inocula "nitrobein" and "Halex-2" containing one or more N-fixing bacteria, respectively. We couldn't find data on the effects of AMF on marjoram plant quality.

Different species of the genus *Mentha* are used in pharmaceutical, food, cosmetics, and beverages (Verma et al. 2010). Their typical flavor and aroma are due to the presence of essential oils, a natural source of menthol, menthone, isomenthone, menthofuran, carvone, linalool, and linalyl acetate (Verma et al. 2010; Bharti et al. 2013). Freitas et al. (2004), testing the effect of four AMF on plants of *Mentha arvensis* L. grown at different P levels, found a higher essential oil content (up to 89 %) in AM plants than in uninoculated ones. Similar results on the production of secondary metabolites were obtained by Gupta et al. (2002) on three cultivars of *M. arvensis* inoculated with *G. fasciculatum*. These results underline that not only different fungi induce different effects on the same cultivar but also that different cultivars produce different metabolites in the presence of the same fungus. In addition to this, as mentioned above, another factor should be considered: mineral nutrition (in particular N, P, K). When no phosphorus is added, AMF inoculation increases biomass production, volatile compound, and menthol content in *Mentha viridis* (Karagiannidis et al. 2011). No increment in essential oil and menthol content occurs at high P level. An enhancement of secondary metabolite production in *Mentha piperita* L. (peppermint) plants, inoculated with AMF in relation to nutrient availability and uptake, was reported also by Silva et al. (2014) and by Arango et al. (2012).

In addition to the plant belonging to the family Lamiaceae, others from the families Asteraceae, Apiaceae, Apocynaceae, Clusiaceae, Geraniaceae, Passifloraceae, and Valerianaceae produce many secondary metabolites of commercial interest, including terpenes, phenols, and alkaloids, whose biosynthesis is influenced

by the presence of microorganisms in the soil or in the growing medium. In particular, AMF increase the production of sesquiterpene lactones in *Arnica montana* (Jurkiewicz et al. 2010), geraniol and linalool in coriander (*Coriandrum sativum L.* – Kapoor et al. 2002a; Farahani et al. 2008), anethol in fennel (*F. vulgare* Mill. – Kapoor et al. 2004), and limonene and carvone in *Anethum graveolens* L. (Kapoor et al. 2002b). Nitrogen-fixing bacteria affect essential oil and carvone content in dill (Darzi et al. 2012).

Moreover, Kapoor et al. (2007), Chaudhary et al. (2008), Awasthi et al. (2011), and Binet et al. (2011) investigated the effects of AMF or PGPR on *Artemisia* plants showing a general improvement of plant mineral nutrition and a stimulation of bioactive compounds production. Mycorrhiza boost artemisinin content increasing the density of glandular trichome in leaves (Kapoor et al. 2007). This is in accordance with a previous study on *Ocimum basilicum* plants inoculated with three different AMF (Copetta et al. 2006).

Hypericum perforatum produces two important molecules including hypericin and pseudohypericin, known to have antiviral activity and inhibitory effects toward the acquired immune deficiency syndrome (AIDS). Zubek et al. (2012) observed that hypericin and pseudohypericin contents significantly increase in the presence of AM symbiosis.

Plants of *Passiflora alata* inoculated with *Gigaspora albida* produce a higher amount of biomolecules in leaves, especially flavonoids, in respect of control plants (Oliveira et al. 2014).

Similar results are reported also in some officinal plants including *Valeriana officinalis* (Nell et al. 2010) and *Angelica archangelica* (Zitterl-Eglseer et al. 2015) largely employed in herbal uses.

Theobroma cacao L. is a small tree of the family Sterculiaceae, native of South America. Its berries contain a variable number of seeds, depending on the plant cultivar. It is known either as drug, for the alkaloid content, mainly caffeine and theobromine, or as oleaginous plant since large amounts of cocoa butter are extracted from its seeds. For these qualities *T. cacao* is used in confectionery, pharmaceutical, and cosmetic industry. There are many studies concerning the extraction methods of alkaloids or cocoa butter and their different uses in industry (Li and Hartland 1996; Schulz 2004), but no studies on the effects of microbe inoculation on cocoa quality are found in the literature except one, in which an increase of nutrient uptake (N, P, K) in *Theobroma* plants inoculated with the AMF *Scutellospora calospora* was reported (Chulan 1991).

Tea (*Camellia sinensis*), belonging to the family Theaceae, is an important crop cultivated in different areas of the world. Chinese people (from which the name "sinensis" was derived) were the first to use tea as medicinal drink (Eden 1958). There are different varieties of *C. sinensis*. Their quality depends on many factors such as organic and inorganic composition of harvested shoots, plant mineral nutrition, and microbe interaction. Changes in the active compounds, flavonoids, amino acids, vitamins (C, E, K), caffeine, and polysaccharides are responsible for the different taste, flavor, and color (Mondal et al. 2004; Bagyalakshmi et al. 2012). It is noteworthy that the vitamin C content in tea leaves is the same one found in lemon (Mondal et al. 2004). Tea consumption has beneficial effects on human health because of its antioxidant and antimicrobial activity, besides protecting cell membranes from oxidative damages, preventing coronary heart diseases and normalizing blood pressure.

In literature, scanty data on the effects of AMF or PGPR inoculations on tea quality are reported. Singh et al. (2010) tested the effect of two different mixed fungal consortia, one containing indigenous populations of fungi and another containing cultivated AMF. The results showed an increase of amino acids, total protein, total polyphenols, sugar, and caffeine content in AM plants with better response in tea plants inoculated with native AMF.

Moreover, in a field experiment, conducted to investigate the effect of *Pseudomonas putida* (an indigenous potassium-solubilizing bacteria) on tea quality, Bagyalakshmi et al. (2012) observed an improvement of the content of

theaflavin, thearubigin, and caffeine and highly polymerized substances, total liquor color, briskness, and color and flavor indexes in plants inoculated with bacteria.

Finally, the effect of AMF on the quality of plants used in traditional medicine of China has been reviewed by Zeng et al. (2013).

5.10 Conclusion

In this chapter we have summarized the effects of soil microbes on different plant parts, concerning storage (roots, bulbs, tubers), vegetative (leaves), or reproductive (fruits) structures. Modifications concerned the accumulation of mineral nutrients, sugars, lipids, and a number of chemically diverse secondary metabolites. In some cases, the reported variations can be explained by the nutritional improvement operated by the microorganisms in favor of the plant, but this is not the rule.

The number of AMF species used in the described experiments is rather small even considering the limited number of Glomeromycota. It is clear that widening the range of tested AM species could only be positive for a better understanding of the effects induced in plants. Also the number of plants species is not extremely large (about 30). This is most likely related to the overwhelming importance of a relatively small number of plant species for agriculture, human and animal nutrition, and industry.

In spite of these limitations, some facts do emerge:

- Soil microbes can affect the quality of crops (often increasing yield as well).
- Even if most experiments have been carried under controlled conditions and on a reduced scale, the relatively rare tests run in the field show that a practical exploitation is feasible and can result in better products in real life agriculture.
- Combinations of different microorganisms often perform better than single inoculants; combinations can sometimes act synergistically, some other addictively.
- Different microorganisms can give different results on the same plant species (for instance, promoting the accumulation of a specific secondary metabolite). This opens to the possibility of using selected isolates or strains according to the environmental conditions or according to the kind of result or product that is expected.

Therefore, a new role and exploitation of AMF and PGPR, as enhancers of crop quality, can be described, in addition to increasing yield, providing valuable ecosystem services and protecting plants from pathogens. The selection of new crop cultivars should consider the ability of plant to interact with the beneficial soil microbes.

Acknowledgments To the memory of my friend Horst Vierheilig, who first prompted me to write this review, sitting by a swimming pool in Marrakech, in 2010. It was originally meant to be submitted to a journal. Then, several things happened, delaying, hampering, and stopping this work. In 2014 I was invited by Raffaella Balestrini to submit a chapter for the present book, and I immediately thought about this topic. Once more, I would not have been able to complete this chapter if it had not been for my fellow authors Elisa and Valeria.

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Part II

Diverse Applications

Biological Potential of Arbuscular Mycorrhizal Fungi

6

Raffaella Balestrini

Contents

Abstract

Microorganisms play a key role in preserving soil fertility in forest and agro-ecosystems. The exploitation of their beneficial traits represents a promising avenue for the development of more sustainable agriculture. In this chapter, attention is focused on arbuscular mycorrhizal (AM) fungi, considering the aspects that have been highlighted through the sequencing of the *Rhizophagus irregularis* genome and on the mechanisms involved in the nutritional exchanges that take place during their interaction with plants. Examples of the use of this group of fungi in applicative projects are also reported.

6.1 Introduction

Modern agriculture and farming practices are mainly based on the cultivation of high-yield varieties, combined with the use of agrochemicals, i.e., fertilizers and pesticides (Schlaeppi and Bulgarelli 2014). Fertilizers provide the main nutrients required by plants and, among these nutrients, phosphorus (P) represents a particularly critical component (Roy-Bolduc and Hijri 2011). As mineral fertilizers (such as phosphate) are derived from nonrenewable natural resources and agrochemicals are often hazardous to the environment, alternative and more

R. Balestrini (✉)
Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche (CNR), Viale Mattioli 25, 10125 Torino, Italy
e-mail: raffaella.balestrini@ipsp.cnr.it

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_6

sustainable practices should be explored (Schlaeppi and Bulgarelli 2014). Microorganisms play a key role in preserving soil fertility in forest and agro-ecosystems. The exploitation of beneficial microbial traits, for the host plant or the environment, represents a promising avenue for the development of a more sustainable next-generation agriculture (Schlaeppi and Bulgarelli 2014). Together with rhizobacteria, the most important biofertilizer microorganisms are mycorrhizal fungi, and in particular arbuscular mycorrhizal (AM) fungi, which form symbioses with the roots of the most important crop species (e.g., tomato, maize, wheat, rice, and potato). Mycorrhizal fungi are usually considered biofertilizers, since their best understood function in the symbiosis is an improvement in plant mineral nutrient acquisition, in exchange for C compounds derived from the photosynthetic process, which results in positive host growth responses as well as in a better response to stress conditions (Balestrini et al. 2015; van der Heijden et al. 2015). They are considered to be essential elements for plant nutrition as their hyphae can extend for many meters and they can uptake the mineral nutrients present in the soil and transfer them to the roots. In recent years, several works have been focused on verifying the effect of AM fungi on the edible portions of plants and on human-relevant nutrients (Giovannetti et al. 2012a; Hart et al. 2014; Sbrana et al. 2014; Zouari et al. 2014; Chap. 5 by Bona et al. in this book).

The characteristics of the soil in which plants grow are the first parameters that can influence the productivity as well as the product quality of plants. The reduction (or even the disappearance) of mycorrhizal fungal propagules, as a result of certain cultural practices in agricultural systems, such as the use of chemicals on the soil or soil treatments, can be considered as an indicator of the decreased quality of the plant–soil system (Giovannetti et al. 2006). AM fungi can be considered the most important microorganisms that need to be monitored in the environmental impact assessments of the cultural practices used in agriculture. Some saprotrophic fungal species can also play a beneficial role on plants and soil, by functioning as natural biocontrol agents. *Trichoderma* spp., the most commonly applied biocontrol fungi, has been studied extensively using a variety of research approaches (Mukherjee et al. 2013). Together with other beneficial microbes, they could be involved in the disease-suppressive phenomenon of soils in which crop plants suffer less from specific soilborne pathogens than expected (Mazzola 2002). In the last few years, the metagenomes of fungal communities associating with plant roots have been revealed, and great interest has been shown in having information about natural soil microbiota (Orgiazzi et al. 2012; Schlaeppi and Bulgarelli 2014; van der Heijden et al. 2015). Here, attention is focused on AM fungi, and in particular on the aspects highlighted through the sequencing of *R. irregularis* genome (Tisserant et al. 2013; Lin et al. 2014), and on the mechanisms involved in the nutritional exchanges that take place during the interaction with the plants. Examples of the use of this group of fungi in applicative projects are also reported.

6.2 Looking Inside the Genome of an AM Fungus

AM fungi are obligate biotrophs that colonize the roots of plants and form highly branched structures (arbuscules) inside the cortical cells of the roots (Fig. 6.1). Although AM fungal

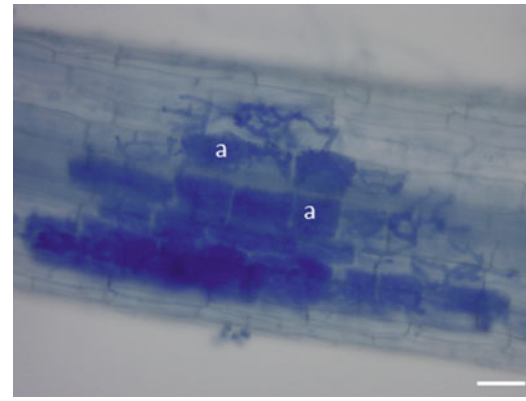

Fig. 6.1 Tomato root colonized by an AM fungus (*Cotton blue staining*), where intraradical hyphae and arbuscules (a) are visible. Bar, 40 μm

spores can germinate in the absence of host plants (pre-symbiotic phase), they depend on the establishment of intracellular symbiosis to complete the fungal life cycle and produce the next generation of spores. The genome of the AM fungus *R. irregularis* DAOM-197198 (formerly *Glomus intraradices*) was the third mycorrhizal genome to be published (Tisserant et al. 2013; Lin et al. 2014). Interestingly, the observed low level of genome polymorphism does not support the presence of highly diverged multiple genomes in the multinucleated *R. irregularis* (Tisserant et al. 2013). A *de novo* genome sequencing of the individual nuclei of *R. irregularis* has revealed a low level of polymorphism between the nuclei (Lin et al. 2014), in agreement with the previously mentioned result. The first three sequenced mycorrhizal genomes, i.e., the ectomycorrhizal *Laccaria bicolor* (Martin et al. 2008) and *Tuber melanosporum* (Martin et al. 2010) and the AM fungus *R. irregularis*, show substantial losses in plant cell wall-degrading enzymes. Recently, new fungal genomes, including ectomycorrhizal, orchid, and ericoid species, have been sequenced with the aim of elucidating the genetic bases of the mycorrhizal lifestyle (Kohler et al. 2015). The latter work has confirmed that a convergent evolution of ectomycorrhizal (ECM) fungi involves a reduced set of genes encoding plant cell wall (PCW)-degrading enzymes, compared to their ancestral wood decayers. In spite of the intraroot habitat, PCW-degrading enzymes lack in *R. irregularis*, thus suggesting that the penetration of the root cells relies on plant enzymes (Tisserant et al. 2013). It can be hypothesized that signal molecules released by the fungus are perceived by the plant cells which, in turn, activate their own enzymes, thus allowing the penetration of the fungus through the cell wall and the intracellular development of the functional structures of the symbiosis, i.e., the arbuscules (Balestrini and Bonfante 2014; Kuo et al. 2014). Invertase or sucrose transporter genes have not been identified in its genome, thus suggesting a great dependence of the fungus on its host for carbohydrates, as well as the key enzymes that are involved in the biosynthesis of fungal toxins and thiamine (Tisserant et al. 2013). *R. irregularis* also has a relatively small repertoire of effector-like proteins (Lin et al. 2014), including five putative secreted proteins with a so-called crinkler (CRN) domain. Secreted CRN domain effectors are abundantly present in the oomycete plant pathogens of the *Phytophthora* genus, and similar proteins have been recognized in *Batrachochytrium dendrobatidis*, but not in other sequenced fungi (Lin et al. 2014). A *R. irregularis* effector protein (SP7) has already been characterized (Kloppholz et al. 2011), and genome analysis has shown the presence of 13 sequences with similarity to SP7. Alignment of these protein sequences conserved features are also present in SP7, which would seem to indicate that these proteins are good candidates for the display of effector functionality (Lin et al. 2014). Transcriptomics has revealed that a battery of mycorrhiza-induced small secreted proteins (MiSSPs) is expressed in symbiotic tissues (Tisserant et al. 2013). Starting from the genome sequence, a monosaccharide transporter (MST2), which is specific to and required for the symbiosis, has already been characterized (Helber et al. 2011), and its expression has been correlated to that of the mycorrhiza-specific plant phosphate transporter gene PT4, which is used as an indicator of a functional AM association.

Taken together, the results show that the obligate biotrophy of this fungus does not seem to be associated with a large reduction in metabolic complexity, as observed in many obligate biotrophic pathogens, and its ability to interact with the soil environment, regarding nutrient uptake, is maintained in the symbiotic fungus (Tisserant et al. 2013).

6.3 New Insights Into Nutrient Exchanges in AM Symbiosis

AM fungi colonize the roots and form highly branched structures inside root cortical cells, i.e., the arbuscules, which are the functional site of nutrient exchange. The formation of symbiosis with AM fungi leads to an improved nutritional status of the host plant (Smith and Smith 2012),

especially under low nutrient availability, allowing the plant to access unexplored soil niches and absorb usually inaccessible nutrient sources. The fungus, in turn, receives photosynthetic carbohydrates. The extraradical AM mycelium captures water and mineral nutrients from the soil and is an important sink for host carbon. Inside the roots, carbohydrates and mineral nutrients are then exchanged across the interface between the plant and the fungus (Balestrini et al. 2015). These aspects have been studied extensively from both physiological and molecular perspectives (Harrison et al. 2002; Bucher 2007; Smith and Smith 2011). It has clearly been demonstrated that plants possess a symbiotic phosphate uptake pathway (Bucher 2007; Smith and Smith 2011). Moreover, ammonium transporters specifically expressed in arbusculated cells have also been discovered (Gomez et al. 2009; Guether et al. 2009a; Kobae et al. 2010). Evidence of the involvement of AM symbiosis in the transfer of mineral nutrients has been obtained in studies of several plant species (Nagy et al. 2005; Paszkowski et al. 2002; Guether et al. 2009b; Hogekamp et al. 2011; Casieri et al. 2013 for a review). On the fungal side, it is worth noting the fact that a phosphate transporter (Balestrini et al. 2007; Tisserant et al. 2012; Fiorilli et al. 2013) and an ammonium one (Pérez-Tienda et al. 2011) have been found as expressed in arbuscules. Transcriptomic data have also shown the upregulation of a putative amino acid transporter in the intraradical mycelium *vs* the extraradical one (Tisserant et al. 2012). Recently, a *R. irregularis* gene sequence, similar to members of the PTR2 family of fungal oligopeptide transporters (called *RiPTR2*), has been identified and characterized (Belmondo et al. 2014). Gene expression analysis has shown that *RiPTR2* is expressed in the extraradical mycelium, thus suggesting a role in the uptake of organic N from the soil. However, a higher expression has been observed in the intraradical stage, i.e., arbuscules, which would seem to suggest a role in the mobilization of organic N in mycorrhizal roots (Belmondo et al. 2014). Considering the plant side, two *Medicago truncatula* PTR genes have also been described as mycorrhiza responsive and their transcripts have been detected in arbusculated cells (Gomez et al. 2009). Overall, these results suggest that the fungus may reabsorb nutrients released at the periarbuscular interface, thus exerting control over the amount of nutrients delivered to the host (Balestrini et al. 2007; Belmondo et al. 2014). A large number of papers have been published on phosphate (Pi) and nitrogen (N) uptake and their transfer during symbiosis (Casieri et al. 2013). Recently, new information has been obtained on sulfur (S) acquisition. The *G. intraradices* fungus has been shown, in monoxenic conditions, to take up both sulfate- and S-containing amino acids and to transfer them to the plant (Allen and Shachar-Hill 2009). On the plant side, recent investigations have revealed that AM symbiosis can influence the expression of plant sulfate transporters and, as a consequence, can improve the S nutritional status of the host plant (Casieri et al. 2012; Giovannetti et al. 2014). The transcriptomic response of *M. truncatula* to different sulfate concentrations in mycorrhizal and non-mycorrhizal plants has been investigated, and it has been found that the regulation of *M. truncatula* sulfate transporters depends on the S concentration and on the mycorrhizal colonization in both the root and shoot (Casieri et al. 2012). Sieh and colleagues (2013) have analyzed the influence of mycorrhizal colonization on the metabolite composition of leaves and the expression of sulfur starvation-related genes in *M. truncatula* grown under different sulfur and phosphate fertilization treatments. Giovannetti et al. (2014) have recently identified and characterized a novel AM-dependent sulfate transporter, LjSultr1; 2, that also responds to S starvation. Its transcript accumulates specifically in the arbusculated cells of mycorrhizal roots. The authors have also shown the importance of this sulfate transporter in the uptake of sulfate in *Lotus japonicus* seedlings (4 and 10 days old), even in the absence of the mycorrhizal fungus. This would seem to suggest that a single gene, *LjSultr1;2*, mediates both direct and symbiotic pathways. Although potassium (K^+) is one of the most abundant elements in the soil, its

availability is very low, limiting plant growth and productivity of ecosystems (Garcia and Zimmermann 2014). The contribution of AM symbiosis to the enhancement of plant K^+ nutrition is not clearly understood yet, and only a few data have been reported on the putative AM symbiosis contribution to K^+ acquisition (Garcia and Zimmermann 2014). Interestingly, it has been suggested that the improvement in K^+ nutrition, due to mycorrhizal symbiosis, could act on the abiotic stress tolerance of the host plant, e.g., under salinity and drought stress conditions (Garcia and Zimmermann 2014 and references therein). The upregulation of several genes involved in nutrient transport, which may be involved in Pi, S, N, and K^+ uptake by the plant from the fungus, has been observed in *L. japonicus* mycorrhizal roots (Guether et al. 2009b). Among them, two putative aquaporin genes, named *LjNIP1* and *LjXIP1*, have been identified. A further gene expression analysis has revealed a good correlation between the expression of *LjNIP1* and *LjPT4*, the phosphate transporter that is considered a marker gene of mycorrhizal functionality. *LjNIP1* transcripts have been localized exclusively in arbuscule-containing cells using laser microdissection (Giovannetti et al. 2012b), and it has been suggested that LjNIP1 could be considered a novel molecular marker of mycorrhizal symbiosis. On the fungal side, two functional aquaporin genes, *GintAQPF1* and *GintAQPF2*, have recently been cloned from the AM fungus *G. intraradices*. Gene expressions were analyzed during symbiosis under drought stress, and the potential transport of water via AMF to the host plants was suggested (Li et al. 2013a, b). Fungal aquaporins could play a role not only in helping AM fungi to resist drought stress but also in plant drought tolerance (Li et al. 2013b). Soil waterlogging is considered a major abiotic stress that affects the growth, development, and survival of numerous plant species, in natural as well as in agricultural and horticultural ecosystems (Parent et al. 2008). The role of AM symbiosis in the tolerance of tomato plants to flooding has recently been reported (Calvo-Polanco et al. 2014). In particular it was shown that, under flooded conditions, the hydraulic conductivity of the roots of plants inoculated with *R. irregularis* increased, and this effect was correlated to a higher expression of the plant aquaporin SlPIP1;7 and of the fungal aquaporin GintAQP1.

Looking at these results, several efforts have been made in the last few years to highlight the role of AM symbiosis in plant nutrient and water uptake (Casieri et al. 2013; Bárzana et al. 2014). The correlation of molecular and biochemical data to ecophysiological data will constitute a major advance (van der Heijden et al. 2015). This could in fact lead to an improvement in the knowledge on the impact of AM symbiosis on plant performances, under different growth conditions, and on the role of AM fungi in plant protection against biotic and abiotic stresses.

6.4 Exploitation of AM Fungi in Sustainable Agriculture

The use of AM fungi in agriculture has not yet been widely adopted in the typical intensive agriculture of Europe and North America, while advances have been made by developing countries, such as Cuba, India, and Mexico, where chemical fertilizers are prohibitively expensive (Roy-Bolduc and Hijri 2011). New methods for the large-scale production of AM fungi (Ijdo et al. 2011) and seed coating using AM fungi (Vosatka et al. 2012) have been developed in recent years. Recently, the use of in vitro produced AM fungi has led to significant yield increases in the globally important food security crop cassava (Ceballos et al. 2013). Roy-Bolduc and Hijri (2011) have reported that two main approaches to manage AM fungi in agriculture programs are possible: using an inoculum of selected AM fungal strains or adopting practices that can enhance indigenous AM fungi. In the latter case, they are well adapted to local conditions, although they might have a lower efficiency than selected strains. In the last few years, several researches have focused on the selection of more efficient AM fungal strains, and a crucial point is that of finding the most efficient plant/AM fungus combinations. The

first step is the identification of AM fungal soil communities from several ecosystems and the study of the shifts in these communities, i.e., under different soil managements (Alguacil et al. 2008; Lumini et al. 2010; Borriello et al. 2012). Several papers have been published in the last few years on the identification of AM fungal communities using both morphological and molecular methods (Balestrini et al. 2010; Al-Yahyaèei et al. 2011; Lumini et al. 2011; Berruti et al. 2014; Borriello et al. 2015), also under abiotic stress conditions, e.g., salinity (Estrada et al. 2013a, b). A major advancement has been made, thanks to the development of "high-throughput" sequencing techniques, such as 454 sequencing platforms, which have greatly changed the analysis of environmental samples in terms of yields, cost, and speed, and has resulted in thousands of sequences being obtained in a short time (Lindahl et al. 2013; Opik et al. 2013; Balestrini et al. 2015 and references therein). The selection of a suitable host plant for the mass production of indigenous AM fungi has also been made (Abdullahi et al. 2014). An increasing number of companies produce and sell certified AM fungal inoculants for horticulture and agriculture, although their success in root colonization is not predictable (Berruti et al. 2013). Several field investigations have shown that different AM fungal species/isolates may lead to different colonization rates and different growth responses in plants. In addition, different plant genotypes can respond differently to AM fungal inoculation (An et al. 2010). Recently, the application of commercial inocula, constituted by an AM fungal isolate (*Funneliformis mosseae*), or a consortium of different fungi and bacteria in pot-cultivated *Camellia japonica*, has shown that the applied AM fungal species/isolates poorly colonized the root system, which would seem to suggest that commercial formulations should be more targeted and host specific to successfully colonize the host root and, as a consequence, potentially express their benefits (Berruti et al. 2013). AM fungal species that were found in association with camellia plants in seminatural ecosystems have been used in a parallel study on C. *japonica* "Nuccio's Pearl," and a higher level of colonization has been obtained. The identification of the AM fungal species that form the arbuscules inside the roots has also been possible using the microdissection approach (Berruti et al. 2013). Moreover, it has been reported that the tolerance of maize to salt stress is enhanced more by AM fungi isolates from saline environments than by the collection ones (Estrada et al. 2013a, b).

The appropriate application of AM fungi to improve food security, by increasing the overall yield of important crops, has been discussed in a recent perspective article by Rodriguez and Sanders (2015). The main challenges of using AM fungi to significantly improve the production of important food security crops, especially in the tropics, have been outlined, with particular attention being paid to ecological aspects related to the introducing of non-native AM fungi, which could have an impact on the existing AM fungal communities. It should be recalled that AM fungal inoculants are difficult to trace in field experiments. However, in the presence of the *R. irregularis* reference genome, the partial genome re-sequencing of multiple isolates from different geographic origins will facilitate the identification of genome-wide markers for future genomic population studies as well as the discrimination between introduced *R. irregularis* strains and native ones (Rodriguez and Sanders 2015).

6.5 Conclusions

In the last years, major advancements have been done in the knowledge of the biology of AM fungi and the ecology of AM symbiosis. As reported by van der Heijden and colleagues (2015), a further frontier will be to develop predictive models that help us to decide when, and under what conditions, application of a treatment based on mycorrhizal fungi is needed and advantageous.

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The Use of Arbuscular Mycorrhizal Fungi in Combination with *Trichoderma* spp. in Sustainable Agriculture

7

Jose Antonio Pascual

Contents

Abstract

Arbuscular mycorrhizal (AM) fungi as well as fungal biological control agent such as *Trichoderma* spp. have shown beneficial effects on plant growth and health. The study of the interactions between AM fungi, fungal antagonists and plants is of great interest. Before using them, it is in fact important to understand whether and how the two different beneficial fungal components can influence each other and/or the host plant. In this chapter, the attention is focused on the plant responses to the inoculation with AM fungi and *Trichoderma* spp., alone or in combination, with particular attention to the impact on the plant hormonal balance.

7.1 Interactions of AMF with Trichoderma: An Example of Microbial Interactions

The use of AM fungi (AMF) as bioinoculants can benefit plant growth and health, and their use could contribute to a reduction in the use of pesticides and other environmentally harmful agrochemical products currently required for optimal plant growth and health (Barea et al. 1997). The degree of the effect on plants depends on the plant species and the AMF (Jeffries and Barea 2001). The successful use of AMF in sustainable agriculture

J.A. Pascual (✉)
Centro de Edafología y Biología Aplicada del Segura, CEBAS-CSIC, PO BOX 4195, Campus Universitario de Espinardo. 25, 30100 Murcia, Spain
e-mail: jpascual@cebas.csic.es

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_7

requires a selection of the appropriate host/fungus combination, infectivity and efficacy (Haas and Krikun 1985; Tarbell and Koske 2007). The study of interactions between some other beneficial organisms associated with plant roots is important, because such interactions could either enhance or inhibit the beneficial effects of individual species. Little is known about the interactions between AMF and beneficial saprophytic fungi, and the few studies published on this topic do not provide any conclusive findings (Green et al. 1999; Vázquez et al. 2000). Even more important, the beneficial effect that is attributed to these interactions under controlled experimental conditions may not be reflected in field experiments (Calvet et al. 1992; McAllister et al. 1997; Fracchia et al. 1998; Vázquez et al. 2000; Martinez et al. 2004). These interactions must be identified and characterised for their successful establishment in plants. AMFs have been shown to cause changes in the rhizosphere that could affect both the quality and quantity of microbial populations and to promote the activity of other microorganisms that could compete with soilborne pathogens (Filion et al. 1999; Barea et al. 2002). It has also been demonstrated that some *Trichoderma* strains may influence AMF activity (Green et al. 1999; Vazquez et al. 2000; Brimner and Boland 2003; Martinez et al. 2004).

Martinez-Medina et al. (2009) hypothesised that the combined use of AMF and *Trichoderma harzianum* (Rifai) could improve plant growth and, at the same time, help to protect against *Fusarium oxysporum* pathogen, within the context of a sustainable soil–plant system. It should be noted that the effects of the interaction between AMF and *T. harzianum* may be very different, depending on the AMF strain and the saprophytic strain (Martinez et al. 2004). Martinez-Medina et al. (2009) found no additional suppressive effect of *T. harzianum* when *Glomus mosseae* was co-inoculated, because the AMF itself caused reduction of soilborne pathogen. This suppressive effect was also shown against other microorganisms such as the co-inoculated *T. harzianum* strain (Azcón-Aguilar and Barea 1996). It has also been reported that the presence of *T. harzianum* significantly increased root colonisation by several AMF species, reaching values significantly higher than the most effective AMF (*G. mosseae*) inoculated alone, in contrast with the absence of effects observed in other studies (Camprubí et al. 1995; Vázquez et al. 2000). On the other hand, *T. harzianum* populations were generally reduced in the presence of AMF, except in the case of the co-inoculation with *Glomus intraradices*. This could be due to the modifications of root exudates or to substances released by the extramatrical mycelium (McAllister et al. 1994; Filion et al. 1999; Martínez et al. 2004). Competition between *T. harzianum* and AMF in the first establishment stages, which could be maintained in time, has been also proposed to explain these findings (McAllister et al. 1994; Green et al. 1999). Competition for host photosynthates or sites, microbial changes in the rhizosphere due to the presence of AMF species, and induction of local and systemic defence responses have been proposed (Caron 1989; Azcón-Aguilar and Bago 1994). By contrast, Filion et al. (1999) observed an improvement in germination of *T. harzianum* conidia by *G. intraradices*, suggesting a complex interaction between *T. harzianum* and the AMF. Recently, the interaction between *Trichoderma atroviride* (PKI1), two AMF species and the host plant has been studied under in vitro culture conditions. Using in vivo imaging of green fluorescent protein-tagged lines, Lace et al. (2014) showed that *T. atroviride* did not activate symbiotic-like responses in the plant cells at the contact points. Furthermore, it has been observed that *T. atroviride* strain parasitized the hyphae of the two AMF species (*Gigaspora gigantea* and *Gigaspora margarita*) through localised wall breaking and degradation, although this was not associated with an evident chitin lysis or the upregulation of two major chitinase genes. *T. atroviride* colonised extensive areas of the root epidermis, in association with localised cell death, and plant colonisation by both the fungal symbionts has also been observed when *Trichoderma* was applied to a pre-established AM symbiosis. Table 7.1 summarises the main interaction effects related to the interaction between AMF and *T. harzianum* in some crops.

Table 7.1 Summary about the interaction between AMF, *T. harzianum* and some plants

	Growth and nutrition	Control against *F. oxysporum*	Induction of systemic response in absence of the pathogen	Induction of systemic response in presence of the pathogen	Differences on root gene expression
T. harzianum	Increase melon growth and nutrition, particularly by solubilising nitrogen to be uptaken by roots (Contreras-Cornejo et al. 2009)	Increase melon resistance against plant pathogens, although it depends on the species (Yedidia et al. 2003; Martinez-Medina et al. 2014)	Increase SA and JA levels (Hanson and Howell 2004)	Response through SA, ACC and ABA due to pathogen was modulated by the presence of the *T. harzianum* strain (Martinez-Medina et al. 2013)	Induction of gene (*LOX* and *PAL*) related to systemic induced resistance (SIR) (Yedidia et al. 2003; Shoresh et al. 2005)
AMF	Increase melon plant growth and nutrition particularly by solubilising phosphorus to be uptaken by roots (Pozo and Azcon-Aguilar 2007)	It can increase plant resistance against plant pathogens, but it is more rarely than *T harzianum* (Pozo and Azcon-Aguilar 2007; Martinez-Medina et al. 2011)	No increase in SA and JA levels (Pozo et al. 2002)	Possible *priming* effect related to jasmonic route (Pozo et al. 2009). Modulated response through ACC and ABA by pathogen action	Induction of gene (*PAL*, *PR1* and *PR3*) related to systemic acquired resistance (SAR) (Vierheilig 2004)
T. harzianum + AMF	Increase but it is dependent on the AMF–*T. harzianum* combination. A good combination observed was for *G. mosseae*–*T. harzianum* (Martinez-Medina et al. 2009)	Reduction of the plant disease but it was not synergic between the different assayed coinoculations (Saldajeno et al. 2008; Martinez-Medina et al. 2009)	Decrease the levels of salicylic and JA in respect to the ones only inoculated with *T. harzianum* (Martinez-Medina et al. 2011; Garmendia et al. 2005)	Pattern similar to *T. harzianum*-inoculated plants (Martinez-Medina et al. 2011)	Decrease in the levels of gene expression related to the ones showed in the AMF-inoculated plants that reflect a loose on SAR (Vinale et al. 2008b)

7.2 The Use of a Combination of AMF–*Trichoderma* to Improve Plant Health

It has been well documented that AMF themselves can control a whole range of plant pathogens, and their effect on plant yield and nutrient uptake depends on the type of species and even on the strain. The alleviation of damage caused by soilborne pathogens, such as *Phytophthora*, *Fusarium*, *Pythium*, *Rhizoctonia*, *Sclerotium* and *Verticillium*, has been reported in mycorrhizal plants (Bi et al. 2007). Martinez-Medina et al. (2009) showed that the percentage of plants infected by *F. oxysporum* in AMF-inoculated soils decreased significantly,

compared to non-inoculated soils, but some AMF, such as *Glomus claroideum* and *G. mosseae*, did not show any suppressive effect on melon plants. If these treatments were also co-inoculated with *T. harzianum*, the percentage of infected plants would have been reduced significantly. As far as suppressing disease incidence is concerned, *T. harzianum* was seen to be more effective than AMF.

Several studies have reported on the biocontrol capacity of *Trichoderma* species (Chet 1987; Chet et al. 1997; de Meyer et al. 1998; Yedidia et al. 2003; Harman 2000; Howell et al. 2000; Soresh et al. 2005; Martínez-Medina et al. 2009). Various biocontrol mechanisms have been reported, such as mycoparasitism, antibiotic production, competition or the induction of local and systemic defence responses (Howell 2003; Yedidia et al. 2003; Harman et al. 2004). However, most studies on the reduction of disease by *Trichoderma* spp. have focused on microbial interactions rather than on host plant responses (Shoresh et al. 2005; Korolev et al. 2008). Moreover, a number of mechanisms have been proposed to explain growth enhancement by *Trichoderma* spp. (Harman et al. 2004; Benítez et al. 2004).

7.3 Crosstalk Between AMF–*Trichoderma* and Plants

Plants react to external abiotic and biotic alterations with a similar response to that of a plant pathogen reaction, which triggers a wide range of defence mechanisms in plants that confer protection against pathogenic invasion. Fungal interactions with phytohormonal signalling and the induction of resistance against pathogens have also been reported as growth-promoting mechanisms (Vassilev et al. 2006). A possible role of indole acetic acid (IAA) in the growth stimulation of tomato plants produced by inoculation with *Trichoderma aureoviride* was proposed by Gravel et al. (2007). Vinale et al. (2008a) observed that some secondary metabolites that are involved directly in the *Trichoderma*–plant interaction may act as auxin inducers. Phytohormone signalling pathways are currently emerging as important regulators of AM establishment (Gutjahr 2014). Mycorrhizal fungi have important physiological implications for the host plants that are mediated through a hormone response that involves an altering in the levels of cytokinins, gibberellins, ethylene, abscisic acid (ABA), jasmonic acid (JA) and auxin (Hause et al. 2002; Ludwig-Müller et al. 2002; Vierheilig 2004; Foo et al. 2013; Fernández et al. 2014). This alteration has been reported not only at a root level but also for the transportation of these hormones to the aerial hosted plant shoots (Toussaint 2007). It has been suggested that phytohormones, such as IAA, cytokinins and ethylene, may alter shoot or root growth (Tsavkelova et al. 2005). In addition, plant hormones, such as jasmonic acid (JA), salicylic acid (SA) and ethylene, may act as signalling molecules during the activation of systemic resistance in plant shoots (Abeles et al. 1992; Sticher et al. 1997; Pieterse et al. 2003). Recent studies have shown a systemic effect in the shoots of mycorrhizal roots (Liu et al. 2007; Fiorilli et al. 2009). The response of the plant differs, depending on which AMF is inoculated. *G. intraradices*-inoculated plants have shown increased shoot IAA and ACC, the ethylene precursor, while a decrease has been observed using *G. mosseae*. These findings reinforce the idea that the effects of inoculation with different fungi on the hormonal balance of plants may vary to a great extent. The analysis of the defence-related hormones, SA, ABA and JA, and the expression of marker genes of the pathways in which they are involved have recently revealed significant changes in the roots of mycorrhizal plants, and the changes seem to be depended on both the plant and AMF that are used (Fernandez et al. 2014). An important role in promoting the association with AMF has been shown for strigolactones, which can be exudated by plant roots (Akiyama et al. 2005). Furthermore, there have also been several studies related to the induction of resistance by AMF, focusing especially on the activation of plant defence mechanisms in roots (Pozo and Azcón-Aguilar 2007; Avis et al. 2008; Pozo et al. 2009).

Several reports have demonstrated that the interaction between AMFs and *Trichoderma* spp. may be beneficial for both plant growth and plant disease control (Linderman 1992; Barea et al. 1997; Saldajeno et al. 2008; Martínez-Medina et al. 2009). A synergistic effect of some saprophytic fungi on AMF spore germination and colonisation has been confirmed (Calvet et al. 1993; McAllister et al. 1996; Fracchia et al. 1998). For example, it has been reported that some *Trichoderma* strains may influence AMF activity (Calvet et al. 1992, 1993; Brimner and Boland 2003; Martinez et al. 2004; Martínez-Medina et al. 2009). Volatile and soluble exudates produced by saprophytic fungi are involved in these effects (McAllister et al. 1994, 1995; Fracchia et al. 1998). Nevertheless, the results of research on the interactions between soil saprophytic and AMF differ widely, even when the same species of saprophytic fungi are involved. For example, *T. harzianum* was found to have antagonistic, neutral and stimulating effects on AMF (Siddiqui and Mohmood 1996; Fracchia et al. 1998; Godeas et al. 1999; Green et al. 1999; Martínez-Medina et al. 2009). The effects of such interactions should be studied because they could either enhance or inhibit the beneficial effects of the individual species (Barea et al. 1997, 2002; Saldajeno et al. 2008).

In general, AMF and *T. harzianum* inoculation has resulted in an improvement in shoot weight and nutritional status (Martinez-Medina et al. 2009). Haggag and Abd-El-Latif (2001) found that the combined inoculation of *G. mosseae* and *T. harzianum* enhanced the growth of geranium plants; the main plausible explanation being that *T. harzianum* by itself solubilises inorganic nutrients and increases plant nutrient uptake (Altomare et al. 1999; De Silva et al. 2000). Altomare et al. (1999) hypothesised that phosphorus could be solubilised and stored in the *Trichoderma* biomass and then released in a readily available form in close proximity to the roots after lyses of the aged mycelium. Inoculation with *T. harzianum* produces a negative effect on nitrogen uptake in low levels of fertilisation doses, because of competition between plant roots and *T. harzianum*, since, in many cases, microorganisms are superior competitors (Kaye and Hart 1997; Hodge et al. 2000). Martinez-Medina et al. (2009) showed a general increase in the plant nitrogen content when co-inoculated with *T. harzianum* and different AMFs. As mentioned before, it was shown that co-inoculation with *T. harzianum* and *G. mosseae* was more effective than any other combination tested with regard to increases in the uptake of nutrients.

A systemic effect of the AMF on the IAA and ACC balance in the host plant has been shown (Meixner et al. 2005). On the other hand, plants co-inoculated with AMF and *T. harzianum* showed a different hormone profile due to mediation of systemic increases in the IAA, ACC and ABA concentrations. Interaction with auxin signalling has been proposed as a plant growth promotion mechanism for *T. virens*, as a dose-dependent effect of IAA on increasing biomass production has been shown by Contreras-Cornejo et al. (2009). It has been proposed that ethylene may affect shoot and root growth through the stimulation of auxin biosynthesis (Ruzicka et al. 2007). It has also been taken into account that *T. harzianum* itself is able to modify the shoot balance of stress- and defence-related hormones to a great extent. Three major signal molecules are known to be involved in the systemic defence responses of plants: SA, commonly shown to be involved in systemic acquired resistance (SAR), and JA and ethylene, which are involved in induced systemic resistance (ISR) (Abeles et al. 1992; Sticher et al.1997; Pieterse et al. 2003). Shoresh et al. (2005) suggested the involvement of JA and ethylene in the protective effect conferred by *T. asperellum* on cucumber, but did not observe any variation in the SA content. Martinez et al. (2001) instead suggested that the SA and ethylene pathways together coordinate the activation of defence mechanisms via an interaction between the two signalling pathways, since SA causes an inhibition of ethylene production. These findings, together with the increased *F. oxysporum* populations observed in the treatment involving inoculation with *T. harzianum*, indicate that the protective effect

against *Fusarium* wilt conferred to melon plants by *T. harzianum* is not only due to direct antagonism but also to a plant-mediated phenomenon (Harman et al. 2004; Shoresh et al. 2005).

The accumulation of SA or pathogenesis-related (PR) proteins, or the expression of marker genes associated with systemic acquired resistance, has not been reported in systemic tissues of AM plants (Pozo et al. 2009). Therefore, it has been suggested that priming is the main mechanism that operates in the induction of resistance by AMF (Cordier et al. 1998; Pozo et al. 2002; Pozo and Azcón-Aguilar 2007; Zhu and Yao 2004; Whipps 2004). It is plausible that AMFs repress SA- as well as JA-dependent systemic pathways stimulated by *T. harzianum* in the host, in order to achieve a compatible interaction. This supports the hypothesis of Pozo and Azcón-Aguilar (2007) that a functional mycorrhiza implies partial suppression of SA-dependent responses in the plant, which is compensated by an enhancement of JA-regulated responses in the roots. Hence, in plants co-inoculated with *G. intraradices* and *T. harzianum*, the additive effect on the suppression of disease caused by *F. oxysporum* seems to be due to a positive interaction between different plant protection mechanisms that are mediated by both agents, which may include a priming response mediated by the AMF as well as a systemic SA-dependent response mediated by *T. harzianum*.

This multiple-player crosstalk normally occurs in nature among plants and rhizosphere microorganisms (Barea et al. 2002; Kohler et al. 2007; Saldajeno et al. 2008; Martinez-Medina et al. 2009). These interactions may result in a different plant status from that associated with the single interaction between the plant and each microorganism (Yedidia et al. 2003; Garmendia et al. 2005; Marra et al. 2006). In nature, in diseased plants, the SA, JA, ACC and ABA concentrations increase in the shoot; *T. harzianum*-inoculated plants show a similar hormonal profile, thus indicating the involvement of similar pathways in both interactions (*T. harzianum* and pathogen plant reaction). There is extensive evidence that *Trichoderma* spp. are able to elicit the defence systems of a number of plant species (Hanson and Howell 2004; Shoresh et al. 2005; Vinale et al. 2008b). ACC and ABA accumulation in plant shoots has been shown to be lower when *T. harzianum* or *G. intraradices* and the pathogen interacted with the plant, compared to the pathogen alone, thus confirming that the presence of antagonists modifies the effect of a pathogen on the hormonal profile of a plant. Moreover, when *T. harzianum* interacts with the pathogen, a decrease in SA is observed in diseased plants. Marra et al. (2006) have shown that, in bean plants, *Rhizoctonia solani* or *Botrytis cinerea* pathogens and *Trichoderma* lead to a change in proteins involved in the response to pathogen attack, when the beneficial fungus is present. These authors observed that when both *Trichoderma* and the pathogen interacted with the plant, several PR proteins were upregulated less than by the pathogen alone. *T. harzianum* plants may have two effects: (a) related to basal resistance, as increased SA and JA concentrations as observed in healthy *T. harzianum*-inoculated plants, and (b) hormone modulating through ABA-, ethylene- and SA-dependent responses to pathogen attack, which resulted in the activation of a more appropriate response. The resistance against *F. oxysporum* mediated by *G. intraradices* instead seems to include mechanisms that are independent of SA and JA signalling, although *G. intraradices* is also able to attenuate the plant defence response to pathogen attack, which results in attenuated ACC and ABA accumulation in the shoot.

Therefore, the synergistic effect observed in plants co-inoculated with *G. intraradices* and *T. harzianum* seems to be due to a positive interaction between different plant protection mechanisms mediated by the two agents, which may include an improvement in plant nutrition, damage compensation, competition for colonisation sites or photosynthates, changes in the root system, changes in rhizosphere microbial populations and activation of plant defence mechanisms (Pozo and Azcon-Aguilar 2007). All the gathered information points out that the mechanisms involved in the control of *Fusarium* wilt by *T. harzianum* in melon plants seem to be

related not only to an induction of plant resistance but also to its capacity to attenuate the hormonal disruption caused by the disease (Martínez-Medina et al. 2010, 2011, 2014). However, the resistance mediated by *G. intraradices* against *F. oxysporum* seems to include mechanisms that are independent of SA and JA signalling. A synergistic effect on disease control has been found for the *T. harzianum–G. intraradices–F. oxysporum* tripartite interaction. However, no synergistic effect has been observed on either the attenuation of the hormone disruption induced by the pathogen or on the SA-, JA- or ACC-mediated plant response. Therefore, the synergistic effect pertaining to biocontrol capacity seems to be the result of numerous modes of action exhibited by each microorganism, which are different from the plant-mediated mechanism.

It has often been reported that the plant response to mycorrhizal fungi can vary according to the AMF species (Martinez-Medina et al. 2009, 2013). Moreover, the accumulation pattern of the transcript of defence-related genes varies among the different plant–AMF symbioses (Pozo et al. 2002; Gao et al. 2004; Grunwald et al. 2009). Campos-Soriano et al. (2012) have shown that rice root colonisation by *G. intraradices* promotes the systemic induction of genes that play a regulatory role in the host defence response. In *Medicago* species, transient increases in different flavonoid/isoflavonoid compounds were found to depend on the plant and fungal genotypes (Harrison and Dixon 1993, 1994; Volpin et al. 1994, 1995). These findings reinforce the idea that differences in the colonisation dynamics of AMF species could explain their dissimilar behaviour as plant bioprotectors (Martínez-Medina et al. 2009).

Arbuscular mycorrhizal (AM) fungi as well as fungal biological control agent such as *Trichoderma* spp. have shown beneficial effects on plant growth and health. The study of the interactions between AM fungi, fungal antagonists and plants is of great interest. Before using them it is in fact important to understand whether and how the two different beneficial fungal components can influence each other and/or the host plant. In this chapter, the attention is focused on the plant responses to the inoculation with AM fungi and *Trichoderma* spp., alone or in combination, with particular attention to the impact on the plant hormonal balance.

7.4 Conclusion

Specific arbuscular mycorrhizal and *Trichoderma* species can be used for sustainable agriculture by utilising their beneficial effects on plant growth and health; but before using them, it is in fact important to understand whether and how the two different beneficial fungal components can influence each other and/or the host plant. It is demonstrated that the interactions between them are direct, and also it is remarkable that the crosstalk between AMF–*Trichoderma* is also through the plant by phytohormonal signalling, such as by salicylic and jasmonic acid, ethylene, ACC, IAA or ABA.

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Bioformulations of Novel Indigenous Rhizobacterial Strains for Managing Soilborne Pathogens

8

Pervaiz A. Abbasi, Salah Eddin Khabbaz, and Liang Zhang

Contents

Abstract

The antagonistic potential of indigenous strains of *Bacillus*, *Paenibacillus*, and *Pseudomonas* isolated from potato rhizosphere or soil and soybean plants to manage soil and root diseases of vegetable crops is presented here as a case study. These bacterial strains were also characterised for production or activities of antibiotics, metabolites, volatiles, phytohormones, and lytic enzymes. In agar plate assays, most of the strains showed broad-spectrum antagonistic activity against various selected fungal and oomycete pathogens. Irradiated peat was found to be a suitable carrier material for preparing formulations of these bacteria and delivering them on seed, root, or substrate. In pot experiments, irradiated peat formulations of these bacteria provided control of *Pythium* damping-off and root rot and *Phytophthora* blight and root rot of cucumber, *Rhizoctonia* damping-off of radish, and *Fusarium* crown and root rot and *Fusarium* wilt of tomato. Bacterial treatments also resulted in higher fresh weights of plants produced in pathogen-infested substrate. In micro-plot

P.A. Abbasi (✉)
Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, 32 Main Street, Kentville, Nova Scotia B4N 1J5, Canada
e-mail: Pervaiz.Abbasi@agr.gc.ca

S.E. Khabbaz • L. Zhang
Southern Crop Protection and Food Research Centre, Agriculture and Agri-Food Canada, 1391 Sandford St., London, Ontario N5V 4T3, Canada

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_8

trials, coating of seed potato tubers with irradiated peat formulation of some of these antagonistic bacteria reduced scab severity on daughter tubers and increased tuber weights when treated tubers were planted in a potato soil with a history of common scab disease. These disease suppression and plant-growth promotion activities of various bacterial strains might be related to the production of different antibiotics, secondary metabolites, lytic enzymes, phytohormones, siderophores, and volatiles. The results of these studies indicate that several of the bacterial strains isolated from local sources showed their potential use as biofungicides for protection of seedling damping-off and root rot and tuber diseases caused by various soilborne pathogens.

8.1 Background

The traditional disease control methods based on chemical fungicides have been very effective in managing soilborne plant pathogens, but they also have ecological and environmental concerns, such as contamination of soil, water, and food material, resistance development in pathogens, and nontarget effects on beneficial soil microbes. During the past 30 years, significant research efforts have been dedicated toward the development of biological-based disease management strategies as alternative to chemical pesticides (Gerhardson 2002; Weller et al. 2002; Fravel 2005; Haas and Défago 2005; Lugtenberg and Kamilova 2009; Olubukola 2010). As a result, biological control has become an ecologically sound and environmentally friendly method for managing diseases caused by soilborne plant pathogens. Plant-growth promoting rhizobacteria (PGPR) have been successfully used as potential biocontrol agents of plant pathogens (Weller 1988; Raaijmakers et al. 2009). These beneficial bacteria can colonise plants and inhibit pathogenic microorganisms by producing an array of antibiotics, metabolites, lytic enzymes, and phytohormones and by fixing nitrogen and solubilising phosphate (Knowles 1976; Neilands 1981; Nautiyal et al. 2002; Kim et al. 2008; Athukorala et al. 2009; Santoyo et al. 2012). Beneficial bacteria can also provide suppression of plant diseases by inducing systemic resistance in host plants (Ramamoorthy et al. 2001; Bakker et al. 2003).

Disease in nature is the exception and not the rule, and natural biological control may be playing a part in that exception. This implies that sources of biofungicides are widespread and their population can either be exploited and enriched or potential candidates can be isolated, tested in vivo and in vitro for broad-spectrum anti-pathogen activities, formulated in some suitable carrier material and evaluated again for disease suppression, and finally redeployed. So the soils that are exposed to pathogens and soils that are not exposed to pathogens can be good source of biofungicides. In soils that are exposed to pathogens, biological control may be occurring naturally leading to low disease occurrence on host plants even though the pathogen inoculum in the soil is high. These are also known as suppressive soils (Cook and Baker 1983), such natural soils suppressive to soilborne pathogens have been discovered worldwide (Cook and Baker 1983; Alabouvette 1999; Weller et al. 2002), and indigenous soil microbial communities generally contribute to disease suppression by reducing and competing with pathogen populations (Weller et al. 2002; Bonanomi et al. 2010). It is also possible to exploit natural disease-suppressive conditions by soil incorporation of organic materials (Mathre et al. 1999; Abbasi et al. 2008; Abbasi 2011; Bernard et al. 2012) and other agronomic practices. Organic soil amendments which act primarily as nutrient source for crop production can also act as disease management tools. Disease management by organic amendments may occur by increasing the populations and activities of biocontrol agents and by enhancing their disease control effect (Mathre et al. 1999).

Soilborne plant diseases such as wilts, scab, damping-off, and root rots are major yield-

limiting factors in agricultural, ornamental, and horticultural crops. These diseases can cause substantial economic losses to growers every year. Chemical fumigants may provide control of some of these diseases but they can be expensive, undesirable, and not always available or effective. Beneficial microbial communities from rhizosphere and endophytes play a central role in improving plant growth and health. In this chapter, we present recent research findings from our laboratory on the isolation and characterisation of beneficial rhizobacteria from indigenous resources as potential biofungicides for managing soil and root diseases of vegetable crops.

8.2 Isolation and Selection of Antagonists

Increasing demand for biological-based disease control strategies has prompted searches for novel strains of biocontrol agents from native resources and to develop them as biofungicides. Biological control is also a key component of organic amendment-mediated disease management, particularly those diseases caused by soilborne pathogens. Incorporation of organic materials to soils prior to planting provides suppression of plant diseases and in most cases disease suppression occurs through enhanced microbial activity in the amended soil (Abbasi et al. 2008; Abbasi 2011). We found that such amended soils are a good source for isolating biocontrol strains (Abbasi 2013).

Thirteen bacterial strains were chosen after preliminary agar plate screening for further characterisation of antagonistic and biocontrol potential (Table 8.1 and Fig. 8.1). Eight of these bacterial strains (Khabbaz and Abbasi 2014; Zhang et al. 2015) were isolated from soil samples collected from potato field plots treated with an organic soil amendment for establishing disease-suppressive conditions against soilborne potato diseases (Abbasi 2013). In addition, two of the bacterial strains were isolated from leaves of soybean plants grown in pots containing soil from a field in a greenhouse, and three previously uncharacterised strains selected were from culture collection (Zhang et al. 2015). The antagonistic activity of these bacterial strains or their volatiles was determined on agar plates in co- or dual-culture or sealed double plate assays against various fungal and oomycete pathogens. The cell-free culture filtrates (CF) of five selected strains were also tested against these plant pathogens on PDA plates. All 13 bacterial stains displaying broad-spectrum antagonistic activities against the selected pathogens in plate assays were identified based on their sequences of the 16S rRNA gene after polymerase chain reaction

Table 8.1 Bacterial strains assessed for antagonistic and biocontrol potential

Biocontrol strain	Reference
Bacillus amyloliquefaciens 1A-48	Zhang et al. (2015)
Bacillus amyloliquefaciens 1B-14	Zhang et al. (2015)
Bacillus amyloliquefaciens 1B-23	Zhang et al. (2015)
Pseudomonas chlororaphis 1B-26	Zhang et al. (2015)
Bacillus subtilis 8B-1	Khabbaz and Abbasi (2014)
Pseudomonas sp. 8D-45	Khabbaz and Abbasi (2014)
Pseudomonas fluorescens 9A-14	Khabbaz and Abbasi (2014)
Bacillus amyloliquefaciens 9A-31	Zhang et al. (2015)
Pseudomonas chlororaphis SL5	Zhang et al. (2015)
Paenibacillus polymyxa SL23	Zhang et al. (2015)
Paenibacillus polymyxa #50	Zhang et al. (2015)
Paenibacillus polymyxa #53	Zhang et al. (2015)
Pseudomonas fluorescens PEF-5 #18	Zhang et al. (2015)

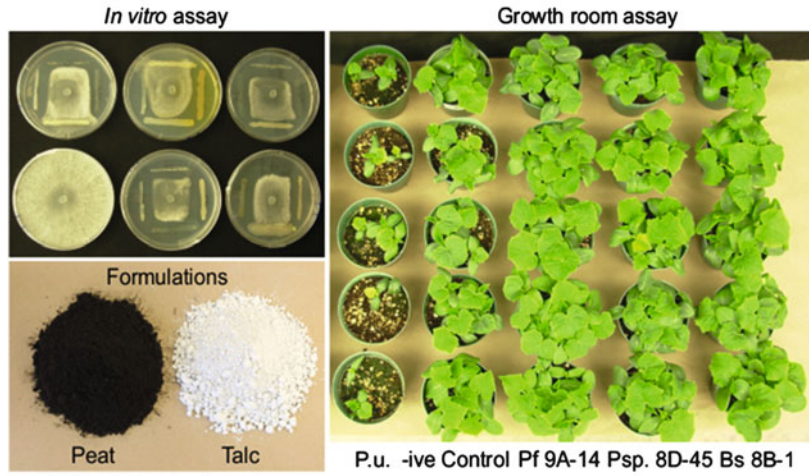

Fig. 8.1 Graphical description for selection and screening of bacterial antagonists as potential biofungicides

(PCR) amplification (Khabbaz and Abbasi 2014; Zhang et al. 2015).

8.3 Characterisation of Bacterial Strains for Antagonistic Activities

An understanding of the mechanisms of pathogen inhibition, disease suppression, and plant-growth promotion by biocontrol strains is necessary for their development as biofungicides. Therefore, all 13 bacterial strains were also characterised for their ability to produce different types of antibiotics, lytic enzymes, secondary metabolites, volatiles, or phytohormones. PCR analysis showed the presence of the genes involved in the biosynthesis of antibiotics such as bacillomycin, bacilysin, iturin A, surfactin, and fengycin in most of the *Bacillus* strains and the genes for biosynthesis of pyoluteorin, pyrrolnitrin, and 2,4-diacetylphloroglucinol (DAPG) in most of the *Pseudomonas* strains. However, the actual production of these antibiotics by bacterial strains was not assayed in this study. Using various agar plate assays, these bacterial strains were also assayed for their ability to produce hydrogen cyanide (HCN), siderophore, β-1,3-glucanase, chitinase, protease, indole acetic acid (IAA), and salicylic acid (SA) and to fix nitrogen and solubilise phosphate (Khabbaz et al. 2015; Zhang et al. 2015).

The results of the agar plate assays indicated that nine of the antagonistic bacterial strains produced protease, eight strains produced HCN, seven strains showed β-1,3-glucanase activity, and four strains showed chitinase activity and produced siderophore (Khabbaz et al. 2015; Zhang et al. 2015). Ten bacterial strains were able to produce phytohormones such as IAA and SA, eight strains showed the ability to fix nitrogen, and three strains showed phosphate solubilisation activity (Khabbaz et al. 2015; Zhang et al. 2015). However, any specific role of these various activities produced by the antagonistic bacterial strains needs to be established for pathogen inhibition, disease suppression, and plant-growth promotion.

8.4 Preparation of Bioformulations of Antagonistic Bacteria

Formulation of antagonistic bacteria in a suitable carrier material is necessary for their effective delivery as well as for improving their shelf life (Vidhyasekaran et al. 1997; Bashan et al. 2014). A suitable carrier material is one that is easily available, economical, and easy to use. A carrier material should stabilise the populations of the injected or inoculated bacteria in the

formulations during production and storage phases giving them a longer shelf life. Formulations can also protect the inoculated organisms from adverse environmental effects and may enhance their activity and effectiveness for plant-growth promotion and disease suppression (Van Veen et al. 1997; Jones and Burges 1998). As described below, the formulations of antagonistic bacteria were prepared in irradiated peat (in most experiments) or talc powder (in some preliminary studies only). Antagonistic bacteria were applied as preplant or post-plant treatments to the potting mix or as seed or root treatments.

8.4.1 Irradiated Peat Formulation

A sterile irradiated peat (Becker Underwood Company, Saskatchewan Canada) was used in most experiments for preparing formulations of antagonistic bacterial strains. Bacterial strains were grown in Luria-Bertani (LB) or nutrient broth-yeast extract (NBY) broth and their cells were harvested by centrifugation and resuspended in sterile distilled water (DW). The concentration of bacteria in the suspension was estimated by spectrophotometer readings and by dilution plating on NBY or LB agar plates. Sterile irradiated peat (120 g) was mixed with autoclaved LB or NBY broth (70 mL) and respective bacterial suspension (5 mL at 10^8–10^9 CFU mL^{-1}) under sterile conditions and incubated for 24 h. The formulated irradiated peat was later air-dried in a laminar flow hood to a workable moisture level and kept in polythene bags and used for the treatments immediately or as needed. To prepare formulation containing a mixture of bacterial strains, the antagonistic bacteria were grown separately in LB or NBY broth and equal volume (v/v) of each bacterial suspension was mixed with irradiated peat and broth as described above.

8.4.2 Talc Formulation

The formulations of antagonistic bacterial strains were also prepared in talc powder for some initial experiments. The talc formulation was prepared with sterilised talc powder as described previously (Vidhyasekaran and Muthamilan 1995). Talc powder (1 kg) was sterilised by autoclaving and mixed with LB broth (400 mL). Calcium carbonate (15 g) to neutralise the pH and carboxymethyl cellulose (10 g) as adhesive were added to the mixture under sterile conditions. Bacterial strains were grown in flasks containing LB or NBY broth as described above and cells were centrifuged, resuspended in sterile DW, and the concentration estimated as described above. Bacterial suspension (10^8–10^9 CFU mL^{-1}) was added to the talc-broth mixture under sterile conditions and incubated for 24 h. The formulated product was dried in a laminar flow hood as described above. Talc formulations containing a mixture of bacterial strains were also prepared as described above.

8.4.3 Shelf Life of Formulated Products

The shelf life of irradiated peat and talc formulations, prepared with three bacterial strains *Pseudomonas fluorescens* 9A-14, *Pseudomonas* sp. 8D-45, and *Bacillus subtilis* 8B-1 and stored at room temperature for 6 months, was determined by following the populations of bacterial strains on NBY agar plates by dilution plating (Khabbaz and Abbasi 2014). One-gram samples were drawn after 10- to 30-day intervals for preparing tenfold dilutions and plating on NBY plates. Colonies on plates were counted after 3 days of incubation and expressed as CFU g^{-1} product.

The results of shelf-life experiments indicated that the initial bacterial populations of the three strains were slightly higher in formulations prepared with irradiated peat (9.89–10.11 log CFU g^{-1}) as compared to in the talc powder formulations (9.86–9.98 log CFU g^{-1}) although

both products were injected with similar bacterial concentrations. The viability of injected bacteria in both irradiated peat and talc formulations gradually declined overtime during the 6-month storage period; however, the population densities of all three bacteria remained above 10^8 CFU g^{-1} in both products throughout the storage period. The viability of antagonistic bacteria did not significantly change during the first 20 days of storage in irradiated peat formulation and during the first 10 days in talc formulation compared to their respective initial populations at day zero. In irradiated peat formulation, the bacterial populations dropped to 9.0 log CFU g^{-1} after 2 months and to 8.0 log CFU g^{-1} after 3 months of storage at room temperature. In talc formulation, the bacterial populations dropped to 9.0 log CFU g^{-1} after 40–50 days and to 8.0 log CFU g^{-1} after 70 days to 3 months of storage. These results indicated that the formulated products should be applied immediately after preparation (irradiated peat within 20 days and talc within 10 days) for achieving maximum initial population densities of antagonistic bacteria.

8.5 Application of Antagonistic Bacteria

The biocontrol potential of antagonistic bacterial strains was evaluated for disease suppression and plant-growth promotion in potting substrates or soil infested with pathogen inoculum in growth-room or greenhouse pot assays (Abbasi et al. 2004), as described below. To achieve this, bacterial strains were grown in NBY broth, and their cell suspensions or bioformulations prepared in irradiated peat and talc were either applied directly to potting substrates as preplant amendment or post-plant drench treatments or as seed or root treatments.

8.5.1 Substrate Application

The potting mix used in growth-room pot assays was a commercial peat-based mix, Pro-Mix BX containing 75–85 % sphagnum peat moss, horticultural grade perlite and vermiculite, macro- and micronutrients, dolomitic and calcitic limestone, and a wetting agent (Premier Horticulture Inc., Rivière-du-Loup, Quebec, Canada). Controlled slow-release fertiliser Osmocote 14-14-14 (N-P-K) (Scotts-Sierra Horticultural Products Co., Marysville, Ohio) was added to the potting mix or soil at 4 g kg^{-1} mix. Bacterial strains were applied to the pathogen-infested potting mix either as cell suspensions in sterile water (100 mL aliquots at 5×10^8 CFU mL^{-1}) or as formulations in irradiated peat and talc (10 % m/m mix) prior to planting (preplanting amendment). Bacterial cell suspensions were also applied as drench treatments immediately after planting. The preplanting treatments were mixed with the infested potting mix and incubated overnight at 24 °C before planting. The post-planting drench treatments of bacteria were diluted with water to the desired concentration (5×10^8 CFU mL^{-1}) and applied as 100 mL aliquots immediately after planting or 24 h after pathogen infestation.

8.5.2 Seed and Root Application

Cucumber (*Cucumis sativus* L. 'Straight Eight') or radish (*Raphanus sativus* L. 'Early Scarlet Globe') seeds were used in all growth-room experiments. Seeds were soaked in the bacterial suspensions (5×10^8 CFU mL^{-1}) for 2 h, air-dried in a laminar flow hood, and immediately planted in the pathogen-infested potting mix. Seeds were coated with talc and irradiated peat formulations of antagonistic bacteria by mixing the pre-wetted seeds with the bacterial bioformulations. The treated seeds were immediately sown in the infested mix. Seeds soaked in sterile water were used as control, whereas seeds treated with metalaxyl 48 % (10 mg L^{-1}) or benomyl 50 % WP (0.6 g kg^{-1}) served as the fungicide control. Roots of tomato (*Solanum lycopericum* L. 'Bonny Best') were treated with irradiated peat formulations of the selected bacterial strains to control *Fusarium* crown and root

rot and *Fusarium* wilt. Tomato seedlings were grown in vermiculite plug trays in a growth room for 4 weeks. They were gently removed from the trays and their roots after washing with running water were treated with the control and inoculated irradiated peat by coating. Treated seedlings were immediately planted in pathogen-infested potting mix or soil in pots and placed in a greenhouse.

8.6 Evaluation of Antagonistic Bacteria for Disease Suppression

The biocontrol potential of the selected antagonistic bacterial strains was evaluated for suppression of seedling damping-off and root rot of cucumber [*Pythium ultimum* Trow and *Phytophthora capsici* Leonian], seedling damping-off of radish [*Rhizoctonia solani* Kühn], *Fusarium* crown and root rot of tomato [*Fusarium oxysporum* Schlechtend.:Fr. f. sp. *radicis-lycopersici* WR Jarvis and RA Shoemaker], and *Fusarium* wilt of tomato [*Fusarium oxysporum* f. sp. Schlechtend.:Fr. *lycopersici* (Sacc.) WC Snyder and HN Hans] in growth-room or greenhouse pot assays and common scab of potato [*Streptomyces scabies* (Thaxter) Waksman and Henrici, syn: *S. scabiei* (Trüper and dè Clari 1997)] in micro-plots. Bacterial treatments were applied as cell suspension or as talc and irradiated peat formulation either directly to the potting mix or on seeds or roots. Disease conditions were created artificially by inoculating potting mix or soil with pathogen inoculum (1.25–3 % m/m) grown on pearl millet seed (Borrego-Benjumea et al. 2014), except for the potato scab study in micro-plots where a naturally infested soil from a field with a history of potato scab was used. The pearl millet seed inoculum of pathogens was mixed with potting mix or soil and incubated overnight prior to planting.

8.6.1 *Pythium* Damping-Off and Root Rot of Cucumber

The biocontrol potential of the three selected antagonistic bacteria, *P. fluorescens* 9A-14, *Pseudomonas* sp. 8D-45, and *B. subtilis* 8B-1, was evaluated for suppression of seedling damping-off and root rot of cucumber as preplant amendment and seed treatments in a *P. ultimum*-infested potting mix under growth-room conditions (Khabbaz and Abbasi 2014). In initial experiments, only cell suspensions of the three bacteria were used either as preplant amendment or post-plant drench treatments. The irradiated peat and talc formulations of the three bacteria were used as preplant amendment treatment, whereas irradiated peat formulation of the three bacteria was used as seed treatment.

8.6.1.1 Preplant Substrate Treatment

Application of cell suspensions of the three antagonistic bacteria to the *P. ultimum*-infested potting mix, either prior to planting as an amendment or after planting as a drench treatment, provided equal control of *Pythium* damping-off and root rot of cucumber seedlings and showed similar increase in plant fresh weights. In comparison to the infested control, the preplanting bacterial treatments showed a 125–290 % increase in healthy cucumber seedlings and a 27–50 % reduction in disease severity, whereas the post-planting bacterial treatments showed a 173–273 % increase in healthy seedlings and a 27–45 % reduction in disease severity. Both preplant and post-plant bacterial treatments to the pathogen-infested mix also significantly increased plant fresh weights compared to the pathogen-infested and noninfested controls.

Bioformulation of the three antagonistic bacteria prepared in irradiated peat or talc was also evaluated as preplant amendment treatments for suppression of *Pythium* damping-off and root rot of cucumber seedlings (Khabbaz and Abbasi 2014). As seen with cell suspensions, treatment of pathogen-infested potting mix with irradiated peat and talc formulations of all three bacteria also showed a significant increase in healthy

cucumber seedlings and plant fresh weights and a significant decrease in damping-off and root rot severity compared to the infested controls. A preplant amendment of untreated control irradiated peat and talc did not provide any protection of cucumber seedlings from damping-off and root rot. The disease protection by bacterial bioformulation and metalaxyl treatments was comparable. The preplant amendment treatment of irradiated peat formulation of Pf 9A-14 showed a significant increase in plant fresh weights of cucumber compared to all other treatments.

8.6.1.2 Seed Treatment

Seed treatment of control irradiated peat and talc did not provide any protection of cucumber seedlings from damping-off and root rot. Under a very high disease condition (97 % diseased plants in the infested control), all three bacterial and metalaxyl seed treatments were equally effective in protecting cucumber seedlings from damping-off and root rot with 66–75 % healthy seedlings with cell suspensions, 78–94 % healthy with talc formulation, 78–97 % healthy with irradiated peat formulation, and 84 % healthy with the metalaxyl seed treatment. Bacterial and metalaxyl seed treatments also showed a 53–69 % reduction in damping-off and root rot severity (Khabbaz and Abbasi 2014) and a four- to sixfold increase in plant fresh weights compared to the infested controls.

Seed treatment of a mixture of two or three strains of antagonistic bacteria in irradiated peat formulation was also investigated for suppression of *Pythium* damping-off and root rot of cucumber. Seed treatments with metalaxyl and irradiated peat formulations of all three antagonistic bacteria as single or mixtures of two or three strains suppressed damping-off and root rot of cucumber seedlings and showed a 590–810 % increase in healthy seedlings, a 43–57 % reduction in damping-off and root rot severity, and a 92–245 % increase in plant fresh weights as compared to the infested controls. The seed treatment with a mixture of three bacterial strains (*P. fluorescens* 9A-14, *Pseudomonas* sp. 8D-45, and *Bacillus subtilis* 8B-1) was the best treatment that showed a 245 % increase in plant fresh weights as compared to the infested control and a 61 % increase as compared to the noninfested control.

The biocontrol potential of another three antagonistic strains, *P. fluorescens* PEF-5 #18, *Pseudomonas chlororaphis* SL5, and *Paenibacillus polymyxa* #50, was also evaluated for suppression of *Pythium* damping-off and root rot of cucumber in growth-room assays. Bacteria were applied on seeds as irradiated peat formulations. All three bacterial treatments showed less disease severity, more healthy plants, and higher plant fresh and dry weights as compared with the non-treated seeds grown in pathogen-infested potting mix (Abbasi and Zhang unpublished data).

8.6.2 *Phytophthora* Damping-Off and Root Rot of Cucumber

The irradiated peat and talc formulations of the three antagonistic bacteria, *P. fluorescens* 9A-14, *Pseudomonas* sp. 8D-45, and *B. subtilis* 8B-1, were also evaluated for suppression of *Phytophthora* damping-off and root rot of cucumber seedlings as preplant amendment and seed treatments in a *P. capsici*-infested potting mix in growth-room pot assays (Khabbaz et al. 2015).

8.6.2.1 Preplant Substrate Treatment

In a *P. capsici*-infested potting mix, preplant treatments with cell suspension or irradiated peat and talc formulations of all three antagonistic bacteria also protected cucumber seedlings from *Phytophthora* damping-off and root rot and produced 69–85 % healthy seedlings compared to 6 % healthy in the infested controls. Disease control by antagonistic bacteria was comparable to the metalaxyl treatment. Plants produced in pathogen-infested potting mix pretreated with cell suspension or irradiated peat or talc formulations of the three bacteria showed significantly higher fresh weights

compared to the plants produced in infested and noninfested control mixes and in infested mix pretreated with metalaxyl.

8.6.2.2 Seed Treatment

Seed treatments of cell suspension or irradiated peat and talc formulations of all three antagonistic bacteria also protected cucumber seedlings from *Phytophthora* damping-off and root rot and showed 72–85 % healthy seedlings compared to 3–6 % healthy in the infested controls. Seed treatment of irradiated peat and talc formulation of *P. fluorescens* 9A-14 provided better disease protection than the metalaxyl seed treatment. All bacterial seed treatments also showed significantly higher plant weights compared to infested and noninfested controls and the metalaxyl seed treatment.

Seed treatments with mixtures of antagonistic bacteria in irradiated peat formulation were also evaluated for suppression of *Phytophthora* damping-off and root rot of cucumber (Khabbaz et al. 2015). Seed treatments with irradiated peat formulations of a mixture of two or three bacteria showed 63–84 % healthy seedlings as compared to 22 % healthy in infested control and 50 % healthy in metalaxyl seed treatment. All bacterial seed treatments also showed a reduction in damping-off and root rot severity and an increase in plant fresh weights compared to the infested controls. The bacterial seed treatment containing a mixture of three bacteria was the best treatment that showed a significant increase in plant fresh weights as compared to all other treatments.

In order to improve the disease suppression by antagonistic bacteria, acibenzolar-*S*-methyl (ASM) or Actigard (50 WP; Syngenta Crop Protection Inc., Guelph, Canada), a chemical activator of disease resistance in plants (Oostendorp et al. 2001; Anith et al. 2004; Park et al. 2013), was used as seed treatment in combination with *P. fluorescens* 9A-14 (Pf 9A-14). The aqueous suspension of ASM (30 mg a.i. L^{-1}) was used as seed treatment alone or in combination with Pf 9A-14 (5×10^8 CFU mL^{-1}). Both as single and mixture treatments, ASM and Pf 9A-14 protected cucumber seedlings from *Phytophthora* damping-off and showed a 280–461 % increase in healthy seedlings, 40–44 % reduction in damping-off severity, and a 112–217 % increase in plant fresh weights as compared to the untreated infested control (Fig. 8.2).

There were no significant differences between Pf 9A-14 and ASM treatments in suppressing *Phytophthora* damping-off of cucumber seedlings when used alone, but Pf 9A-14 seed treatment resulted in higher plant fresh weights over ASM treatment (Fig. 8.2). The mixture treatment (Pf 9A-14 + ASM) did not show a significant improvement over the individual treatments for increasing healthy cucumber seedlings or reducing disease severity, but showed a significant increase in plant fresh weights over the individual treatments (Fig. 8.2).

The effectiveness of four additional antagonistic bacterial strains, *P. chlororaphis* SL5, *B. amyloliquefaciens* 1A-48 and 1B-14, and *P. fluorescens* PEF-5 #18, was also evaluated as seed treatment for suppression of *Phytophthora* damping-off and root rot of cucumber in pathogen-infested potting mix. After 2 weeks of planting, plants in pathogen-infested potting mix sown with seeds treated with irradiated peat formulation of all four bacteria consistently showed less disease severity, more healthy plants, and higher plant fresh and dry weights compared to the plants in pathogen-infested mix sown with untreated control seeds (Abbasi and Zhang unpublished data).

8.6.3 *Rhizoctonia* Seedling Damping-Off of Radish

The biocontrol potential of three antagonistic bacteria *P. fluorescens* 9A-14, *Pseudomonas* sp. 8D-45, and *B. subtilis* 8B-1 was also evaluated for suppression of damping-off of radish seedlings as preplant amendment and seed treatments in a *R. solani*-infested potting mix under growth-room conditions (Khabbaz et al. 2015).

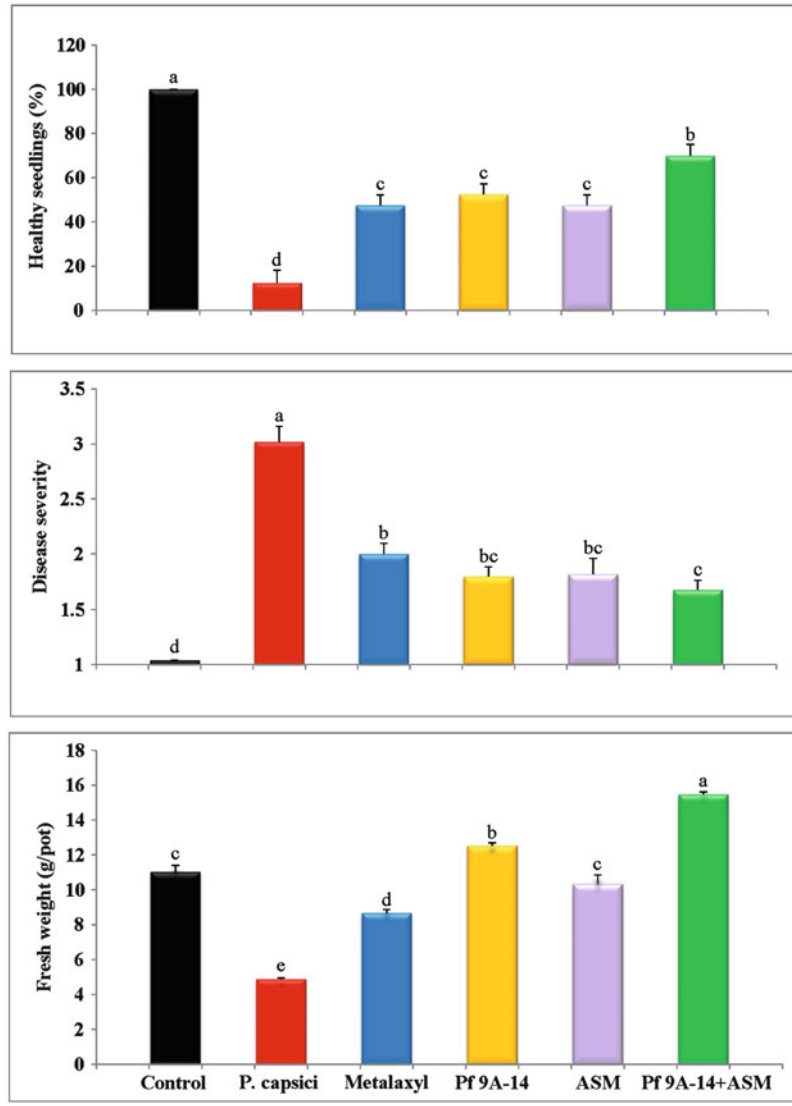

Fig. 8.2 Effect of seed treatment of acibenzolar-*S*-methyl (*ASM*) and *P. fluorescence* Pf 9A-14 alone or in combination on healthy seedlings, *Phytophthora* damping-off and root rot severity, and plant fresh weights of cucumber in an infested peat mix under growth-room condition. Values (± SE) are the mean of four replicates. Means with a common letter are not significantly different according to Fisher's protected least significant difference test at $P \leq 0.05$

8.6.3.1 Preplant Substrate Treatment

Preplant application of cell suspension or irradiated peat and talc formulations of all three antagonistic bacteria to the *R. solani*-infested potting mix showed a modest protection of radish seedlings from damping-off with 18–27 % healthy seedlings in potting mix treated with bacteria as compared to the 5 % healthy in the infested controls. Comparatively, benomyl preplant treatment was more effective in increasing the percentage of healthy seedlings than the antagonistic bacteria. Preplant treatments of all three antagonistic bacteria and benomyl also increased fresh weights of radish plants compared to the infested controls.

8.6.3.2 Seed Treatment

The irradiated peat formulations of three antagonistic bacteria applied alone or in mixture as seed treatments also showed a modest protection of radish seedlings from damping-off. In pots sown with bacterial-treated seeds, 25–51 % seedlings were healthy as compared to the 5 % healthy in the infested controls. All bacterial seed treatments showed a reduction in *Rhizoctonia* damping-off severity and an increase in plant

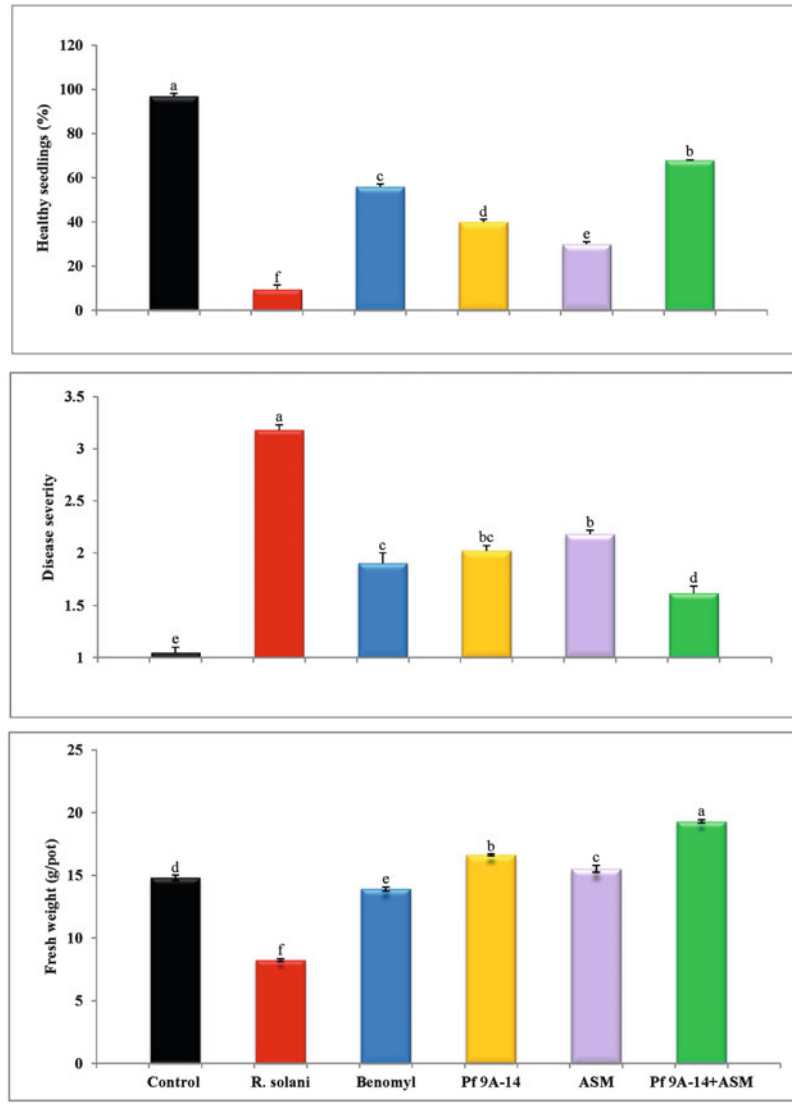

Fig. 8.3 Effect of seed treatment of acibenzolar-*S*-methyl (*ASM*) and *P. fluorescens* Pf 9A-14 alone or in combination on healthy seedlings, *Rhizoctonia* damping-off and root rot severity, and plant fresh weights of radish in a *R. solani*-infested potting mix under growth-room condition. Values ± (SE) are the mean of five replicates. Means with a common letter are not significantly different according to Fisher's protected least significant difference test at $P \leq 0.05$

fresh weights compared to the untreated infested controls. The bacterial seed treatments containing mixtures of any two antagonistic bacteria showed similar percentage of healthy seedlings. However, the seed treatment containing a mixture of three bacteria significantly increased plant fresh weights as compared to all other treatments.

As disease protection with antagonistic bacteria was only modest, an improvement in suppression of *Rhizoctonia* damping-off of radish by antagonistic bacteria was attempted with combined seed treatment of ASM and Pf 9A-14 in growth-room pot assays. Actigard or ASM was dissolved in water at 30 mg a.i. L^{-1} and used as seed treatment alone or in combination with Pf 9A-14 (5×10^8 CFU mL^{-1}). Radish seeds treated with ASM and Pf 9A-14 alone or as mixture significantly increased the percentage of healthy seedlings, decreased damping-off severity, and increased plant fresh weights compared to the infested control (Fig. 8.3). The combined ASM and Pf 9A-14 seed treatment showed a significant improvement over the individual treatments and protected radish seedlings from damping-off by increasing the percentage of healthy radish seedlings, reducing disease severity, and increasing plant fresh weights (Fig. 8.3). The disease protection achieved with the mixture

treatment (ASM + Pf 9A-14) was also better than benomyl seed treatment (Fig. 8.3).

8.6.4 *Fusarium* Crown and Root Rot (FCRR) of Tomato

The biocontrol potential of the six selected antagonistic bacterial strains (*P. fluorescens* 9A-14, *B. subtilis* 8B-1, *Pseudomonas* sp. 8D-45, *P. polymyxa* #53, *P. chlororaphis* SL5, and *P. fluorescens* PEF-5 #18) was investigated for suppression of FCRR in *Forl*-infested potting mix and a field soil in greenhouse pot assays (Zhang et al. 2015). Bacteria were applied as irradiated peat formulation on roots of tomato seedlings which were immediately planted in the infested potting mix or soil.

Tomato seedlings treated with irradiated peat formulation of the six bacteria and grown in *Forl*-infested potting mix for 25 days showed a 40–63 % reduction in FCRR severity, a 169–274 % increase in plant height, and a 101–126 % increase in root length as compared to the non-treated control seedlings grown in *Forl*-infested mix. The bacterial roots treatments also showed a significant increase in plant fresh and dry weights as compared to the untreated infested controls. The effects of most bacterial treatments were better than or comparable to the benomyl treatment for plant height, root length, fresh or dry weights, and FCRR severity. The plants treated with bacterial strains *Pseudomonas* sp. 8D-45 and *P. fluorescens* PEF-5#18 and grown in *Forl*-infested mix showed a 63–66 % increase in fresh and 29 % increase in dry weights as compared to untreated plants grown in noninfested mix.

Similar results were also achieved in *Forl*-infested field soil (Zhang et al. 2015). Tomato plants treated with irradiated peat formulation of bacteria and grown in *Forl*-infested soil for 25 days showed a 40–60 % reduction in FCRR severity, a 17–53 % increase in plant height, and a 265–300 % increase in root length as compared to the untreated plants grown in *Forl*-infested field soil. All bacterial and benomyl treatments also showed similar results for FCRR severity, root length, and plant fresh and dry weights in field soil as well. Plants treated with bacterial and benomyl treatments and produced in *Forl*-infested soil showed higher fresh and dry weights as compared to the untreated control plants produced in *Forl*-infested soil, and such plants also had longer roots as compared to untreated control plants grown in pathogen noninfested soil.

8.6.5 *Fusarium* Wilt of Tomato

The biocontrol potential of ten antagonistic bacteria (Table 8.2) was also evaluated for suppression of *Fusarium* wilt in greenhouse pot assays. Tomato seedlings were treated with irradiated peat formulation of the selected bacterial strains and grown in *Fol*-infested potting mix for 25 days before evaluating for disease severity and growth parameters. Plants treated with bacterial formulations and planted in *Fol*-infested potting mix showed a 61–74 % reduction in severity of *Fusarium* wilt (Table 8.2). These treated plants also showed a 607–673 % increase in fresh weights and a 300–400 % increase in dry weights as compared to the untreated plants grown in pathogen-infested mix. Plants treated with bacterial formulations and planted in *Fol*-infested potting mix showed a 17–28 % increase in plant fresh weights and a 25 % increase in plant dry weights (six bacteria) compared to the untreated plants grown in pathogen noninfested control soil (Table 8.2).

8.6.6 Common Scab of Potato

Common scab and *Verticillium* wilt are two main soilborne diseases of potatoes (*Solanum tuberosum* L.) causing significant crop losses to growers and both these diseases often occur in the same field. These two diseases and the potato plant are a good model system to evaluate disease control effects of biofungicides and other products. Seed potato tubers also offer good surface area for application of microbial biocontrol agents.

The biocontrol potential of the selected ten antagonistic bacterial strains was also assessed

Table 8.2 Effect of irradiated peat formulation of selected antagonistic bacterial strains as tomato root application on plant fresh and dry weights and severity of *Fusarium* wilt in a potting mix infested with *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) in a greenhouse

Treatment	Fresh weight (g)	Dry weight (g)	Disease severity
Control	9.1 ± 0.4 b	0.4 ± 0.0 b	0 ± 0.0 c
Fol	1.5 ± 0.1 c	0.1 ± 0.0 d	4.6 ± 0.0 a
Benomyl	10.5 ± 0.5 a	0.4 ± 0.0 b	1.4 ± 0.2 b
Paenibacillus polymyxa #50	10.9 ± 0.4 a	0.5 ± 0.0 a	1.4 ± 0.2 b
Paenibacillus polymyxa #53	11.2 ± 0.6 a	0.4 ± 0.0 b	1.8 ± 0.2 b
Bacillus amyloliquefaciens 1A-48	11.2 ± 0.3 a	0.5 ± 0.0 a	1.2 ± 0.4 b
Bacillus amyloliquefaciens 1B-14	11.6 ± 0.4 a	0.5 ± 0.0 a	1.6 ± 0.2 b
Bacillus amyloliquefaciens 1B-23	11.4 ± 0.4 a	0.4 ± 0.0 b	1.4 ± 0.2 b
Pseudomonas chlororaphis 1B-26	10.7 ± 0.4 a	0.5 ± 0.0 a	1.8 ± 0.2 b
Bacillus subtilis 8B-1	11.3 ± 0.6 a	0.5 ± 0.0 a	1.6 ± 0.2 b
Pseudomonas sp. 8D-45	11.2 ± 0.8 a	0.5 ± 0.0 a	1.8 ± 0.2 b
Pseudomonas fluorescens 9A-14	10.6 ± 0.5 a	0.4 ± 0.0 b	1.6 ± 0.2 b
Bacillus amyloliquefaciens 9A-31	10.9 ± 0.6 a	0.4 ± 0.0 b	1.8 ± 0.2 b

Values ± standard errors are the average of five replicates, if followed within a column by a common letter are not significantly different according to Fishers protected least significant difference test at $P \leq 0.05$

for suppression of common scab of potato in the micro-plots. A sandy-loam soil for these studies was brought from a commercial potato field with a history of common scab disease. Seed potato 'Yukon Gold' tubers were coated with irradiated peat formulation of the bacterial strains prior to planting in a potato soil. Untreated tubers or tubers treated with non-inoculated control irradiated peat served as control. Five treated and untreated tubers were planted in early June in each of the four replicate micro-plots per treatment. Daughter tubers were harvested in late September and assessed for disease severity and deep-pitted lesions (Abbasi 2013). Harvested tubers were also weighed to determine total yield.

The disease level on daughter tubers harvested from the control micro-plots was very high and most of the tubers had deep-pitted lesions. Under these high disease conditions, seven bacterial treatments provided a modest disease control and showed a 13–39 % reduction in severity of common scab on daughter tubers as compared to the control treatment. Three bacterial strains showed no reduction of scab severity on daughter tubers. Seed treatment of most bacterial strains increased tuber weights compared to the untreated control treatment. Only one strain showed a reduction in deep-pitted lesions on tubers (Abbasi et al. unpublished data).

8.7 Conclusions and Next Steps

Indigenous antagonistic bacterial strains displayed the potential to be developed as biofungicides to control various plant diseases caused by soilborne pathogens. Under controlled-environment conditions, bacterial strains provided good control of *Pythium* or *Phytophthora* damping-off, root rot of cucumber, *Fusarium* crown and root rot and *Fusarium* wilt of tomato. The disease control effect of bacterial strains against *Rhizoctonia* damping-off was only modest. Under micro-plot conditions, some bacterial strains provided only modest control of common scab of potato in a very high disease level natural soil. Irradiated peat was found to be a suitable carrier material for delivery of biocontrol bacteria on seed, root, or directly to potting substrate as an amendment. Antagonistic bacteria also showed a significant effect on plant-growth promotion and increased plant fresh or dry weights. In some cases, the disease protection and plant-growth promotion effect by antagonistic bacteria was comparable or even better than the chemical fungicides. Most of the *Bacillus* and *Paenibacillus* strains showed the capacity to produce antibiotics such as bacillomycin, bacilysin, iturin A, surfactin, and fengycin, whereas most of

the *Pseudomonas* strains possessed antibiotic biosynthetic genes for pyoluteorin, pyrrolnitrin, and 2,4-DAPG. However, the actual production of these antibiotics by antagonistic bacterial strains was not confirmed. Most bacterial strains showed production of lytic enzymes, HCN, IAA, and SA. Some strains also produced siderophores and volatile compounds. Antagonistic and plant-growth promotion activities of these bacterial strains might be related to the production of several types of antibiotics, lytic enzymes, phytohormones, secondary metabolites, siderophores, and volatile compounds.

Although the biocontrol potential of antagonistic bacteria for suppression of soilborne diseases was demonstrated under controlled-environment, unpasteurized or unsterilized potting substrate and field soil was used in all pot experiments and should be containing the natural competing organisms. The bioformulations of these antagonistic bacterial strains prepared in irradiated peat can now be evaluated under field conditions. Therefore, future studies should be directed at confirming the disease control potential of these antagonistic bacteria under natural conditions of disease development in the field. Studies should also be aimed at confirming the specific modes of disease suppression and plant-growth promotion by antagonistic bacteria and integrating these biocontrol agents with other disease management options.

Acknowledgements Technical assistance was provided by Brian Weselowski, Bruce McPherson, and Igor Lalin. The funding for the research work reported in this book chapter was provided by Agriculture and Agri-Food Canada.

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Bio-based and Reduced-Risk Strategies for the Management of *Phytophthora* Blight and Root Rot of Pepper

9

Yan Ma, Qiujun Wang, Yun Cao, Guangfei Wang, Dejie Guo, and Pervaiz A. Abbasi

Contents

Abstract

Phytophthora blight and root rot caused by *Phytophthora capsici* affects several solanaceous and cucurbit hosts worldwide. The disease has become a constraint in cultivation of chili pepper in China and is an emerging problem in Canada and the USA affecting pepper, tomato, cucumber, and other cucurbits. Several bio-based approaches such as biofungicides; soil amendments of rapeseed meal, composts, and manures; soil solarization; grafting; and reduced-risk chemicals have been investigated for the management of *Phytophthora* blight and root rot of pepper. The biocontrol potential of fungal antagonists such as *Penicillium striatisporum* Pst10, *Trichoderma harzianum*, and *Trichoderma hamatum* 382 and bacterial antagonists such as *Bacillus amyloliquefaciens* BS211, *Pseudomonas corrugata*, and other *Pseudomonas* and *Bacillus* strains was shown for suppression of pathogen growth and disease. Soil amendment of rapeseed meal, manures, and composts also provided suppression of *Phytophthora* blight and root rot under greenhouse and field conditions. Some combinations of biocontrol agents and soil amendments provided enhanced disease control. Soil solarization can be very effective in reducing pathogen inoculum from the top

Y. Ma • Q. Wang • Y. Cao • G. Wang • D. Guo
Institute of Agricultural Resource and Environment, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China

P.A. Abbasi (✉)
Atlantic Food and Horticulture Research Centre, Agriculture and Agri-Food Canada, 32 Main Street, Kentville, NS B4N 1J5, Canada
e-mail: Pervaiz.Abbasi@agr.gc.ca

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_9

10-cm layer of soil, but this strategy may be only effective in countries where summer months are very hot. Anaerobic soil disinfestation using wheat straw as the carbon source followed by flooding for 2–3 weeks reduced pathogen populations in soil and also disease incidence on pepper. Reduced-risk chemicals such as phosphonates and Actigard and grafting of resistant rootstocks also provided disease control and offer additional sustainable methods for the management of *Phytophthora* blight and root rot of pepper. Integration of various management strategies may be more effective for managing disease problems such as *Phytophthora* blight and root rot.

9.1 Introduction and Background

Phytophthora blight and root rot is caused by a soilborne oomycete pathogen *P. capsici* Leonian. The pathogen has a wide host range infecting a variety of plant species including pepper, tomato, eggplant, cucumber, and other cucurbits worldwide and causing symptoms such as damping-off, blight, and crown, root, or fruit rots depending on the developmental stage of the plant at the time of infection (Ristaino and Johnston 1999; Hausbeck and Lamour 2004). *Phytophthora* blight and root rot is also a common and destructive disease of greenhouse and field-grown chili and sweet peppers (Fig. 9.1) in China and worldwide. In China, the disease has become a serious constraint in cultivation of chili peppers causing serious economic losses to growers in several provinces every year. This disease situation may have been established due to continuous cultivation of peppers in greenhouses for many years. *Phytophthora* blight and root rot is an emerging disease in Canada and the USA affecting several solanaceous and cucurbit crops both under field conditions and in commercial greenhouses. With the phasing out of methyl bromide and due to concerns of negative impacts of chemical pesticides on the environment and human health, the development of sustainable, eco-friendly, and alternative methods of managing soilborne diseases such as *Phytophthora* blight and root rot has become a top priority (Colla et al. 2012).

Phosphorous acid and the phosphorous acid-based fungicides such as phosphonates or phosphites are reduced-risk chemicals as there are no known harmful effects associated with their use (Guest and Grant 1991). Phosphorous acid and phosphonates fall under the category of natural products. Phosphonates and phosphites have been very effective against diseases caused by oomycete plant pathogens. Although their compatibility with microbial biofungicides for disease suppression has not been reported, phosphonates have been shown to induce symbiotic infections by mycorrhizal fungi (Jabaji-Hare and Kendrick 1987; Howard et al. 2000). Application of a phosphonate formulation as a pre-planting amendment, post-planting drench, or seed treatment provided effective control of *Pythium* damping-off of cucumber (Abbasi and Lazarovits 2005, 2006a) and as an application to soil prior to planting or after planting provided suppression of clubroot of bok choy and cabbage under micro-plot and field conditions (Abbasi and Lazarovits 2006b). Acibenzolar-*S*-methyl (ASM) or Actigard is a chemical activator and inducer of systemic resistance in plants (Oostendorp et al. 2001). Both these chemicals can be used as sustainable disease management approaches.

There is also a growing interest in the use of microbial-based biofungicides as bioformulations and agricultural by-products as biofumigants for replacement of chemical pesticides to manage soilborne diseases and pests. There have been considerable efforts devoted toward developing biofungicides for managing soilborne diseases, and several bacterial and fungal antagonists have been reported as potential candidates (Ezziyyani et al. 2007). Similarly, soil incorporation of organic material containing agricultural by-products and manures and grafting also show tremendous potential of managing such soilborne diseases (Gilardi et al. 2013). In the case of biofumigation, the suppression of soilborne pathogens and pests is mainly accomplished by release of inhibitory chemicals during microbial

Fig. 9.1 *Phytophthora* blight and root rot affecting sweet pepper (**a**–**d**) and chili pepper (**e** and **f**)

decomposing of organic material (Matthiessen and Kirkegaard 2006). Several plant species including mustards, oilseed radish, rapeseed, Sudan grass, and pearl millet have been used as rotation or cover crops for their biofumigation effect (Hansen and Keinath 2013). As cover crops, these plants can be disked back into the soil as green manure followed by plastic mulching to retain volatiles for maximum effect. Most of the nutrients will also be retained in the soil.

Alternative and sustainable strategies have also been used for the management of *Phytophthora* blight and root rot of pepper, and considerable research efforts and resources were put forward in search for potential biofungicides or suitable soil amendments from locally available resources. Soil solarization and anaerobic soil disinfestation are also used as alternative strategies for the management of *Phytophthora* blight and root rot of pepper. The reduced-risk chemicals such as phosphonates and Actigard, as

well as grafting technology, were also investigated as alternative management solutions for this disease. In this chapter, an overview of the work on the management of *Phytophthora* blight and root rot of pepper with bio-based approaches such as biofungicides; soil amendments of rapeseed meal, composts, and manures; solarization; grafting; and reduced-risk chemicals is discussed.

9.2 Management of *Phytophthora* Blight and Root Rot of Pepper

Control of *Phytophthora* blight and root rot is very complicated, and several sustainable disease management options have been investigated including cultural practices, soil amendments, resistant cultivars, biofungicides, solarization, and grafting (Ristaino and Johnston 1999; Granke et al. 2012; Gilardi et al. 2013). Some of these sustainable disease management strategies are described in this review. For instance, all the disease management work on chili pepper was carried out in China using locally available resource materials such as bioformulations with indigenous biofungicides, soil biofumigation with locally available agricultural by-products such as rapeseed meal and manures, and soil amendment of wheat straw followed by flooding. The disease management work on bell or sweet pepper was reported from Canada, the USA, Europe, and Korea.

9.2.1 Bioformulation of Fungal Antagonists

During the search for potential biocontrol agents, an isolate of *P. striatisporum* Pst10 was recovered from the rhizosphere of chili peppers grown in a greenhouse in Nanjing, China (Ma et al. 2008). On agar plate assays, the isolate Pst10 was shown to inhibit the growth of mycelium of *Phytophthora* spp., *Cladosporium cucumerinum*, and *Sclerotinia sclerotiorum*. In subsequent in vitro pathogen inhibition tests, the culture filtrates (CF) of Pst10 were used. The CF of Pst10 was obtained after growing the fungal culture in potato dextrose broth in a shaker for a week and by removing mycelium and spores after filtration and centrifugation. In in vitro tests, the CF inhibited the mycelium growth and also inhibited formation and/or germination of sporangia and spores of *P. capsici*. The CF completely inhibited the growth of mycelium, and a 20-fold dilution of CF inhibited formation and germination of sporangia and spores. A 100-fold dilution of CF also caused abnormal effects on the pathogen mycelium. The antifungal substances from organic solvent extracts of CF of Pst10 were isolated using thin-layer chromatography (TLC) approach. Three potential compounds isolated from TLC plate also showed antifungal activity against *P. capsici*.

The next challenging work was how to exploit the biocontrol potential of this native isolate obtained from the rhizosphere of chili peppers to manage *Phytophthora* blight and root rot. Both fungal propagules and CF of Pst10 showed the potential to be used as active ingredients for growth suppression of *P. capsici*. The ability of Pst10 for suppression of *Phytophthora* blight and root rot in chili peppers was tested in greenhouse pot assays with soil infested with sporangia of *P. capsici*. Conidia and CF of Pst10 were mixed with the pathogen-infested soil. Pots were planted with chili pepper seedlings cv. Sujiao No. 2 and placed in a greenhouse. Plants were assessed for disease incidence after 7 and 14 days. The treatment of pathogen-infested soil with conidia and CF of Pst10 significantly reduced the incidence of *Phytophthora* blight and root rot of chili. More than 70–80 % plants were still healthy with both Pst10 treatments after 14 days of planting.

A suitable substrate was needed that could serve as a delivery material for Pst10 and enhance its colonization and effectiveness. Composted pig manure was the obvious and likely choice as it was easily available from local sources. The colonization of composted pig manure by Pst10 was assessed in pots containing soil from a local vegetable farm. The soil was inoculated with Pst10 at 4×10^6 conidia

per g dry soil and amended with composted pig manure at 1 % on a weight basis. The pots were also planted with chili pepper seedlings. The colony-forming units (CFUs) of Pst10 were determined in soil samples taken from pot experiments on a selective medium (Ma et al. 2008) after 3, 10, 20, and 40 days of planting. We found that the composted pig manure slowly increased the colonization of the chili rhizosphere by Pst10. The CFUs of Pst10 were 3 times higher after 10 days and 17 times higher after 20 days of planting in composted pig manure-amended soil as compared to Pst10-amended soil. However, after 40 days of planting, the Pst10 CFUs in composted pig manure-amended soil decreased but still 4 times higher than the Pst10-amended soil.

Composted pig manure seems to enhance or at least maintain the original inoculated population density of Pst10 in the amended soil. This may be a key factor for a successful biological control agent to establish and proliferate in soil and rhizosphere. It was interesting to know the impact of combined treatments of Pst10 and composted pig manure on the incidence of *Phytophthora* blight and root rot of chili. In greenhouse pot experiments, the simultaneous or combined application of Pst10 (conidia + CF) and composted pig manure to pathogen-infested soil was proved to be the best treatment that significantly reduced the incidence of *Phytophthora* blight and root rot of chili. More than 92 % plants were still healthy with the combined treatment after 14 days of planting.

An isolate of *T. harzianum* showed some success in suppressing the growth of *P. capsici* and controlling *Phytophthora* blight and root rot of pepper in Spain (Sid Ahmed et al. 1999). This antagonistic isolate also showed synergistic activity with a bacterial antagonist *Streptomyces rochei* as a combined treatment greatly reducing the pathogen population from soil (Ezziyyani et al. 2007). Treatment of pepper seeds and roots with *T. harzianum* reduced stem necrosis caused by *P. capsici* (Sid Ahmed et al. 2000). Several biocontrol mechanisms for the suppression of pathogen growth and disease by *T. harzianum* have been suggested including direct inhibition of pathogen growth by production of inhibitory compounds, competition for nutrients and niche, and induction of resistance in pepper plants against pathogen (Sid Ahmed et al. 2000).

Another fungal antagonist *T. hamatum* 382 was reported for its biocontrol potential against *P. capsici* in a greenhouse study from Ohio (Khan et al. 2004). In this study, *T. hamatum* 382 was inoculated onto a compost-amended substrate. The fungal antagonist reduced the severity of *Phytophthora* damping-off and root rot of cucumber, and induced resistance was suggested as a mechanism of this disease reduction.

9.2.2 Bioformulation of Rhizobacteria

An antagonistic strain of *B. amyloliquefaciens* BS211 was isolated from the root of healthy pepper in a field severely affected by *Phytophthora* blight and root rot in Huaian, Jiangsu province of China (Wang et al. 2014a). In dual-culture plate assays, the strain BS211 was antagonistic to mycelium growth of *P. capsici* (Wang et al. 2014a) and other plant pathogenic fungi. The biocontrol potential of BS211 was evaluated in greenhouse pot experiments for suppression of *Phytophthora* blight and root rot disease of chili pepper in a pathogen-infested soil. Soil used in these pot experiments was collected from a pepper field in Huaian and had a sandy loam texture with pH 7.8. The soil was first inoculated with spore suspension of *P. capsici* at 10^3 CFU g^{-1} soil, and the pathogen-infested soil was later mixed with the biocontrol bacteria BS211 at 10^7 CFU g^{-1} soil. The seedlings of chili pepper cv. Sujiao No. 5 grown in a greenhouse mix up to four-leaf stage were transplanted into pots. Plants were rated visually for the incidence of *Phytophthora* blight and root rot disease after 7, 15, 25, and 35 days of transplanting and the percentage of infected plants calculated. After 7 days of planting, all plants grown in *P. capsici* + BS211-treated soil were healthy compared to plants grown in *P. capsici*-infested soil only (0 vs. 15 % disease incidence,

respectively). The incidence of *Phytophthora* blight and root rot gradually increased in *P. capsici*-infested soil reaching 100 % after 35 days of planting. Disease incidence also steadily increased in the *P. capsici* + BS211 treatment reaching a maximum of 20 % after 35 days of planting (80 % plants were still healthy).

The pathogen *P. capsici* has a broad host range, and it also affects cucumbers, melons, and other cucurbits. The same pathogen also causes seedling damping-off and root rot diseases in cucumbers. The cucumber-*Phytophthora* has been a very good model system to assess biocontrol potential of rhizobacteria, and during that screening process, we found several novel strains of rhizobacteria belonging to genera *Pseudomonas* and *Bacillus* that showed biocontrol potential for suppression of *Phytophthora* damping-off and root rot of cucumber (Khabbaz et al. 2015). The formulations of the bacterial strains were prepared in irradiated peat or talc powder and applied directly to the pathogen-infested potting substrate or on seed. A more detailed description of this work can be found in Chapter No. 8 in this book.

Strains of rhizobacteria were also investigated for suppression of *Phytophthora* blight and root rot on bell pepper in Korea. In this work, bacteria were screened using radicle and seedling assays and tested for suppression of *Phytophthora* blight and root rot of pepper under artificial and natural inoculation conditions (Sang et al. 2008). Bacteria were applied as drench and root treatments. Artificial inoculations were made with *P. capsici* zoospores 5–7 days after bacterial treatments. The results of both controlled environment and field conditions consistently showed less incidence and severity of *Phytophthora* blight and root rot on bell pepper plants treated with the two *P. corrugata* strains CCR04 and CCR80. This reduction in disease was later shown to be related to bacterial colonization of pepper roots (Sang and Kim 2014).

Another study from Korea used a field-effective biocontrol strategy against *Phytophthora* blight and root rot complex of pepper caused by three pathogens (Kim et al. 2008). In that study, a combination of three strains of chitinolytic bacteria was selected based on their activity against three pathogens in the complex to control *Phytophthora* blight complex under greenhouse and field conditions. The combination of three bacterial strains was more effective than any single strain in suppressing *Phytophthora* blight and root rot in pot assays. Bioformulations of the combined strains prepared in a chitin-based growth medium resulted in effective control in field applications. The study also suggested that the effectiveness of the formulated product for the management of control of *Phytophthora* blight and root rot of pepper could be further improved by integrating with other management strategies such as crop rotation and soil solarization and by altering timing of application.

9.2.3 Biofumigation of Rapeseed Meal

Incorporation of *Brassica* plant residues or seed meal to soil has been shown to suppress soilborne pathogens and pests by the effect of biofumigation (Matthiessen and Kirkegaard 2006). To determine the biofumigation effect of rapeseed meal on *Phytophthora* blight and root rot of pepper, a field experiment was conducted in a pepper-growing area of Huaian, Jiangsu province of China. The rapeseed meal used in the field experiment was obtained by grinding dried seed containing rapeseed pods in a warring blender. This seed meal had a total glucosinolate content of 85.71 μmol g^{-1} and 5.04 % of total N. The rapeseed meal (0.4 %, w/w) and dazomet (0.03 %, w/w) were incorporated in the field plots to a depth of 20 cm using a rotary tiller, and plots were immediately irrigated to ensure high soil moisture for hydrolysis of glucosinolates. Subsequently, the plots were covered with plastic sheets for about 20 days before transplanting with 5-week-old red pepper seedlings. Plots were irrigated immediately after planting and daily after that as needed. Pepper seedlings were monitored for disease development, and the cumulative number of infected plants was calculated from the day

after transplanting until pepper harvest. Plants were designated infected with *P. capsici* if they showed pathogen signs such as white mycelia at the stem base and symptoms commonly associated with the disease, such as yellowing of leaves and wilting. Disease incidence from each plot was calculated and expressed as the percentage of diseased plants.

The results of the field experiment indicated a positive effect of biofumigation with soil incorporation of rapeseed meal on suppression of *Phytophthora* blight and root rot disease of chili pepper. The plots treated with rapeseed meal showed less disease incidence and an increase in pepper yield as compared to the control and dazomet treatments (Wang et al. 2014b). The effects of soil amendment on communities of bacteria and fungi in soil were also assessed by denaturing gradient gel electrophoresis, and the results indicated that the biofumigation of rapeseed meal increased bacterial diversity and decreased fungal diversity (Wang et al. 2014b). The incidence of disease on chili peppers was negatively correlated with bacterial diversity in soil and positively correlated with fungal diversity in soil. The evaluation of chemical properties of the amended soils indicated that rapeseed meal amendment increased total N and nitrate-N contents of soil as well as available P and K contents. The field study also found a significant correlation between microbial community structures in the amended soil and chemical properties of the soil.

Overall, the results of this study indicated that soil biofumigation of rapeseed meal reduced incidence of *Phytophthora* blight and root rot disease of chili pepper. This disease control effect could be due to release of fumigants (Larkin and Griffin 2007) after breakdown of rapeseed meal in soil that may be toxic to pathogen and also by indirect effects of induced changes in the structure and composition of soil microbial communities. On another note, rapeseed meal is a high-nitrogen-containing product, and its amendment to soil could also lead to release of ammonia and nitrous acid which can kill *Phytophthora* propagules (Tsao and Oster 1981). This needs to be further investigated as it depends on the application rate of the nitrogenous amendment and other soil factors (Tenuta and Lazarovits 2002). An accurate mode of action of a soil amendment will be very helpful for its optimal use and for maximizing its disease-controlling effect.

9.2.4 Combinations of Biofumigation and Bioformulation

The effects of a combination of rapeseed meal and biofungicide *B. amyloliquefaciens* BS211 for control of *Phytophthora* blight and root rot of pepper on soil bacterial community structure were determined in greenhouse experiments (Wang et al. 2014a). The soil for the pot experiment was collected from a field in Huaian, Jiangsu province, where the field had been under repeated pepper cultivation since 1980. The soil was infested with spore suspension of *P. capsici* at a concentration of 10^3 CFU g^{-1} soil. At 1 day after infesting soil with *P. capsici*, the rapeseed meal was incorporated into soil at a rate of 4 g kg^{-1} dry soil, and the appropriate quantity of water was added to adjust soil moisture content to 50 % of the water holding capacity. This moisture level was maintained by regularly adding water when required. Amended soil was later transferred to 5-L pots and covered with double-layered plastic film to minimize fumigant emissions. Pots were transferred in a growth room and incubated at 25 ± 2 °C for 20 days. The plastic cover was maintained for 20 days, and after its removal, the soil was gently mixed to ensure the release of any residues of isothiocyanate. Antagonistic bacteria BS211 was grown separately in nutrient broth in 5-L fermentation tanks. The bacterial cells were suspended in 0.01 M phosphate buffer, and the concentration of bacteria was adjusted at 10^9 CFU ml^{-1} using a spectrophotometer. Immediately after rapeseed incorporation, the suspension of BS211 was added to the soil at a final concentration of 10^7 CFU g^{-1} soil.

Analysis of the disease incidence showed that the rapeseed meal alone and in combination with BS211 significantly reduced disease incidence of

Phytophthora blight and root rot in pepper plants relative to the control treatment (Wang et al. 2014a). The disease incidence in the control treatment was 26.7 %, whereas there was no disease in the rapeseed meal and rapeseed meal + BS211 treatments after 15 days of soil treatment. After 20 days of soil treatment, 66.7 % of the pepper plants in the control treatment were diseased, whereas the disease incidence in the rapeseed meal treatment was 12.5 % and in the rapeseed + BS211 treatment was 6.3 %.

9.2.5 Soil Amendments of Composts and Manures

Anaerobic digestion of manures for biogas generation is a promising way to treat large amounts of animal manures. The slurry obtained after digestion should be safe to apply in the fields for crop production. Manures and manure slurries have been shown to promote plant growth and control plant-parasitic nematodes (Jothi et al. 2003). In China, the effects of locally available manures for suppression of *Phytophthora* root rot of chili pepper were evaluated in greenhouse pot assays. Their impacts on mycelium growth and spore germination of *P. capsici* were investigated in agar plate tests.

The results indicated that the anaerobically digested slurry (ADS) can inhibit the mycelium growth and zoospore germination of *P. capsici* and application of slurry to infested soil significantly reduced the incidence of *Phytophthora* blight and root rot in pepper plants (Cao et al. 2013, 2014). The effects on zoospore germination and disease incidence were greater with anaerobically digested pig slurry than anaerobically digested dairy slurry (Cao et al. 2014). While application of raw pig and dairy slurry had no significant suppressive effect on *Phytophthora* blight and root rot, pepper plants grown in soil treated with anaerobically digested pig and dairy slurries showed significantly higher shoot biomass than those grown in soil treated with corresponding raw slurry and negative control. ADS also significantly increased the numbers of soil bacteria, fungi, actinomycete, and antagonistic microorganisms including *Pseudomonas fluorescens* and *Trichoderma* spp. The PCR-DGGE analysis also showed an improvement of the bacterial and fungal diversities in the ADS treatments. Anaerobically digested pig slurry application could enhance the activities of peroxidase and catalase and hydrogen peroxide content while decreasing malondialdehyde content in chili pepper leaves (Cao et al. 2014).

Similarly, composts or compost extracts have been shown to suppress *Phytophthora* blight and root rot of pepper (Kim et al. 1997a; Sang et al. 2010). Composts and various soil amendments showed variable results in disease suppression at Florida (Kim et al. 1997a). In greenhouse pot assays, various composts and soil amendments were incorporated into soil prior to planting pepper seedlings and inoculations with *P. capsici* zoospores to assess their impact on *Phytophthora* blight and root rot. Chitosan, crab shell waste, and citrus pulp with molasses reduced incidence and severity of the disease on bell pepper, but there were no reductions in soil populations of *P. capsici*. There was also high microbial activity in the amended soils. It was speculated that the observed disease reductions may have been due to induced resistance in pepper and increased microbial activity. However, some of these same composts and soil amendments yielded variable results under field conditions (Kim et al. 1997b). Some amendments were not incorporated in the field soil. For instance, roots of pepper seedlings were dipped in chitosan (0.2 % w/v) solution and planted in the field.

Extracts of composts from six different commercial compost facilities in Korea were investigated whether they can be used to control root and foliar infections in pepper plants by *P. capsici* (Sang et al. 2010). In in vitro assays, four out of 47 compost extracts inhibited zoospore germination, germ tube elongation, mycelium growth, and population of *P. capsici*. The selected compost extracts also showed a reduction in incidence and severity of the disease in the seedling and plant assays.

In a greenhouse study from Spain, Nunez-Zofio et al. (2011) tested semi-composted

mixture of horse manure and chicken litter and a non-composted mixture of sheep manure and chicken litter followed by plastic mulching for reduction of *Phytophthora* blight and root rot of pepper (Nunez-Zofio et al. 2011). They found a reduction of 86 % and 65 % in disease incidence, and this reduction was linked to an increase in soil microbial activity. Organic soil amendments in conjunction with plastic mulching can be a good strategy for the control of *Phytophthora* crown and root rot of pepper particularly under temperate climate. The study also suggested that the reduction in disease incidence was partly due to a decrease in viability of pathogen oospores possibly by the production of ammonia. An increase in soil microbial activity and functional diversity may also have resulted in pathogen suppressiveness.

9.2.6 Soil Solarization

Soil solarization is another sustainable approach applied in several countries where possible with an aim to reduce the pathogen inoculum from soil and reduce *Phytophthora* blight and root rot of pepper. In some cases, soil solarization was also used in combination with soil organic amendments for better results. Soil is actually heated using a clear plastic as a mulch cover by trapping the sun's energy. Solarization may be very effective in killing pathogen inoculum at the top soil layer to a depth of 10 cm or less. The soil disinfestation is only possible during hottest summer months and in abundant sunshine. In the open fields, the use of soil solarization may be limited to small areas only. However, solarization is gaining popularity in greenhouses or plastic houses and high tunnel hoop houses for vegetable production. In Italy, soil solarization has been very effective to control *Phytophthora* blight in plastic houses (Polizzi et al. 1994).

In a 2-year field study from Turkey, soil solarization with clear plastic for 8 weeks was shown to reduce the incidence of *Phytophthora* blight and root rot of pepper comparable to the methyl bromide treatment (Yucel 1995). Soil temperature in the top 5-cm soil was recorded as 47 °C in their study. Similar soil solarization temperatures were also reported to a depth of 10 cm from a Florida study, and at that depth, the solarization treatment reduced *P. capsici* populations similar to the methyl bromide treatment (Coelho et al. 1999). Soil temperature dropped to 41 °C at 25-cm depth, and soil solarization was not effective in reducing pathogen populations at those depths (Coelho et al. 1999). In another study from Florida, soil solarization was examined in combination with soil amendment of urban plant debris (Chellemi 2006). *Phytophthora* blight was significantly less in solarized plots.

9.2.7 Anaerobic Soil Disinfestation

Anaerobic soil disinfestation is another bio-based and nonchemical alternative method of soil fumigation. This preplant treatment works by creating anaerobic conditions in the soil. The soil is incorporated with easily decomposable organic material such as fresh plant material, crop residue, wheat or rice bran, rice straw, and molasses and later irrigated or flooded. After treatment of soil with anaerobic soil disinfestation, it can be solarized with plastic mulch. This combination can provide very effective control of soilborne plant pathogens. Anaerobic soil disinfestation can be applied in cloudy periods or in areas where sunlight is low as it does not require high solar radiation. In that way, it has advantage over soil solarization. In a US field study, a combination of anaerobic soil disinfestation using molasses as the carbon source and solarization was suggested as an effective strategy to maintain pepper and eggplant yields in the absence of soil fumigants (Butler et al. 2014).

Soil incorporation of wheat straw followed by flooding for extended periods of time is a common practice for chili pepper production in China. A study was carried out to determine the impact of soil incorporation of wheat straw and flooding on soil properties, population of *P. capsici*, and the growth and yield of chili pepper in high tunnel fields (Gu et al. 2014). In

a laboratory experiment, the effects of different rates of wheat straw on soil physical properties and *P. capsici* population were studied under different flooding times. Wheat straw and flooding significantly decreased soil EC but increased organic matter, available phosphorus, available potassium, organic acids, and phenolic acid contents. The number of *P. capsici* was reduced significantly in both flooding only and flooding plus wheat straw in comparison with the moist soil. Application of 0.25 % wheat straw with flooding for 10 or 14 days strongly inhibited the growth of *P. capsici*. However, inhibition of *P. capsici* declined with increasing rates of wheat straw. Field tests showed that soil incorporation of wheat straw at 400 kg per 667 m^2 followed by flooding for 20 days not only increased contents of ammonium nitrogen, available phosphorus, and available potassium but also effectively reduced disease incidence of *Phytophthora* blight and root rot on chili pepper, promoting pepper growth and enhancing yield of chili pepper by 12 %.

9.2.8 Reduced-Risk Chemicals

Phosphonates are systemic and very stable in plants so they required less application. Although phosphonates are categorized as natural products, they are currently not registered for organic crop production. The disease management work on sweet or bell pepper was carried out in Canada with a new liquid formulation of phosphonate. In our previous work, phosphonate provided effective control of some soilborne diseases of vegetable crops when applied as an amendment prior to planting or as a drench after planting or as seed treatment (Abbasi and Lazarovits 2005, 2006a). It also suppressed clubroot on bok choy and cabbage when applied to the infested soil before or after planting (Abbasi and Lazarovits 2006b). Phosphorous acid can be taken up by plants as phosphonate ions and can provide control of oomycete diseases of agronomical and horticultural crops; however, it is not a substitute for phosphate fertilization (Förster et al. 1998).

The effectiveness of phosphonate to control *Phytophthora* blight and root rot on sweet or bell pepper was shown in a greenhouse potting mix or sandy loam soil artificially infested with soil inoculum of *P. capsici* in growth room pot assays (Abbasi et al. 2011). Phosphonate treatments (0.05 %, 0.1 %, and 0.2 % active ingredient (a.i.)) were applied as post-planting drenches after transplanting pepper seedlings in the infested potting mix or soil. The pathogen inoculum was incorporated into potting mix or soil, and the infested mix or soil was then placed into plug trays. Six-week-old pepper seedlings "Early Calwonder" were transplanted and immediately treated with phosphonate drench solutions. Trays were kept in a growth room, and plants were rated 6 weeks later for incidence and severity of *Phytophthora* blight and root rot using a modified 1–4 scale (Silvar et al. 2005). Disease incidence was expressed as a percentage of plants showing disease symptoms.

The bell pepper plants grown in the infested potting mix or soil that received a drench application of phosphonate showed less incidence and severity of *Phytophthora* blight root rot compared to the control plants receiving no treatment (Abbasi et al. 2011). In both peat-based mix and soil, disease protection increased with the increasing phosphonate concentration. The 0.2 % phosphonate treatment provided the best disease control with more than 90 % of the plants remaining healthy after 6 weeks of treatment compared to none in the control. In the untreated infested control, all the plants were diseased, and most were dead within 2 weeks after transplanting.

Another chemical Actigard is an activator of resistance in plants against diseases, and its potential for the management of *Phytophthora* blight and root rot disease on peppers was investigated as foliar sprays (Matheron and Porchas 2002). In greenhouse pot experiments, the pepper plants were either inoculated with *P. capsici* or grown in soil naturally infested with the pathogen and sprayed with Actigard. The plants sprayed with Actigard and grown in naturally infested field soil showed better survival compared with non-treated plants. These

greenhouse trials showed potential of Actigard as chemical management tool for *Phytophthora* blight and root rot on peppers, but the efficacy of this product needs to be confirmed under field conditions. Phytotoxicity to pepper plants may be another issue that needs to be addressed as well.

9.2.9 Grafting of Resistant Rootstocks

Grafted vegetable technology is one option which has the potential to increase resistance in the vegetables against plant diseases and, thereby, reducing pesticide usage. The use of grafted plants can dramatically increase the productivity of field-grown vegetables under conventional and organic management. This technology is currently popular in Asia but is gaining interest in Europe and North America. The high cost of grafting may be the main reason of slow adoption of this technology for broader applications. In recent years, the interest in the use of resistant rootstock particularly for grafting has significantly increased for crops such as tomato, pepper, and melon. In countries like Canada and Italy, a big number of grafted plants are used every year but mostly for greenhouse vegetable production.

Grafting is mainly used to increase host resistance against root rots and wilt diseases and to increase yield. In a study from Italy, pepper plants grafted onto partially resistant and resistant rootstocks were investigated for the management of *Phytophthora* blight and root rot disease under artificially and naturally infested field conditions (Gilardi et al. 2013). Grafting of pepper plants on resistant rootstocks provided the best control of *Phytophthora* blight and root rot under both conditions. The study also looked at improving the disease control effect of plants grafted on susceptible and partially resistant rootstocks in combination with compost soil treatment. Compost soil amendment did not show a significant effect under artificially infested conditions, but was a significant factor in reducing disease incidence and improving disease control under naturally infested field conditions. Therefore, there is always possibility to integrate compost or other soil amendments with grafting, particularly when cultivars susceptible to *Phytophthora* blight are grown or when pepper plants grafted onto partially resistant rootstocks were used.

9.3 Conclusions and Next Steps

Bio-based approaches including indigenous biofungicides as bioformulations; rapeseed meal, composts, and manures as soil biofumigation; grafting technology; and reduced-risk chemicals such as phosphonates and Actigard can be used as additional disease management strategies to control *Phytophthora* blight and root rot of pepper. Some fungal and bacterial antagonists isolated from pepper rhizosphere showed biocontrol potential for the management of *Phytophthora* blight and root rot disease of pepper. Soil amendments of rapeseed meal, composts, and manures also provided suppression of *Phytophthora* blight and root rot under greenhouse and field conditions possibly by altering the composition and structure of soil microbial communities and by direct toxicity to pathogen. Similarly, some combinations of soil amendments and biofungicides also showed less disease incidence on pepper plants in greenhouse pot assays and in the field. Soil solarization can be very effective in reducing pathogen inoculum from the top 10-cm layer of soil, and its effect can be enhanced with other strategies such as soil amendments. This strategy will be only effective in hot summer months. Anaerobic soil disinfestation using wheat straw as a carbon source followed by extended periods of flooding is another effective way of reducing pathogen inoculum from soil and also reducing disease incidence on plants. Anaerobic soil disinfestation can also be combined with solarization for enhanced effect. Grafted plants on resistant rootstocks also offer sustainable management solution if the associated costs could be lowered. Phosphonate post-planting treatments and Actigard foliar sprays also showed a reduction in the severity and incidence of *Phytophthora*

blight and root rot of pepper under growth room conditions. Adoption of any single method for managing diseases such as *Phytophthora* blight can be a challenge. Therefore, future considerations should be focused on developing a holistic approach including several management options built in the production system to reduce overall losses from soilborne diseases such as *Phytophthora* blight and root rot.

Acknowledgments Technical assistance in China was provided by Anyi Hu and Yueding Xu, and the research work reported here was completed by funding from the research programs in nonprofit industries (agricultural sector) and Ministry of Agriculture of China (201103004, 201203050). Technical assistance in Canada was provided by Brian Weselowski and several summer students (Antoine Hnain, Bethany Bradford, Halema Khan, Lindsay Knezevich, Nicole Korba, Meghan Braun, and Victoria Hewelett), and the funding for research was provided by Agriculture and Agri-Food Canada.

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Formulation of *Pochonia chlamydosporia* for Plant and Nematode Management

10

Aurelio Ciancio, Mariantonietta Colagiero, Isabella Pentimone, and Laura Cristina Rosso

Contents

Abstract

Soil microorganisms play a key role in plant nutrition and health, interacting with soil pests with beneficial, antagonistic effects. The nematode parasitic and root endophytic fungus *Pochonia chlamydosporia* has been extensively studied in the last years for exploitation, due to its multiple behaviours in soil and the rhizosphere. The fungus has a complex biology and can act as a biological control agent of phytonematodes, as a plant growth promoter or as a soil saprotroph. In this review we consider several aspects concerning its production and application as a nematode and plant management tool, including biodiversity and trophic specialisation. Formulations of *P. chlamydosporia* have already reached the industrial stage. Commercial products are available for biological control of root-knot or cyst nematodes or plant growth promotion in intensive to peri-urban cropping systems. Aspects related to the fungus biology, production substrates, industrial scale-up and conservation methods are examined. Finally, potential in nematode management is discussed.

A. Ciancio (✉) • M. Colagiero • I. Pentimone • L.C. Rosso
Istituto per la Protezione Sostenibile delle Piante (IPSP), Consiglio Nazionale delle Ricerche, Via G. Amendola 122/D, 70126 Bari and URT IPSP, 86035 Larino (CB), Italy
e-mail: aurelio.ciancio@ipsp.cnr.it

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_10

10.1 Introduction

Technical progress in soil management and plant protection relies on knowledge concerning many microorganisms, including the use and application of biological control agents (BCAs) as possible alternatives to pesticides. Biological pest management is a promising technology in crop protection since it can provide sustainable and safer alternatives to many chemicals. This approach also became necessary due to some circumstances, related to consumers' demand for organic food or to the recent withdrawal by the European Union (EU) and other agencies of many pesticides and nematicides, due to environment and human safety issues.

Although promising, an organic approach is often difficult to apply in plant protection. In fact, in spite of the number of BCAs or plant growth-promoting microorganisms (PGPMs) investigated in the last decades, and of the efforts underpinning effective application protocols, many problems/pests remain yet difficult to manage. Furthermore, basic background data on the biology, behaviour and biochemistry of useful soil BCAs are often lacking or limited to a restricted number of species.

Among nematode BCAs, *P. chlamydosporia* (Goddard) Zare and W. Gams (*Hypocreales*: *Clavicipitaceae*) has been investigated for more than three decades. It is a widespread soil fungus, acting as a facultative parasite of phytonematode eggs, often associated to many phytonematodes of economic importance. It has also been found as a parasite of animal parasitic nematodes, of other soil invertebrates like snail eggs or of oospores (Kerry 2000). *P. chlamydosporia* is a rhizosphere inhabitant and also an endophyte, capable to colonise epidermal and cortex root cells (Bordallo et al. 2002; Maciá-Vicente et al. 2009; Lopez-Llorca et al. 2010). Studies carried out in vitro or in field conditions showed that it also acts as a PGPM (Maciá-Vicente et al. 2009; Escudero and Lopez-Llorca 2012).

This species is present in two forms, *P. chlamydosporia* var. *chlamydosporia*, isolated from many regions worldwide, including Northern Europe and Mediterranean areas, and *P. chlamydosporia* var. *catenulata* (Kamyschko ex Barron and Onions) Zare and Gams, found in Latin America (Kerry 2000; Franco-Navarro et al. 2008; Manzanilla-López et al. 2013). Variability for parameters like egg parasitism or rhizosphere colonisation was experimentally shown among isolates, and a selection for parasitism capacity or other properties (i.e. soil or root colonisation or propagules produced) may be needed for practical exploitation and prior introduction in soil (Bourne et al. 1996; Kerry 2000; Franco-Navarro et al. 2008; Siddiqui et al. 2009; Vieira Dos Santos et al. 2013). The fungus has been tested as a BCA of many nematode pests, including the cereal cyst nematode (CCN) *Heterodera avenae* (Kerry et al. 1984; Kerry 2000), root-knot nematodes (RKNs) *Meloidogyne* spp. (de Leij and Kerry 1991; Verdejo-Lucas et al. 2003; Bontempo et al. 2014) and the false root-knot nematode *Nacobbus aberrans* (Flores-Camacho et al. 2007; Franco-Navarro et al. 2008).

The efficacy of *P. chlamydosporia* as a BCA of *H. avenae* was indirectly demonstrated by applying formalin (at a concentration lethal only to fungi) to soil parcels in which the fungus was present. Subsequent measures of nematode densities during the following months showed the eventual increase of the *H. avenae* population, as resulting from the elimination of the *P. chlamydosporia* antagonistic action (Kerry 2000). Applications with this fungus are at an advanced phase and in some cases already allowed an industrial production. First formulations have been made commercially available in EU (Italy, Spain), Latin America (Cuba, Brazil), Australia or Asia (India, China) (Kerry and Hidalgo-Díaz 2004; Mo et al. 2005).

10.2 Biology of *P. chlamydosporia*

10.2.1 Isolation and Detection

P. chlamydosporia was originally described as a member of the genus *Verticillium*, due to the presence of verticillated phialidic conidiophores each bearing at their apex one cluster of subglobose, ovoid to ellipsoidal conidia

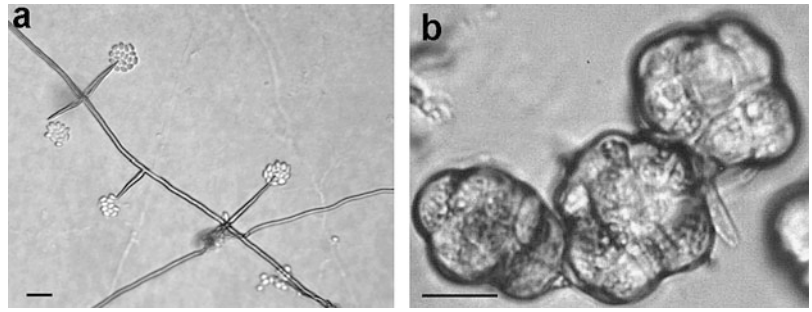

Fig. 10.1 Hyphae of *P. chlamydosporia* var. *chlamydosporia* with conidiophores bearing apical conidial clusters, visible as adhering to the agar surface. (**a**) Chlamydospores. (**b**) *Scale bars* = 10 μm

measuring 2.5–5.5 × 1.5–3.0 μm (Fig. 10.1a). A specific character of the fungus, helpful for its identification and detection on water agar (WA, 8–10 g agar L^{-1} distilled water) or other culture media, is the production of dictyochlamydospores, durable resting propagules also known as chlamydospores (Fig. 10.1b), which confer a yellowish-creamy colour to the mycelium, after prolonged culture.

The fungus taxonomy and phylogenetic position were clarified through studies based on ribosomal RNA sequences, which allowed the erection of the new genus *Pochonia* in which the species was finally placed (Zare et al. 2001). These studies showed that the new genus was phylogenetically distinct from the true *Verticillium*, which includes only plant-pathogenic lineages. Recent studies based on whole-genome sequence and transcriptome data also revealed the close proximity of *Pochonia* to the clavicipitaceous genera *Metarhizium*, which includes endophytic and insect parasitic species, and *Epichloe*, an endophyte of winter grasses (Larriba et al. 2012, 2014).

P. chlamydosporia can be easily isolated from hyphae emerging from nematode eggs or fragmented cysts or egg masses, as well as from root debris and soil particles, once these have been spread on the surface of a nutrient poor medium, i.e. WA, allowing a slow growth of fungi to have a clear view of the developing hyphae. A semi-selective medium was also developed to facilitate the isolation of *P. chlamydosporia* from soil or roots or for counting the number of colony-forming units (CFUs), a procedure needed when studying the fungus density and multiplication rate (de Leij and Kerry 1991).

For production of monoconidial isolates, the clusters of conidia have to be located using a stereoscope. They are visible at the apex of the conidiophores (Fig. 10.1a) formed along the hyphae that emerge from the inoculum, i.e. egg masses, soil particles or root debris. The hyphae often protrude on the WA surface, forming aerial networks or isolated hyphal bridges. After inspecting the plate and once locating the fungus, a conidial cluster can be gently intercepted with a needle holding at its tip a glued eyelash. This simple tool may be sterilised by a quick immersion in a few ml of 95 % ethanol and air dried for a few seconds before use. Alternatively, a tungsten wire (obtained from a broken bulb) can be fused on the glass stirred tip of a Pasteur pipette and then sterilised by heating on a flame before use. It is necessary to rapidly cool the wire by a quick immersion in a few ml of 95 % ethanol or sterile distilled water (SDW) and let it become dry and cool for about 10 s, before aseptically touching the conidial cluster. The intercepted conidia adhering to the sterile eyelash or wire tip can be rapidly smeared and dispersed on a suitable culture medium (usually potato dextrose agar (PDA) or cornmeal agar (CMA)) on which they will germinate, originating new colonies in the following 3–4 days once stored at 25–26 °C. At this moment the small colonies can be observed on the Petri dish using a stereoscope. To produce a monoconidial isolate, a marginal fragment from each of the smallest, isolated colonies appearing on the medium can be removed with a sterile needle tip, to be transferred on a new plate with a suitable growth medium. The colonies can be identified at this moment as a new entry in the collection by

assigning them a unique reference code, adding related data and other information to the collection database (collector's name, date, original sample location, substrate type, etc.).

10.2.2 Metabolism

Several factors like soil nutrients and root exudates affect *P. chlamydosporia* growth and behaviour. Like other parasitic fungi, the excess of compounds rich in C may affect production of key enzymes involved in nematode infection, whereas a high organic matter content of soil may stimulate growth, without any increase in biological control efficacy (Kerry and Bourne 1996). Carbon (C) sources readily available for metabolism and unfavourable pH in the rhizosphere or nematode egg mass may compromise nematode parasitism (Ward et al. 2012).

Studies on parasitic fungi showed that the chemical composition of the host surface is a key inducer for production of specific hydrolytic enzymes during infection by nematode-trapping fungi (Tunlid and Jansson 1991). In the *P. chlamydosporia*–nematode interaction, the molecular response of the fungus involves many genes, including an alkaline serine protease (VCP1), an enzyme specifically degrading proteins from the outer vitelline layers of the egg. This single-copy gene is expressed early during infection (Segers et al. 1996; Morton et al. 2003) and shows single nucleotidic polymorphisms associated to different host nematode species that reflect a long-term, genetic adaptive process that occurred on evolutionary scales (Morton et al. 2003). VCP1 induction is one of the first steps in a complex cascade of regulated genes leading to egg parasitism (Lopez-Llorca et al. 2010; Ward et al. 2012).

Given the sensitivity of the fungus to external biochemical signals affecting its behaviour, it is important to know the nature and effect of these stimuli to apply the mycelium or propagules in conditions that are most suitable for nematode biological control. Two isolates of *P. chlamydosporia* with different host origins, IMI 380407 from RKN and IMI 331547 from the potato cyst nematode (PCN) *Globodera pallida*, were assayed in their saprotrophic-to-parasitic transition, with varying nutritional conditions and sampling times. Treatments included nutrient-rich or nutrient-poor media or a poor medium with PCN or RKN eggs. Expression studies showed that the transcript-derived fragments (TDFs) obtained were affected by different nutritional conditions (starvation in the presence/absence of eggs or nutrient-rich medium). The changes in gene regulation reflected specific systems or functional links, with differences in TDF levels between pathogenic and saprophytic growth. The genes expressed during parasitism for both isolates were involved in cellular signal regulation and transport, regulation of other genes, DNA repair and other functions. Multivariate analysis showed co-expression of genes associated to egg parasitism, together with constitutive genes, related to general metabolism. The differential expression of parasitism-related genes suggested a network of induced/repressed products, playing a role in fungal signalling and infection (Rosso et al. 2011).

Clusters identified by a multivariate analysis in *P. chlamydosporia* IMI 331547 included a phytase expressed in starvation and early after contact with RKN nematode eggs, not linked to other metabolic pathways, and probably accounting for a distinct activity. Parasitism-related TDFs also included a phospholipase D1, expressed in rich nutritional conditions and early after contact with eggs, and a monooxygenase expressed after contact with eggs. Starvation and presence of nematode eggs also induced a bZIP transcription factor not related to parasitism (Rosso et al. 2011).

Assays on healthy or RKN-parasitised plants also showed the induction, 4 and 8 h after incubation of *P. chlamydosporia* with eggs, of the bZIP transcription factor and the phytase-related gene. Mycelial growth stimulation was observed in the presence of 5–10 ppm of phytic acid, suggesting that the fungus can use this molecule as a P source. Nematode-free tomato plants alone induced, at 4 h incubation, a phytase

transcription sixfold higher than control. Changes in transcription were not significant when the fungus was in the presence of nematode-parasitised plants. The expression of a phospholipase D was, after 8 h incubation in the presence of nematode eggs, 900-fold higher than in fungus alone. Further assays performed at different pHs or in the presence of glucose and NH_4^+ showed that the bZIP and phytase transcripts reflect early changes in the fungus metabolism (Rosso et al. 2014).

Parasitism-induced bZIP transcription factors are dimers that regulate genes active in early infection events by recognising DNA palindrome sequences (i.e. CREB, ATF2 or CCAAT/enhancer-binding protein (C/EBP)) (Thiel et al. 2005). These proteins transactivate genes containing cAMP response element (CRE) motifs in their regulatory regions, connecting cellular stimulation with transcription of CRE-containing genes (Bakker and Parker 1991). Studies on *Metarhizium anisopliae* showed that cAMP is involved in the formation of the appressorium, together with Ca^{+2} (Clarkson and Charnley 1997). St Leger et al. (1991) proposed that hyphal differentiation begins after a change in the membrane potential is induced by a contact, possibly through a mechanosensitive ion channel resulting in a disruption of the apical Ca^{+2} gradient required for polar growth. Elevated levels of cAMP coincided with the formation of the appressorium and of a protein kinase identified in the plasma membrane of *Metarhizium anisopliae*, whose substrate could be a CREB factor promoting genes involved in the appressorium formation. In *M. anisopliae* this is a PR1 serine protease, whose level increased tenfold within 24 h of contact with the insect cuticle (Clarkson and Charnley 1997). The bZIP transcript might hence be involved in a similar mechanism in *P. chlamydosporia*, including the activation of specific egg-degrading enzymes (Ward et al. 2012). In line with this possibility, the bZIP expression is reduced in the presence of D-(+)-glucose and enhanced by NH_4^+ (Rosso et al. 2014).

Nematode parasitism and P mobilisation open intriguing scenarios for practical exploitation of *P. chlamydosporia*, suggesting that multiple potential benefits can be derived by this species. Recently, *P. chlamydosporia* genes expressed when endophytic on barley showed production of several proteases, hydrolases and other secreted proteins involved in transport during root colonisation (Larriba et al. 2014). Observations suggest a long-term, stable evolutive adaptation of *P. chlamydosporia* to the soil and rhizosphere environments, characterised by specific interactions with the host plant and complex relationship involving contacts with roots and/or nematode eggs.

The phylogenetic proximity of *P. chlamydosporia* to *Metarhizium* spp. also suggests possible ecological and metabolic similarities. Studies on *M. robertsii* revealed that these entomopathogens have a complex endophytic relationship with their host plant, involving the return to the colonised roots of the N metabolites acquired by the insects when feeding on them. This task is achieved by means of a translocation carried out through the *Metarhizium* endophytic phase, once the fungus parasitises the insects' larvae present in the rhizosphere (Behie et al. 2012). We hypothesise that a similar behaviour might be present in *P. chlamydosporia* when in the presence of (or in contact with) phytoparasitic nematodes, with a direct role in plant nutrition, a part of its direct biological control capacities. If further data will support this hypothesis, then parasitism must be considered as part of a more complex lifestyle, unifying the tri-trophic root, nematode and fungus interactions in a nutrient-based, cyclic relationship sustaining the plant growth.

Considering the feasibility of massal production of *P. chlamydosporia* and that chlamydospores and other propagules may facilitate conservation and dispersal in soil, the fungus holds potential both as a BCA and PGPM. However, for optimal production in fermenters or other solid substrates, data on metabolic preferences of isolates must be produced, since differences may occur for some basic parameters, i.e. growth rates (Fig. 10.2a).

The capability of isolates to metabolise sugars and C sources commonly used in substrates may

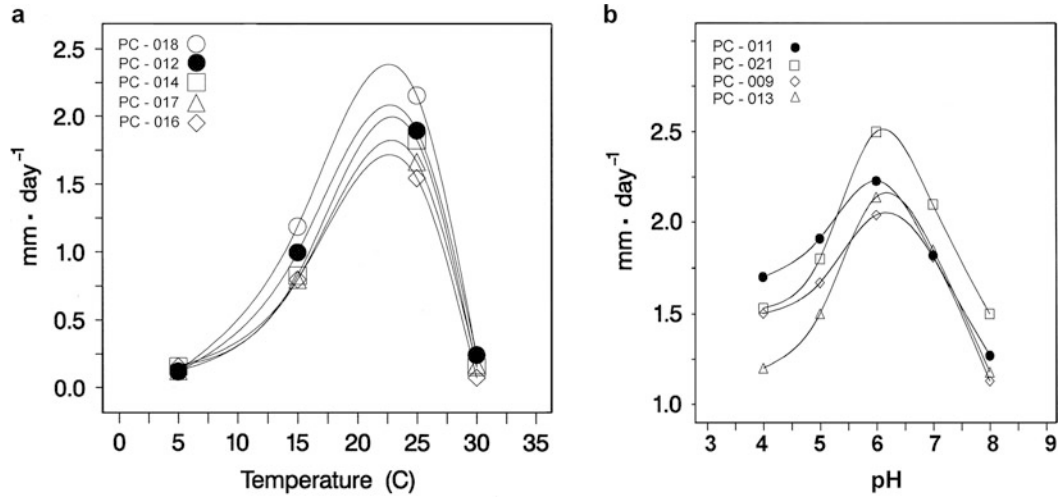

Fig. 10.2 Effect of (**a**) temperature and (**b**) pH on radial growth on cornmeal agar of some isolates of *P. chlamydosporia* var. *chlamydosporia* collected in Italy

yield variations that can affect production. In *P. chlamydosporia* fermentation assays with isolate collected in China and carried out with different C sources, highest conidial production was observed at a C/N ratio of 10:1 (pH 3.7), whereas maximum biomass production was obtained with a C/N ratio of 40:1 (pH 6.8). When testing the fungus growth, sporulation of *P. chlamydosporia* HSY-12-14 was highest on media with 6 g l^{-1} C (C/N = 40:1), or 8 g·l^{-1} C (C/N = 20:1 or 40:1) for conventional production on a single medium, and with 8 g·l^{-1} C (C/N = 10:1) when using two different sequential media (Gao and Liu 2010). Initial pH also affects mycelial growth and conidial production, and optimal values reported were 3.5–4.5 for sporulation and 5–6 for growth. Maximum conidial production was obtained with initial pH 4.0 whereas the maximum biomass was achieved at pH 6.0 (Mo et al. 2005). The latter was also the preferential pH for optimal radial growth observed for some isolates collected in Italy (Fig. 10.2b).

C and N sources affect *P. chlamydosporia* metabolism, with consequences on its growth rate and conidiation. Among the 18 different N sources tested on an Arkansas isolate, casein showed best growth rates on Blackburn and Hayes (1966) solid medium, whereas L-aspartic acid and DL-glutamic acid showed highest conidiation. In liquid cultures, peptone showed highest growth, whereas ammonium nitrate reduced growth and conidiation in solid medium, with minimal growth of *P. chlamydosporia* also in liquid medium (Liu and Chen 2003). Among C sources, D-(−)-fructose, D-(+)-galactose, melibiose and D-(−)-ribose showed highest growth rates and conidiation of *P. chlamydosporia*, followed by D-(+)-glucose, D-(+)-xylose, D-mannitol, sucrose, D-(+)-trehalose and maltose. Among nine vitamins tested, folic acid showed highest growth rate and myo-inositol the optimal conidiation (Liu and Chen 2003).

Like many other groups of invertebrate parasitic fungi, *Pochonia* spp. also are characterised by the production of biologically active secondary metabolites. These include pochonins, a group of antiviral and antiparasitic compounds also known as monordens or resorcylic acid lactones (also referred to as radicicol). They were exclusively produced in submerged cultures by *P. chlamydosporia* and some other species of the genus (Stadler et al. 2003). Isolate P0297 of *P. chlamydosporia* var. *catenulata* produced monorden in methyl-alpha-D-glucopyranoside (MGP) medium, with yields of 15–20 mg·L^{-1} (Hellwig et al. 2003). Further secondary metabolites of members of the genus

include a phomalactone with nematicidal properties (Khambay et al. 2000), aurovertin B and citreoviridin A produced by *Pochonia suchlasporia* var. *catenata* (Gams and Dackmann) Zare and Gams (Stadler et al. 2003) and four aurovertins produced by *P. chlamydosporia* (Niu et al. 2010). Isolate TAMA 87 of *P. suchlasporia* var. *suchlasporia* also produces pochonicine, a pyrrolizidine alkaloid acting as a potent inhibitor of beta-N-acetylglucosaminidase, a chitinolytic enzyme involved in degradation of oligomers produced by chitinase, with potential in defence from insects or other fungi (Usuki et al. 2009).

10.3 Biological Control and Management

10.3.1 Soil Biodiversity and Crop Production

Thanks to their richness in microbial species, soil habitats sustain a web of complex trophic networks (Sleator et al. 2008). However, the effects of this complexity are often neglected, in spite of the many factors and benefits present in the rhizosphere. The positive interaction between soil microbial species and plants is indeed a fundamental component of soil fertility underpinning crop productivity. In spite of the advancements achieved in the last century through the use of chemical fertilizers and pesticides, food production is still dependent on the soil microbiota. Furthermore, several elements that play a fundamental role in sustaining plant nutrition will face, in this century, a worrying shortage, and first of all will be the phosphate sources. It is expected that solutions to emerging problems in plant nutrition and soil pest management will possibly arise by the broad range of soil microbial species and/or by enhancing the activity of beneficial microorganisms selected and applied for this purpose (Altieri 1999; Avis et al. 2008; Adesemoye et al. 2009; Pareg and McMillan 2015). This assumption further underlines the actual need for producing detailed information, either on the density regulation mechanisms active in the rhizosphere or on the biology, ecology and biochemistry of the species involved in these interactions.

DNA-based studies on soil microbial diversity showed that it is extremely rich in species and that their numbers can even appear underestimated, due to many taxa not yet described or difficult to culture (Fierer at al. 2012; Hirsch and Mauchline 2012). Recent advances in soil metagenomic studies showed a range of operational taxonomic units (OTUs) varying in the order of 10^3 to $10^4 \cdot g^{-1}$ of soil (Roesch et al. 2007; Lauber et al. 2009; Frisli et al. 2013). A lower level of phylogenetic diversity, however, is found in soil when compared to other environments, i.e. marine or sediment habitats (Lozupone and Knight 2008). In any case, due to its biodiversity, soil is a rich container for a wide range of microbial by-products and metabolites, including enzymes, antibiotics or siderophores (Martínez-Viveros et al. 2010; Kumar et al. 2012). All these components play a role in the rhizosphere and affect the success of practices aiming at the introduction and establishment of fungi like *P. chlamydosporia* or other nematophagous species. However, soils often show a buffering capacity as concerns their microbial constituents. A mycostasis effect has been often invoked to explain failures in establishing a nematophagous species or the reduced efficacy of selected isolates, in comparison to the promising results found in greenhouse or semi-controlled assays (Lopez-Llorca et al. 2008).

There are several convergent observations on plant growth promotion effects exerted by *P. chlamydosporia*. This fungus can survive in soil in absence of nematodes as a saprotroph for several months. It is also an efficient coloniser of the root surface (Fig. 10.3) and a true root endophyte (Lopez-Llorca et al. 2002; Maciá-Vicente et al. 2009; Manzanilla-Lopez et al. 2011, 2013). When endophytic, either direct or indirect effects may underpin growth stimulation, including the early induction of a wide range of plant defence and resistance genes (Rosso et al. 2013).

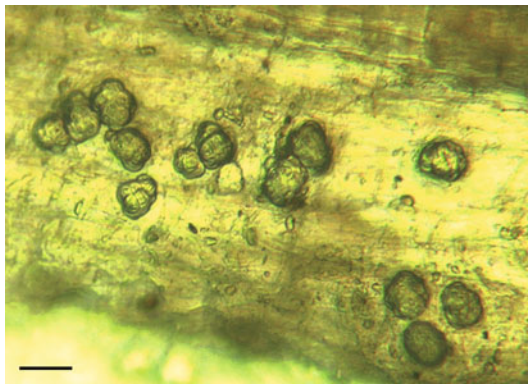

Fig. 10.3 Chlamydospores of *P. chlamydosporia* var. *chlamydosporia* colonising a tomato root. *Scale bar* = 25 μm

10.3.2 Plant Protection and Nematode Management

For decades, since the late 1940s, phytonematodes have been managed by means of chemical treatments based on a variety of nematicides like chlororganics, carbamates, fosforganics, halogenated hydrocarbons, methyl bromide or chloropicrin. These tools, although effective in controlling nematode pests in the short term, often showed a series of negative feedbacks, the most common of which involved the density changes, if not the extinction, of one or more microbial soil components, responsible for root nutrition and/or natural pest regulation. The effects of 2,4-D-(1,3-dichloropropene) in decreasing the numbers of nitrifying bacteria has been documented at this regard (Elliot et al. 1977), as also the mass extinction of any living organism in soil, including beneficial species, due to methyl bromide.

Microbial predators and parasites have been applied since the early 1970s in attempts aiming at safer and effective methods relying on biological control of nematodes. However, soil environment is highly variable and complex, and the reproducibility of results, as usual in agriculture, has been difficult to achieve. This effect is mainly due to the occurrence of several, distinct factors affecting the success (or failure) of the strategies applied. Apart from mycostasis, an important variable involves the microorganism and isolate applied. For *P. chlamydosporia*, a scrutiny of published data shown in the literature for nematode management shows different levels of biological control efficacy, related to the isolate used, the target host species or the application method and dosage (Table 10.1).

P. chlamydosporia is often associated to parasitise nematode eggs, from which it can be easily isolated. The fungus also colonises the egg masses of RKNs as well as the cysts and egg sacs of PCN, CCN and other cyst nematodes. The egg parasitic activity starts through hyphal penetration and eventual digestion of the eggshell, occurring early after contact of the hyphae with the egg surface. The contact eventually originates fungal appressoria allowing the penetration of the vitelline layers and the formation of an internal swollen bulb, from which new invasive hyphae are formed. The egg invasion step is biochemically very active and characterised by the induction and production of specific enzymes, i.e. chitinases and VCP1, specifically involved in the process (Lopez-Llorca et al. 2008). The invasion of the embryo then follows that is rapidly lysed and destroyed by the developing mycelium (Fig. 10.4).

P. chlamydosporia has also been applied to control animal parasitic nematodes (Ferreira et al. 2011; Silva et al. 2011) or other plant-pathogenic fungi. Together with *Pochonia rubescens* and *Lecanicillium lecanii*, isolate 4624 was capable to reduce root colonisation and damage caused by the fungus *Gaeumannomyces graminis* var. *tritici*, a severe pathogen of wheat, barley and other crops and causal agent of take-all disease (Monfort et al. 2005).

Apart from nematode eggs, there are also reports of parasitic activity on adult females, in particular for CCN and other cyst nematodes whose early infection was found to be correlated to higher levels of biological control (de Leij and Kerry 1991; Kerry 2000). Occasionally, parasitism of juveniles may be observed, in relation to the invasion of eggs that are close to hatching.

Density-dependent mechanisms may explain nematode regulation by the soil microflora or by specific antagonists and provide a basic perspective when evaluating BCAs like

Table 10.1 Nematode regulation effects observed for various treatments with different isolates of *Pochonia chlamydosporia* and target nematodes

Treatments	Target nematode	Efficacy[a]	References
P. chlamydosporia	*Heterodera avenae*	24–98 (E)	Kerry et al. (1984)
P. chlamydosporia CMI 334168	*Meloidogyne incognita*	68–72 (J + E)	de Leij et al. (1992a)
		81 (F)	
P. chlamydosporia IMI 331547	*Meloidogyne incognita*	27–61 (E)	de Leij et al. (1992b)
		13–45 (J)	
P. chlamydosporia	*Meloidogyne incognita*	37–44 (E)	Kerry and Bourne (1996)
		81 (F)	
P. chlamydosporia with *Hirsutella rhossiliensis*	*Meloidogyne hapla*	74 (G)	Chen et al. (1999)
P. chlamydosporia CMI 334168	*Meloidogyne hapla*	29–57 (J2)	Viaene and Abawi 2000
		52–71 (E)	
P. chlamydosporia var. *catenulata* Res 392	*Meloidogyne incognita*	17 (J2)	Atkins et al. (2003)
P. chlamydosporia IMI 331547 with oxamyl	*Meloidogyne javanica*	20–25 (G)	Verdejo-Lucas et al. (2003)
		10–16 (E)	
P. chlamydosporia with Surinam grass	*Meloidogyne javanica*	28 (G)	Dallemole-Giaretta et al. (2011)
P. chlamydosporia	*Globodera rostochiensis*	65 (E)	Muthulakshmi et al. (2012)
		53 (F)	

Target stages: *E* eggs, *F* females, *J2* juveniles, *G* gall index
[a]Impact of treatments expressed as percentage of corresponding controls.

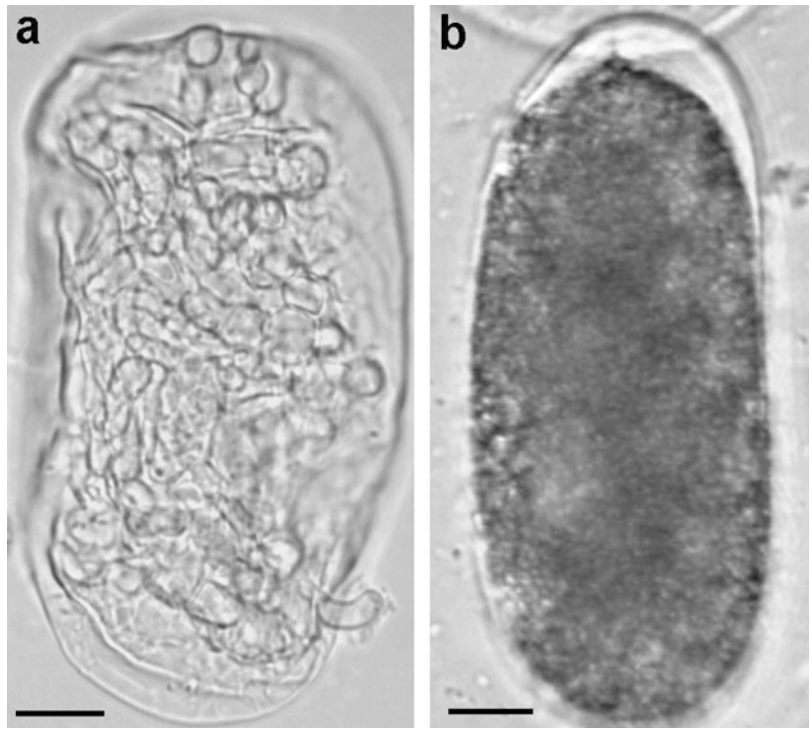

Fig. 10.4 Hyphae of *P. chlamydosporia* var. *chlamydosporia* within a (**a**) RKN-parasitised egg and (**b**) healthy egg with developing embryo. *Scale bars* = 10 µm

P. chlamydosporia (Kerry 2000). There are several reports confirming the beneficial effects of treatments with *P. chlamydosporia* applied in field conditions. Replicated data reinforce the observations carried out in controlled trials and the beneficial effects of the fungus observed in Northern Europe, in CCN-infested cereal fields and in Mediterranean crops mainly for RKNs (Kerry 2000). However, no antagonist may produce a mass extinction of its host in a short term, unless its density is artificially increased through inundative treatments, the host and parasite coexistence being the most common rule. Also, depending on the soil structure and texture, varying saturation thresholds may be imposed to the fungus by self-density dependence and other physical factors that may limit the total number of propagules and active mycelial parts that can be present and active in the rhizosphere. After this threshold is achieved, it is non-useful to increase the density of propagules, since the biomass introduced will be lost, becoming food for other soil organisms or undergoing other processes determining a density decline. The extent of the microbial competition in the rhizosphere is a factor that must be evaluated, as well as the marginal yield increase or benefits achievable by the application, that will start to decline after a given density threshold has been reached.

In general, users must be aware that the nematode population changes need a given time period before any effect of *P. chlamydosporia* can be scored. In any case, it is almost impossible to eradicate a nematode after its spread in a cultivated field, in particular for RKNs that are polyphagous and tend to remain in infestation foci all around the cultivated parcels, on weeds or volunteer plants. Differing from chemicals, BCAs are living forms subject to their own limiting factors and as sensitive to soil conditions as many other soil microorganisms. Their use should then aim at keeping the residual nematode population far below its damaging threshold density, for a time period covering the different plant phenologic stages.

Combined use of *P. chlamydosporia* with chemicals and other microorganisms has also been tested. When applied on potato at 10 kg·ha^{-1} for PCN management with *Pseudomonas fluorescens* and *Trichoderma viride*, with or without carbofuran, an Indian isolate of the fungus showed a 70 % tuber weight increase and a cyst population decrease by around 72 % (Muthulakshmi et al. 2012). In a 2-year study with alternated tomato and lettuce crops infested by *Meloidogyne javanica*, *P. chlamydosporia* IMI 331547 was used alone or in combination with oxamyl. The fungus, applied at a rate of 1.3–2.4·10^6 chlamydospores per plant, was re-isolated from parasitised nematode eggs up to 9 months after application to crops. Treatments with oxamyl showed the highest reduction of galls and egg densities when compared to the fungus alone or untreated controls (Table 10.1). Results showed, however, low rates of egg parasitism and variability between the two experimental sites investigated (Verdejo-Lucas et al. 2003).

Growth promotion effects have been reported either in pot studies or field conditions, with or without nematodes. In a pot study, increased shoot and root weights were observed in barley (Maciá-Vicente et al. 2009). In field conditions in Southern Italy, an increase of around 30 % of yield, achieved through a higher fruit size, was found for zucchini plants treated in a greenhouse with a liquid commercial product based on isolate DSM 26985, given at transplant and at 15- or 30-day intervals (Pietrantonio et al. 2013).

10.4 Industrial Production, Quality Control and Storage

Essential components for biological control programmes are the quality and availability of adequate amounts of inoculum. These may be obtained through primary mass production systems that should be compatible with low industrial production costs for subsequent commercial development and field application. The production of experimental formulations may be carried out starting from inoculum mainly produced in flasks for work in field assays (Fig. 10.5). A larger-scale production system has to be used instead to start an industrial

Fig. 10.5 Small-scale production of mycelium and propagules of *P. chlamydosporia* var. *chlamydosporia* on a sand–maize mix in flasks, for subsequent use in an experimental biological control assay of RKN

production cycle. In the latter case, more intensive and economically viable methods must be followed to achieve market sales. The production processes for anamorphic fungi commonly found to be practical on a pilot scale is the diphasic liquid–solid fermentation technique, in which the fungus is first grown in liquid media and then added to a solid substrate for conidiation (Lomer et al. 1997).

10.4.1 Liquid Culture

In mass production cycles, a liquid starting culture is useful to achieve a rapid mycelial growth that will produce the inoculum for the subsequent solid-phase cycle. The advantages of this procedure are (a) a fast-growing and therefore less expensive production method; (b) enhanced competitiveness due to the possibility of controlling the fungus, reducing the risks that contaminating microorganisms reach the solid substrate; (c) an easier quality check of the liquid culture when testing for contaminations originating from the slope culture; (d) a more rapid colonisation of the solid substrate when the actively growing liquid culture is distributed on it; and (e) an even coverage of the substrate, resulting in homogeneous growth and maximum conidiation.

The industrial production of mycelium often starts in liquid culture in large fermentation vessels which have electronic controls and monitoring. However, aerial conidia are not produced in liquid but require an air-substrate interface on which the propagules can be formed. For *P. chlamydosporia* the chlamydospores are difficult to produce in liquid, but can be obtained from a solid-phase substrate inoculated with conidia or chlamydospores (Montes de Oca 2004). However, a patent (US-20080213867-A1) claimed a method for liquid fermentation in a medium including triglycerides and other fats and C sources that allows production of more than 10^6 chlamydospores ml^{-1}.

It is important to check if, in the early growth phases, distinct clumps of mycelium are formed within an otherwise clear broth. This form of growth is less productive than a homogenous broth colonisation obtained by evenly dispersed mycelial and hyphal fragments. To encourage even distribution of mycelium and hyphal fragments in the liquid broth, the temperature should be tightly controlled and a spore suspension should be used to inoculate the flasks/containers in preference to agar blocks. Fermentation systems could be set up when a large quantity of liquid inoculum is required and a filtered aeration unit (air pump or compressor) is important for optimal production. A simple system would consist of a single unit capable of running multiplex fermenter vessels at any one time (Taborsky 1992).

10.4.2 Solid Substrates

For mass production a solid phase may provide a physical support for the fungus to produce aerial conidia or chlamydospores (the propagules which are best suited for storage and formulation in oil). Usually, the substrate is a cereal or a mix

with a cereal by-product such as rice, millet and maize or wheat bran. Since these are natural products, their nutrient status and microbial components are undefined. The fungus will use a certain proportion of the nutrients supplied by these products during growth and conidiation. In some ways, the structure of the substrates is more important than the nutrients they supply. An ideal substrate will provide a high surface-to-volume ratio, with individual particles kept separated to provide interparticle spaces for aeration and formation of conidia and other propagules. For this reason, broken sterilised cereals are often the preferred substrate, providing a large conidiation surface. If prepared carefully, the grain fragments remain separated from each other after autoclaving and inoculation (Hadapad and Zebitz 2006).

For chlamydospore mass productions, several media can be used, based on barley, rice, wheat or corn. A production medium for *P. chlamydosporia* can be obtained by mixing milled barley with fine sand in 1:1 proportion, followed by autoclaving and drying to the appropriate moisture level (Kerry and Bourne 2002). After inoculation, the fermenter bags/vessels are sealed and turned several times to distribute the inoculum evenly and break up any lumps. Fermenter bags/vessels are inspected for contamination and turned for at least two times during the incubation period to increase aeration and break up any clump eventually formed during the incubation process. The effect on final yields of turning/mixing the substrate during incubation should be evaluated. Good records should be maintained regarding the time at which bags are manipulated during the production process (Jenkins et al. 1998).

Whatever formulation is required, the product must first be dried to ensure that the propagules remain viable during storage. This process can be speeded up by a drier or an air-conditioning system in the incubation room or by accelerating ventilation with a fan. This will depend on the local environmental conditions, being careful to operate the dryer in a cool room to prevent overheating of the conidia during the process. Conidiation typically takes place after approximately 10 days, and for *P. chlamydosporia* on solid substrate, the medium must be kept for 1 month before chlamydospore extraction. Once the conidiation process is complete and spores have been formed over the whole surface of the solid substrate, the next step in the process will depend largely on the intended use of the end product. At this point it is important to ensure that the method of extraction is compatible with the formulation. If, for example, the conidia are to be formulated in oil for ultralow-volume (spraying) application, a very fine powder with uniform particle size is required. Therefore, the extraction process must be either efficient or selective for conidia that have to be separated from the cereal dust. In case the intended use is for direct application to soil, the conidia may be left on the substrate and the whole product of the fermentation may be used for direct application.

For chlamydospore extraction, the colonised medium can be homogenised and washed in the feeding tank of an appropriate separation apparatus, and the spores can be then stored at 4 °C. For conidia-producing fungi, when a minimal moisture content (5 %) is required, the conidia powder can be extracted by filtering or other kinds of devices and packaged in moisture-proof packaging such as aluminium laminate sachets. In this way, conidia have a long shelf life and do not require storage below 0 °C. For *P. chlamydosporia* a formulation of pellets can be produced using a mycelium suspension mixed with Na alginate and diatomite clay at a 10 % (w/v) final concentration (Duan et al. 2008; Walker and Connick 1983).

10.4.3 Culture Maintenance

Effective maintenance of stock cultures is essential for quality control, validation of the production methodology applied and research purposes. Maintaining backup sources of inoculum as a master culture is essential for many reasons, since the isolate original culture may be required for (a) research purposes, (b) preparation of inoculated samples and specimens for quality

control and training purposes, (c) conservation of reference strains for development and validation of any new methods or (d) production of cultures for microbiological assays. There are a large number of long-term culture maintenance techniques that can be employed for the preservation of valuable fungal material. Freeze-drying lyophilisation is the most satisfactory method since it is a form of suspended metabolism. A simple and reliable method (used for entomopathogenic fungi) consists in drying a large number of infected insect cadavers prior to re-isolation of the fungus of interest. The sporulated cadavers should be dried over silica gel to a constant weight and stored in a freezer in a sealed plastic bag, always with silica gel. This material is likely to remain viable for many years and single insects can be removed when a fresh source of inoculum is required (Grivell and Jackson 1969). However, the equivalent for *P. chlamydosporia* would be the storage of RKN egg masses or cysts colonised by the fungus, a method that may appear unsatisfactory given the small dimensions of nematodes and, considering their habitat, the possibility of external contaminations by other soil microorganisms.

Sterile soil has found wide use for stock culture maintenance of spore-forming microorganisms and appears particularly suitable for preservation of fungi. For storage of *P. chlamydosporia* at room temperature, sterilised soil can be used (Green 2008). The fungus can also be stored by pouring a spore suspension in sterile distilled water (SDW) on silica gel particles, later dried and stored at 80 °C.

A simpler method based on SDW, originally described by A. Castellani in 1939 and applied to many fungal groups (Burdsall and Dorworth 1994; Nakasone et al. 2004; Diogo et al. 2005), is also suitable for *P. chlamydosporia*. It has been successfully applied in our laboratory also for *Hirsutella* spp. and other soil fungi. The method is based on agar discs that are aseptically removed from a culture dish using the large end of a Pasteur pipette, flame sterilised. The plugs are then packed in cryo vials for prolonged storage at room temperature, tightly capped to avoid mite contaminations. The vials allow the fungi to be recovered after one or more years. In alternative, a spore suspension in SDW can be used.

10.4.4 Quality Control (QC)

Quality control (QC) should be carried out either during the production process or on the resulting product at the end of the multiplication cycle. Both checks are essential for successful production of high-quality and viable propagules that have to be free of potentially dangerous contaminants. One important objective of the QC procedures carried out during production and related inspection and monitoring is to avoid entrance of contaminants into the system. QC protocols must be incorporated in the routine of the production cycle in order to identify the contaminants and their source, when detected. QC carried out on the final product is designed to ensure that it meets predetermined standards, including parameters like propagule and mycelium viability, virulence and other characters, i.e. colour, moisture content (for long shelf life) as well as those directly related to the cycle efficiency, like the number of propagules g^{-1} of formulation or substrate.

10.4.5 In-Process QC

QC procedures should also be used to monitor/exclude the presence of contaminants entering the system during the downstream processing and/or formulation phases and to provide evidence, through the corresponding analysis reports that they did not occur nor multiply during the fermentation process. Standard QC procedures include measures for detecting large-scale problems such as sterilisation failure and the introduction of contaminants due to in-process handling of the solid substrate. It is important to ensure that contamination is detected as early as possible and that its origin is identified so that subsequent problems can be avoided. A formal set of QC checks must be incorporated into the production process. During

the incubation and manipulation phases, any contamination present in the fermenter must be rapidly identified, and contaminated batches and containers must be discarded (Jenkins et al. 1998).

Analyses include monitoring of the conidial inoculum purity, screening of the liquid media or autoclaved cereal substrates for sterility prior to inoculation and purity checks on liquid or solid substrates postinoculation. Liquid cultures can be checked by aseptically taking a small sample for examination under the microscope for visible signs of contamination. The sterility of the solid substrate can be verified using un-inoculated bags as controls for each autoclave and batch. Usually sterility screenings take place by plating on generalist media. Agar plate cultures are checked visually for the presence of contaminants before use as inoculum for liquid cultures. For propagule yields, although counting *P. chlamydosporia* CFUs on its selective culture medium may be possible, this approach may show some difficulties, i.e. species-/strain-specific differences that should be known. Furthermore, the procedure is extremely time-consuming, and the results are not immediately available.

Accurate identification and quantification protocols are the starting point to develop standards for biological purity, including assessment of the production process to establish that harmful microorganisms or other *P. chlamydosporia* isolates with distinct characteristics, proceeding from other production cycles, are unlikely to be present in the final product. DNA-based technologies involving the use of the polymerase chain reaction (PCR) allow the development of detection techniques that are independent from the culture properties and/or density of target fungi. PCR-based detection assays have been described and applied to a wide range of fungi, including some *P. chlamydosporia* isolates (Hirsch et al. 2001; Ciancio et al. 2005; Atkins et al. 2005, 2009; Cordier et al. 2007; Rosso et al. 2007). To check for bacterial contaminants, PCR with universal 18S bacterial primers like 27F-1492R may be used.

However, for routine use of PCR methods with the fungus isolate, due to the reaction sensitivity and specificity, appropriate selective target regions and primer sets unequivocally assigning the amplicon to the specific isolate produced are needed. Data also must be known on best reaction conditions. Variability is known in *P. chlamydosporia* as concerns specific genes that can be used for detection. Single nucleotidic polymorphisms (SNPs) were found in VCP1. This enzyme is active during the digestion of the egg vitelline and chitinous layers and its sequence is therefore a useful parameter in the choice of the isolate to use. It can also be used for species confirmation. When used in PCR-based assays, this single-copy gene has important practical implications for the identification of the fungus. Isolates of *P. chlamydosporia* proceeding from eggs of *M. incognita*, *Heterodera schachtii* or *Globodera rostochiensis* have SNPs in their VCP1 gene reflecting variations in their amino acid composition and enzyme molecular structure, which depend on the host they were isolated from (Morton et al. 2003; Rosso and Ciancio 2005). This gene may hence be exploited as a specificity marker in production, as well as in monitoring, after the fungus is released in the soil environment (Rosso and Ciancio 2005).

A further source of genetic variability is represented by the presence and possible activation of transposable elements. Genome-wide reshufflings induced by transposons may be difficult to detect, since their activation and effects may change the functionality of genes without a direct phenotypic evidence. At least two different transposons, a polyprotein (closest GenBank acc. CAB91877, with function as protease, integrase, reverse transcriptase) and a Fot5 transposase (closest GenBank acc. CAE55867), have been initially detected in *P. chlamydosporia* (Rosso et al. 2011). However, further transcriptomic studies on endophytic metabolism of *P. chlamydosporia* DSM 26985 showed 154 - transposon-related transcripts, with 6 terms for "transposon", 142 for "transposase-like protein" and 6 for "retrotransposable element-like" (Ciancio et al. 2013).

10.4.6 Record Keeping

Records must be kept for each production batch since the initial step of inoculum production and substrate inoculation. Batches are split according to the autoclave used for sterilisation of the solid substrate, and separated records must be kept for each. Details must be provided on date of inoculation, number of bags and unique tags, results of QC analyses and incidence of contamination during incubation, type of contaminant and final yield of conidia from all bags extracted. It should also be possible to trace each batch produced back to the original stock culture. Details are normally transferred and stored in a digital format, following packaging of each batch, so that production data are archived either as hard or electronic copies. They can be analysed periodically to evaluate, i.e. the cycle efficiency and the effects of minor substrate or process differences, on the final yield.

10.4.7 QC on Final Product

Efficacy is the most critical factor to ensure product performance and long-term acceptance by customers. Knowledge on the isolate ecology and on the effects of environmental factors is essential to ensure reliable field performance and targeted application of the highest-quality product. In general, some simple tests for viability may be set up to measure the product efficiency. A relative quick and accurate procedure to test viability is to determine the propagule germination rate. A relatively quick and accurate method to test viability of *P. chlamydosporia* is to determine the percentage of conidia and chlamydospore germination by plating a diluted suspension of propagules on sorbose agar with antibiotics. The medium is based on 12 g agar, 2 g sorbose, distilled water up to 1 L, adding after autoclaving and cooling 50 mg of each antibiotic (streptomycin sulphate, chlortetracycline and chloramphenicol). If testing for presence of contaminating bacteria, the antibiotics should not be used. Viable and nonviable spores can be counted under a microscope following their incubation at 25–26 °C for a week (Kerry and Bourne 2002).

The virulence of *P. chlamydosporia* can be verified in a bioassay using nematode eggs (Kerry and Bourne 2002). Parasitism is determined by spreading 200–300 RKN or PCN eggs on the surface of a WA culture with antibiotics and then incubated at 24–26 °C for a week. The WA surface must be covered with 2–3-day-old fungal colonies previously inoculated by smearing a small culture block with a sterile loop. Parasitism can be assessed by counting the first 50–100 eggs encountered when scanning the dish under a light microscope at 125–250×, scoring the number of eggs with evidence of parasitism (Fig. 10.4). Variability in bioassays depends on a number of factors including the natural diversity of the isolates or of the nematode host, and the original purity of the population used, that may be contaminated by other indigenous fungal populations or bacteria. Care should be taken to avoid use of nematode populations with other associated parasites. The eggs should be preferably obtained by dissolving egg masses of possibly pure nematode populations in NaOCl (Hussey and Barker 1973), followed by several washings with SDW, before aseptical transfer to the WA. Prior inspection of eggs on a water temporary slide in light microscopy at 125–250× will allow evaluation of incidence of other associated fungal or bacterial species. Some dishes without *P. chlamydosporia* inoculation should also be used as control, to exclude contaminations during the subsequent handling.

10.4.8 Storage

Shelf life is a critical limiting factor for biological control products. It should last as long as possible and, preferably, should not require refrigeration or special treatments (Jones and Burges 1998). Many factors affect the shelf life of fungal products, including nutrition, culture conditions during fermentation,

methods of downstream processing and harvesting, product moisture content, formulation and packaging (Magan 2001). Longevity of fungal products in storage can be determined through equations and experimental tests defining constants for propagules, as per standard methods for longevity of other propagules, i.e. seeds (Roberts and Ellis 1989; Hong et al. 1997). Effects of storage conditions on the survival for *P. chlamydosporia* were measured monthly for 6 months and shelf life was significantly improved at low temperatures and low water activity (a_w) (<0.33). Vacuum and N increased the fungus viability, whereas CO_2 increased the activity, as compared to ambient air. Optimal parameters for *P. chlamydosporia* in formulation of alginate pellets included a temperature range of 4–20 °C, $a_w = 0.12$, and a N-filled atmosphere (Duan et al. 2008).

10.5 Formulation and Application

The number of patents reporting one or more nematode BCAs increased during the last decade, in relation to market demand and potential of microorganisms for industrial and commercial exploitation (Fig. 10.6). Although some of these records refer other uses of the species of interest (i.e. the patents listing *Cordyceps* for medical uses that also report its imperfect stage *Hirsutella*), patent trends reflect changes in the approaches for phytonematode control, and the growth of industrial sectors vacated to promotion and exploitation of alternative management methods. Formulates containing propagules and hyphae of *P. chlamydosporia*, alone or in combination with *Arthrobotrys oligospora* or other fungi, have been produced and tested in Italy either as a dry powder (Fig. 10.7a) or liquid products with vegetable oil (Fig. 10.7b). For field or greenhouse treatments, the fungus may be given to plants by a variety of methods, i.e. liquid sprays, direct addition of chlamydospores or with water suspensions of mycelial extracts to roots. The density of application suggested in many studies is 5×10^3 chlamydospores g^{-1} of soil (Kerry 2000). Hence, for equivalent field applications, an estimate of the soil volume explored by the roots is required. An aqueous mixture of the inoculum may be applied for direct plant inoculation keeping soil moisture at field capacity and dispersing the product on soil or roots, after their exposure.

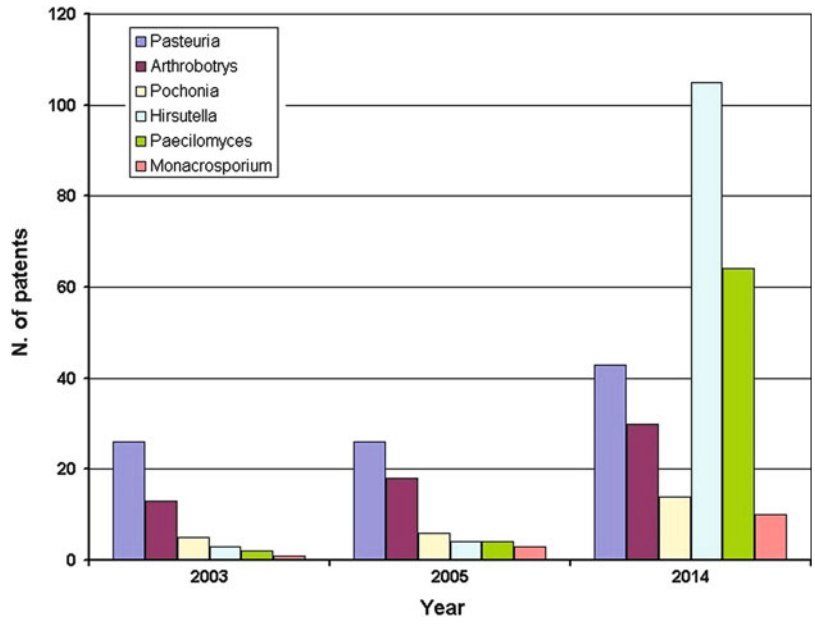

Fig. 10.6 Number of patents retrieved using genera of nematode BCAs (*inset*) as query term per year (Source: http://www.european-patent-office.org/index.htm)

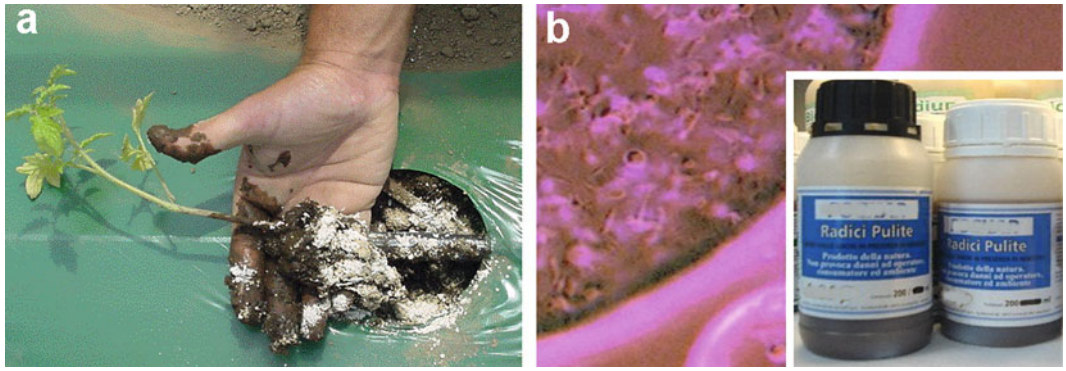

Fig. 10.7 Treatment of tomato seedlings using a powder formulate with *P. chlamydosporia* propagules. (**a**) Liquid commercial products with propagules and (**b**) hyphal fragments suspended in an oil drop as seen at 250×, sold in sealed plastic containers (*inset*)

P. chlamydosporia has no phytotoxicity nor does it exert negative effects on the plant, and treatments may be also performed on seedlings before or during the transplant operations. In a greenhouse, for post-transplant application or for treatments of more advanced phenologic stages, the product may be suspended in water and distributed through the irrigation system with a low operating pressure.

Commercial and market issues for nematode BCAs are related to the market dimension, to the presence of other commercial products and/or microorganisms, to the type of formulations available and to the farmers' perspectives for a successful replacement of synthetic pesticides. Market demands to be met concern technical tools to sustain plant nutrition and manage pests and pathogens (cyst and root-knot nematodes, in particular). To develop, on a microbiological basis, innovative plant growth-promoting products based on fungi like *P. chlamydosporia*, it is necessary to produce a large amount of information and to deploy research efforts on the occurrence, ecology, biology, production and testing of the isolates available, as well as on effective formulations and field efficacy. The type of market that the project aims to reach is represented by the sector of technical tools (biopesticides and/or soil amendment products) for farmers and producers of the agricultural sectors of specialised horticulture, including either organic or conventional crops. In the latter, a wide market space has been opened as a consequence of the ban of methyl bromide and other pesticides. As concerns the organic sector, attention is focussed on producers that, although representing (with a number of farmers around, i.e. 50,000–60,000 only in Italy) a minor fraction of the global market, are more interested in these products and potentially more receptive to their use. Finally, a detailed knowledge is required on the *P. chlamydosporia* isolates to use or produce, since their specificity may become a source of concern if the fungus is applied to control an unsuitable host or it is distributed in suboptimal operational conditions.

10.6 Conclusions

Our current knowledge on the biodiversity, role and potentials of rhizosphere microorganisms is still far from being exhaustive, and a significant effort will be required to get deeper and detailed information, useful for application in a wide range of agroecosystems. The exploitation of root functional species like *P. chlamydosporia* aims at restoring soil equilibrium or enhancing nematode control capacity, but still requires many research efforts. Data on behaviour in soil are fundamental for industrial exploitation of the fungus. To complete this task, a great help proceeds from the fast advancements in transcriptome and genome sequencing (Ciancio

et al. 2013; Larriba et al. 2014). This knowledge will highlight the many factors underpinning the specific activity of *P. chlamydosporia* isolates, as well as provide a knowledge-based platform to sustain its use in food production in many cropping systems, including low-input agriculture.

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Improvement of Crop Protection and Yield in Hostile Agroecological Conditions with PGPR-Based Biofertilizer Formulations

11

Dilfuza Egamberdieva and Anthony O. Adesemoye

Contents

Abstract

Intensive research attempts are underway to mitigate the impacts of climate change, improve salt tolerance and disease resistance of plants using organic farming practices, including biofertilizer which eventually improve degraded soils. In addition, it will form part of integrated environmentally friendly approach for nutrient management and sustainability in ecosystem functions. The use of such microbial inoculants as biofertilizers or biopesticides portends a great promise for controlling disease, improving plant health and soil productivity under environmentally stressed conditions. Stress-tolerant microorganisms with plant-stimulating properties are being discovered, selected and tested under field conditions and the number of successful applications is increasing. Formulation of microorganisms with various carrier materials enables long-term storage and protects them from various stress factors. This review summarizes the current status of microbial inoculants usage and prospects in crop cultivation and crop stress management, with particular attention to arid stress agro-ecological conditions.

D. Egamberdieva (✉)
Leibniz Centre for Agricultural Landscape Research (ZALF), Institute for Landscape Biogeochemistry, Müncheberg 15374, Germany
e-mail: dilfuza.egamberdieva@zalf.de

A.O. Adesemoye
Plant Pathology Department, West Central Research & Extension Center, University of Nebraska–Lincoln, North Platte, NE 69101, USA
e-mail: tony.adesemoye@unl.edu

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_11

11.1 Introduction

The increase in harsh abiotic stresses such as drought, salinity and abrupt changes in temperature is part of the main consequences of climate change and has led to the loss of soil organic matter and other forms of soil degradation that negatively affect agricultural productivity (Ahmad et al. 2012). Agricultural development is considered as the main sector in reducing hunger and poverty but the decrease in productivity of agricultural lands due to abiotic stresses has a direct impact on the standard of living of the more than seven billion people around the world (Munns and Tester 2008). Drought and salinity continue to have significant impacts in both developed and developing countries and are regarded as major factors in crop losses and diseases (Mantri et al. 2012; Hameed et al. 2014). Salinity alone affects 33 % of the potentially arable land area of the world, whereas 950 million ha of salt-affected lands occur in arid and semi-arid regions (UNEP 2008). Major factors increasing salinity include irrigation of cultivated lands with saline water, poor cultural practices and low precipitation. Climate change will result in a change towards a more arid climate, which is conducive to salt accumulation (Othman et al. 2006).

Another important consequence of climate change (especially temperature) and abiotic stresses is increased infection/infestation from pathogens and pests (Korus et al. 2015). For example, high temperatures may make crops to grow faster with reduced seed maturity time and reduced yield and may help certain diseases. On the other hand, low temperatures promote some other diseases and extremely low temperatures and/or snow may lead to frost bite that could predispose crops to diseases afterwards. Scientific projections have indicated that climate change will continue to have major impacts on crops productivity and yield across the world. Recently, a Global Alliance for Climate-Smart Agriculture was launched by world leaders during the United Nations Climate Summit in New York on September 23, 2014, involving multiple stakeholders, farmers, 20 governments and 30 organizations for incorporation of climate-smart approaches within food and agriculture systems (http://www.un.org/climatechange/summit/action–areas). Also, intensive research attempts are underway to mitigate the impacts of climate change, improve salt tolerance and management of plant diseases using farming practices, such as application of farm manure, compost, biofertilizer, recycling of crop residues and/or green manures or a combination of these practices in integrated systems, which will eventually improve soils (Pathma and Sakthivel 2012; Adesemoye and Egamberdieva 2013).

These farm practices have been considered as supplements or possible alternatives to chemical fertilizers. The practices will increase soil health and productivity, crop production in sustainable organic farming and hold a great promise to improve crop yields through environmentally sustainable and better nutrient supplies and use (Wu et al. 2005; Adesemoye et al. 2008; Hashem et al. 2014). Bacterial fertilizers are usually prepared as carrier-based inoculants containing effective microbes which include rhizobia and plant growth-promoting rhizobacteria belonging to genera such as *Bacillus, Pseudomonas*, *Azotobacter*, *Azospirillum*, *Bradyrhizobium*, *Enterobacter*, *Mezorhizobium*, etc. (Stephens and Rask 2000; Rinu and Pandey 2009; Egamberdiyeva et al. 2004; Egamberdiyeva 2007). Formulation of microorganisms with various carrier materials enables long-term storage and high effectiveness of biofertilizers (Marin 2006; Trivedi et al. 2012).

The microorganisms are able to colonize plant roots, stimulate root growth, make soil nutrients available to the plant root, protect plants from various diseases and enhance plant tolerance to various environmental stresses including drought, salinity, high or low temperatures and heavy metals (Egamberdiyeva and Hoflich 2002, 2003; Adesemoye and Kloepper 2009). In addition, they may help change nutrients that will otherwise be bound up in soil to available forms for plants, through solubilization of minerals and

increase the concentrations of P, N, K and Mg in plant root and shoots (Son et al. 2006). Based on these properties, microbial inoculants are considered as environmentally friendly microbial technologies for sustainable agriculture (Fravel 2005; Hashem et al. 2014). However, extreme environmental conditions such as high or low temperature, water and salt stress are deleterious for the survival and effective functioning of the introduced microbial inoculants (Pandey et al. 1998).

The different carrier materials for bacterial inoculants such as peat, charcoal/biochar, methyl ethyl cellulose and vegetable oils that support higher populations of bacteria and display much longer shelf lives are important for plant production in arid and semi-arid soils, which may also suffer from high salinity, water stress, temperature stress, low organic matter and nutrients (Stephens and Rask 2000; Ardakani et al. 2010).

The application of bacterial inoculants for improvement of plant may not always be successful or could produce variable results in different locations and cropping systems. In extremely cold conditions, many gram negative bacteria may not survive to enhance crop performance and gram positive bacteria may become dormant through sporulation. Low population of the inoculated bacterial strain in the rhizosphere of plants grown under hostile environmental conditions may occur. It is reported that salinity and desertification cause a great disturbance to plant–microbe interactions and inhibit microbial activity in the soil (Vardharajula et al. 2011; Egamberdieva 2012).

It is notable that microbes which are adapted to stress conditions will be beneficial to the growth and yield of various agriculturally important plants, including in salinated arid and semi-arid soils (Fu et al. 2010; Turan et al. 2012; Berg et al. 2013). Identifying such microbial strains and/or using such microbial inoculants as biofertilizers or biopesticides portends a great promise for controlling disease and improving plant health and soil productivity under environmentally stressed conditions. In addition it will form part of the needed environmentally friendly approach for nutrient management, climate-smart agriculture and sustainability of ecosystems. This review summarizes the current status of microbial inoculants usage and prospects in crop cultivation and crop stress management, with particular attention to arid and semi-arid agro-ecological conditions.

11.2 Beneficial Microbes

The plant root is associated with different microbial communities, and these microbes can be beneficial, neutral or pathogenic and compete for nutrients and niches under strongly varying conditions (Lugtenberg and Kamilova 2009; Berg et al. 2013). Beneficial rhizosphere bacteria are of two general types, those forming a symbiotic relationship with the plant and those that are free-living in the soil and root (Hameed et al. 2014; Egamberdieva et al. 2013, 2014). Plant growth-promoting rhizobacteria (PGPR) are free-living and play important roles in crop production as they: (i) stimulate root growth, (ii) make soil nutrients available to the plant root and/or fix nitrogen from the air to improve soil fertility, (iii) suppress soil-borne pathogens and (iv) alleviate abiotic stresses in plants (Egamberdiyeva and Hoflich 2004; Hashem et al. 2015; Shrivastava et al. 2015).

Some of the plant beneficial bacteria which can enhance plant growth, induce systemic resistance against diseases and enhance tolerance of plants to salt stress include species of *Pseudomonas*, *Bacillus*, *Azotobacter*, *Azospirillum*, *Flavobacterium*, *Stenotrophomonas* and rhizobia (Nabti et al. 2010). The utilization of root-associated bacteria that interact with plants by mitigating the effects of various stresses opens a novel, inexpensive and advanced technology for combating the problems of salinity (Arshad et al. 2008) and other climate related problems; thus, it continues to stimulate research and commercial interests. It has been shown that most PGPR isolates from environmental stress conditions showed certain enhancement of tolerance e.g., salt tolerance due to efficient metabolic processes that help them to adapt under these environments (Egamberdiyeva 2005; Wu

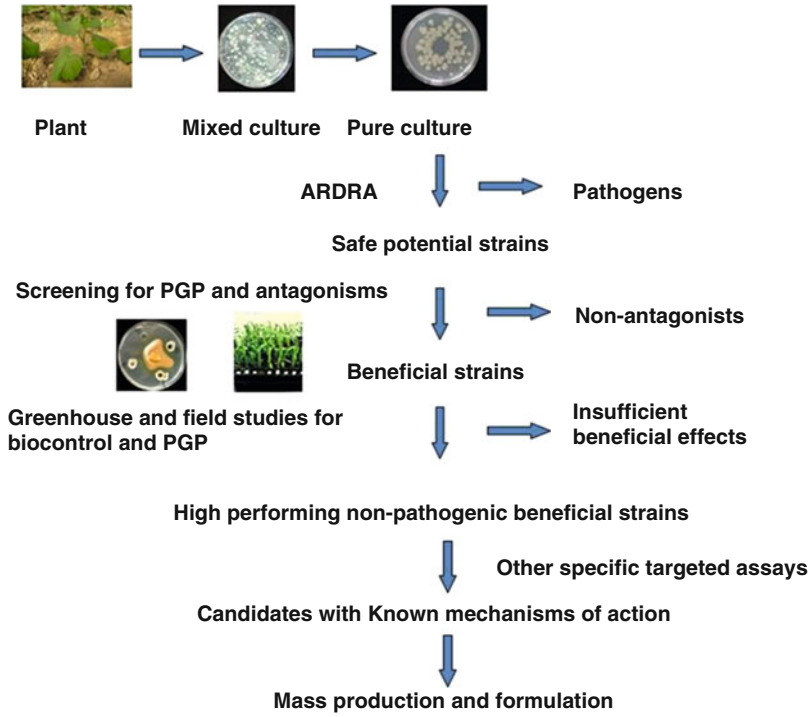

Fig. 11.1 Screening for plant growth promoting and biocontrol strains for the development of microbial inoculants

et al. 2011; Garg and Baher 2013). However, there is concern with variability in the effectiveness of biologicals when used in different conditions or cropping systems. The success of microbial inoculants applied as plant growth stimulator or biocontrol agents to multiple conditions therefore depends on the collection strategy and the screening process (Fravel 2005; Adesemoye personal communication). The screening flowchart (Fig. 11.1) is an effective procedure that could be used for identifying salt-tolerant bacterial inoculants, which are competitive colonizers, as well as for drought- or cold-tolerant bacteria for the formulation of microbe-based technology and could also be adapted to other environmental conditions.

There are many reports on the improvements of plant growth, development and nutrient uptake of cotton (*Gossypium hirsutum*) (Egamberdiyeva and Hoflich 2004), basil (*Ocimum basilicum*) (Golpayegani and Tilebeni 2011), wheat (*Triticum aestivum*) (Nabti et al. 2010; Turan et al. 2012) and goat's rue (*Galega officinalis* L) (Egamberdieva et al. 2013) under drought, cold and/or salt stress conditions. The plants treated with salt-tolerant PGPR contained lower Na^+ and Cl^- contents and higher nitrogen, phosphorus, potassium (NPK) contents compared with un-inoculated plants under salt stress (Yildirim et al. 2011). In another study, Mishra et al. (2011) selected a total of 12 pseudomonads, which were the top PGPR strains from a total of 534 psychro-tolerant bacteria collected at high altitudes in north-western Indian Himalayan region and tested them on wheat. They reported that the strains promoted the growth of wheat and helped in tolerance to cold stress. It has also been observed that mycorrhizal fungi and *Trichoderma* inoculants significantly increased plant height, dry weight, number of nodules, pods and yield of chickpea in comparison to un-inoculated control under stress conditions (Patel et al. 2012; Garg and Baher 2013; Hameed et al. 2014).

Several mechanisms have been proposed to be used by PGPR for plant growth promotion such as nitrogen fixation, synthesis of phytohormones, osmoprotectants, exopolysacharides and 1-aminocyclopropane-1-carboxylate (ACC) deaminase and solubilization of minerals such as phosphorus and potassium (Berg et al. 2013;

Goswami et al. 2013; Sharma et al. 2013). Stimulation of plant growth and yield of various plants by inoculation with PGPR having indole acetic acid (IAA) producing ability has been repeatedly documented, particularly when the plants were subjected to stressful growth conditions (Nadeem et al. 2010). There are also several reports which indicate improved plant growth and development by co-inoculated PGPR (Valverde et al. 2005; Egamberdieva et al. 2010, 2013). Combined inoculation of rhizobacteria strains which produce IAA and have ACC–deaminase activity with *Bradyrhizobium* enhanced the nodulation and plant growth in mung bean (Shaharoona et al. 2006), goats rue (Egamberdieva et al. 2013) and chickpea (Khurana and Sharma 2000) compared with single inoculation.

There are also many reports on the plant growth promoting and biological control activity of *Bacillus* species (Marques et al. 2010; Araujo et al. 2012). The organisms were able to tolerate high temperature, pH and osmotic conditions and were well adapted to the arid- and salt-affected environments (Islam et al. 2013; Ashwini and Srividya 2014). This enabled these bacteria to be effective as plant growth stimulators and biocontrol agents under many environmental conditions (saline, drought, heavy metal contamination, etc.). Other mechanisms of PGPR include exhibition of antagonistic activity against phytopathogenic microorganisms by producing siderophores, cell wall-degrading enzymes, antibiotics and cyanide; induction of systemic resistance; and competition for nutrients and niches (Lugtenberg and Kamilova 2009; Glick 2010; Egamberdieva et al. 2011). Possible mechanisms involved in the establishment of a successful interaction between PGPR and plant roots under hostile environmental conditions have been reviewed and discussed by Adesemoye and Egamberdieva (2013).

11.3 Carrier-Based Preparations of Microbial Inoculants

Arid and semi-arid soils are mostly characterized by water deficiencies, prone to salinity, high or low temperatures and poor nutrients which pose additional challenges for the use of bio-inoculants (Bashan 1998; Egamberdiyeva 2007). The survival and colonization of microbial inoculants in the plant root is critical for them to be able to have significant effect on plant growth and health of crops under hostile environments (Rekha et al. 2007; Khavazi et al. 2007). In arid soils, due to poor bacterial survival in the soil, failure of plant growth promotion and biological control of plant diseases by microbial inoculants are often observed (Van Dyke and Prosser 2000). Improved methods of formulation and use of carrier materials need to be considered to assure efficient delivery of bio-inoculants in crop production systems in stressful conditions (Bashan 1998; Egamberdieva et al. 2011).

The carrier materials used for the bacterial inoculants protect them from various stress factors and prolong shelf life (Kumar et al. 2007; Ardakani et al. 2010). Therefore, carrier is important for the viability of the inoculum in an appropriate formulation. Several materials have been evaluated as potential carriers for bacterial inoculants, including alginate beads (Trivedi et al. 2005), peat (Albareda et al. 2008), biochar (Hale et al. 2015), perlite (Daza et al. 2000; Khavazi et al. 2007) and vermiculite (Sangeetha 2012). Arora et al. (2014) studied other materials including bagasse, saw dust and wood ashes as possible microbial inoculant carriers, and they found that coriander husk, saw dust and bagasse materials were able to sustain the highest viable cell number of rhizobial and *Pseudomonas* strains. Calcium alginate (CA) gel, a biodegradable microcapsule, is another material that has been widely utilized as a carrier for bacterial immobilization, which may protect the cells and has long-term effects under hostile environments (Wu et al. 2011).

The survival rates of microbial inocula in the carrier materials differ, e.g., perlite inoculants can maintain a higher population of microorganisms such as *Rhizobium leguminosarum* bv. phaseoli, *Rhizobium tropici*, *Bradyrhizobium japonicum* and *Bacillus megaterium* than peat inoculants at room temperature for 6 months period (Daza et al. 2000). Trivedi et al. (2005) observed maximum

numbers of alginate-based PGPR *Bacillus subtilis* and *Pseudomonas corrugata* in the rhizosphere of 6-week grown maize (*Zea mays*), compared to a charcoal-based formulation. Wu et al. (2011) studied the improvement in the survival rate of *Klebsiella oxytoca* Rs-5 under salinity stress conditions through the preparation of microcapsules using sodium alginate and calcium chloride. They observed that the bacteria released from microcapsules reached up to 10^{10} cfu/g when immersed in physiological saline for 3 weeks. When biochar (pine wood) was used as a carrier, significantly greater *Enterobacter cloacae* UW5 populations were detected in soil after 4 weeks as compared to direct soil inoculation with liquid inoculums (Hale et al. 2015). Peat moss which contains high availability of labile carbon and high nitrogen content was the best for survival of *E. cloacae* UW5 after 4 weeks in non-sterile soil (Hale et al. 2015). The talc powders containing 1 % carboxy methyl cellulose were also used as carrier for bacterial inoculants with broad-spectrum antifungal activity and suppression of fungal pathogens (Negi et al. 2005).

Albareda et al. (2008) studied the survival of several PGPR strains including *Bradyrhizobium*, *Mesorhizobium* and *Sinorhizobium* strains in different carrier materials and liquid formulations such as bagasse, cork compost, attapulgite, sepiolite, perlite and amorphous silica. They observed that compost and perlite support higher survival rate of PGPR strains compared to other materials, whereas the viable numbers of *Sinorhizobium fredii* SMH12 and *B. japonicum* USDA110 strains were greater than 10^9 and $5x10^8$ cells g^{-1}, respectively.

Ben Rebah et al. (2002) studied wastewater sludge as a carrier for *Sinorhizobium meliloti* and observed that dewatered sludge supported rhizobia survival. Sludge was considered as non-toxic and low cost raw material for microbial inoculants. Ardakani et al. (2010) used bentonite as a mineral carrier for the formulation of *Pseudomonas fluorescens*, which supported more stimulatory effect compared to peat and rice bran formulated inoculants.

11.4 The Use of Microbial Inoculants as Biofertilizers in Hostile Environmental Conditions

The positive effects of PGPR have been observed on various agricultural crops and medicinally important plants under different hostile environmental conditions, including arid- and salt-affected soils (Bano and Yasmeen 2010; Berg et al. 2013; Egamberdieva and Lugtenberg 2014). In a study by Wu et al. (2011) mentioned earlier, the effects of inoculation with free and alginate-encapsulated cells of *K. oxytoca* Rs-5 on cotton germination, root, shoot length and dry weight under salinity stress were examined. They observed that microcapsulated Rs-5 inoculant increased plant growth and biomass as compared with those uninoculated plant and free cells of Rs-5.

Bharathi et al. (2004) studied talk-based formulation of antagonistic bacteria for the management of fruit rot infection in chillies. The combined inoculation of *P. fluorescens* (Pf-1) with *B. subtilis* increased germination of chille by 43 % compared to uninoculated seeds. In addition talk-formulated bacterial strains increased shoot length (56.14 %), number of flowers (270.59 %), fruits (133.3 %) and also reduced fruit rot infection in chillies caused by *Colletotrichum capsici* by 51 % over control. The increase in chitinase, β-1, 3 glucanase, peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and phenol accumulation in plants treated with mixed formulation were also reported (Bharthi et al. 2004).

Evidently, the use of combined inoculation for crop production could result in a significant improvement in various growth parameters (Egamberdieva et al. 2013; Yasmin et al. 2013). Mixed applications of inoculants have been shown to be more effective as biocontrol agents and plant growth stimulators under various environmental conditions (Adesemoye et al. 2008; Adesemoye et al. 2009; Malusá et al. 2012). Formulated microbial inoculants (AMF and *Pseudomonas*) increased plant growth and

uptake of nitrogen (N), potassium (K), phosphorus (P), calcium (Ca) and magnesium (Mg) in sunflower under saline (EC = 7.6 dS m^{-1}) calcareous soil condition. In addition, microbial preparation reduced Cl uptake by the plants which enhanced the nutrition condition of the plant (Shirmardi et al. 2010). Sharma et al. (2013) observed increased germination, root and shoot length, fresh weight and proline content of chickpea seedlings by *Bacillus* sp. and *Pseudomonas* sp. under osmotic potential of up to 0.4 MPa over un-inoculated control. The strains were able to produce IAA and showed P solubilizing activity.

Elkoca et al. (2010) demonstrated increased P, K and micro-nutrient in common bean as a result of *Bacillus* and *Azospirillum* inoculations. In another study, formulation of PGPR which produced ACC deaminase showed positive effects on plant growth of chickpea (Roopa et al. 2012) resulting in increased number of nodules, root, shoot growth and yield of plant under stress conditions. The peat formulated *Mezorhizobium ciceri* significantly induced nodule numbers on the roots of chickpea cultivars, which showed a high correlation with shoot and root weights (Egamberdieva et al. 2014). Inoculation of plants with *M. ciceri* IC53 formulation significantly increased shoot and root dry matter by an average of 20 %, (pod number and yield of chickpea) above the untreated plant under arid saline conditions.

Karlidag et al. (2013) also observed positive effect of microbial inoculants on strawberry growth and yield under salt-affected soil conditions consecutively in 2 years of field experiments. The highest efficiency in alleviating salinity stress on the yield and nutrient (N, P, K, Ca, Mg, sulfur (S) and manganese (Mn)) uptake of strawberry plants was obtained from *Kocuria erythromyxa* EY43 treatment. The combined application of compost and microbial inoculant (*Pseudomonas*) increased the plant growth and uptake of minerals like P, Mn, K, Zn and Fe in chickpea plants (Sahni et al. 2008). Peat-based inoculants of *P. fluorescens* increased plant height (58.1 %), number of cobs per plant (19.5 %) and number of grain rows per cob (28.2 %) of corn as compared to the control plant grown under drought conditions (Zafar-ul-Hye et al. 2014). N'cho et al. (2013) studied the effects of microbial inoculants including *Bradyrhizobium* spp. (RACA 6), arbuscular mycorrhizal fungi (Rhizatech) and *Trichoderma harzianum* (Eco-T) on soybean growth and yield in northern Guinea savannah. Microbial inoculants increased plant growth, yield and nodulation of soybean up to 45 %.

Rajput et al. (2013) evaluated microbial inoculant *Planococcus rifietoensis* for the improvement of plant growth and yield of wheat (*T. aestivum*) improvement under salinity stress conditions. According to their observations, salinity reduced growth and yield parameters of wheat (up to 60 %) in field experiments, whereas microbial inoculants enhanced plant height (29 % over the control), biomass (36 % over the control), growth (37 % over the control), straw weight (50 % over the control) and yield (38 % over the control) by alleviating the toxic effects of salinity. Under salt stress arid conditions, corn–molasses based microbial inoculants including *Pseudomonas extremorientalis* TSAU20, *P. fluorescens* PCL1751 and *Stenotrophomonas rhizophila* e–p10 caused statistically significant increases in plant height and yield of cucumber fruit up to 32 % compared to the un-inoculated control plants (Egamberdieva et al. 2011). In wheat and barley, Turan et al. (2012) tested five boron treatments and four PGPR strains – *B. megaterium* M3, *B. subtilis* OSU142, *Raoultella terrigena* and *Azospirillum brasilense* sp245 – at 5/2 °C 8-h (day/night) temperature and showed that the PGPR treatments alleviated the deleterious effect of low temperature on both crops.

In a study to identify novel PGPR that can retain activity at low and warm temperatures and help winter wheat in cold-hardiness, Adesemoye (personal communication) carried out a year round collection and screening of authochtonous bacteria from wheat roots in the western part of Nebraska, United States. The region produces high amount of wheat yearly and it is a semi-arid climate where frost bite/damages sometimes occur. Organisms of interest collected so far and

being tested in the ongoing study include strains of *Bacillus safensis*, *B. megaterium* and *Bacillus pumilus*. The use of microbial inoculants in arid climatic conditions continues to expand. For example in Iran, Arvin et al. (2012) reported that *Pseudomonas* inoculants increased yield and seed oil content of *Brassica* oilseed rape in field trials under arid soil conditions. Also, microbial preparations based on *Azotobacter* strain Azo-8 increased plant growth and yield of wheat grown under arid conditions (Singh et al. 2013). In another study, *Azospirillum* inoculants combined with P-solubilizing bacteria improved plant growth, fiber yield and fiber quality of cotton under field conditions (Dhale et al. 2011).

In previous field experiments, we have observed plant growth stimulating efficiency of phosphate solubilizing bacterial (PSB) inoculant *Rhizobium meliloti* combined with phosphorite on cotton (Poberejskaya et al. 2003). The PSB combined with phosphorite showed a significant effect on dry matter accumulation in leaves, shoot and root of cotton (Table 11.1).

Compared to the control and fertilizer treatments, the PSB combined with phosphorite was superior over the other treatments. Higher effects were found at the maturity stage. In field experiments, all treatments increased yield of cotton in comparison to control plants (Fig. 11.2). Higher yield obtained after treatment with PSB *R. meliloti*. The yield of cotton

Table 11.1 The effect of phosphate-solubilizing bacteria (PSB) *Rizobium meliloti* URM1 combined with phosphorite on dry matter of cotton (field experiments[a], g/plant)

	Tillering		Flowering			Maturity		
Treatments	Leaves	Stem	Leaves	Stem	Bud	Leaves	Stem	Bud case
Control	8.1	7.0	46.5	10.5	15.5	54.6	39.5	35.3
$NP_{superp}K$	9.5	6.3	47.0	18.7*	17.1	53.0	42.1	36.0
$NP_{phosphorite}K$	8.9	6.0	46.9	18.0*	18.4*	57.4	51.0*	38.3*
$NP_{PSB}K$	14.6*	8.7*	47.0	18.9*	23.4*	89.1*	64.8*	49.5*

[a]Recommended rates of phosphorus (140 kg P h^{-1}, as phosphorite and superphosphate), nitrogen (200 kg N $ha^{-1,}$ as ammonium sulphate) and potassium (60 kg K $h^{-1,}$ as potassium sulphate) were applied. Treatments were: Control – plants without treatments, $NP_{superphosphate}$ K, $NP_{phosphorite}$ K, $NP_{phosphorite+PSB}$ K, (PSB – phosphate-solubilizing bacteria, *Rizobium meliloti* URM1), *significantly different from control plants at $P < 0.05$

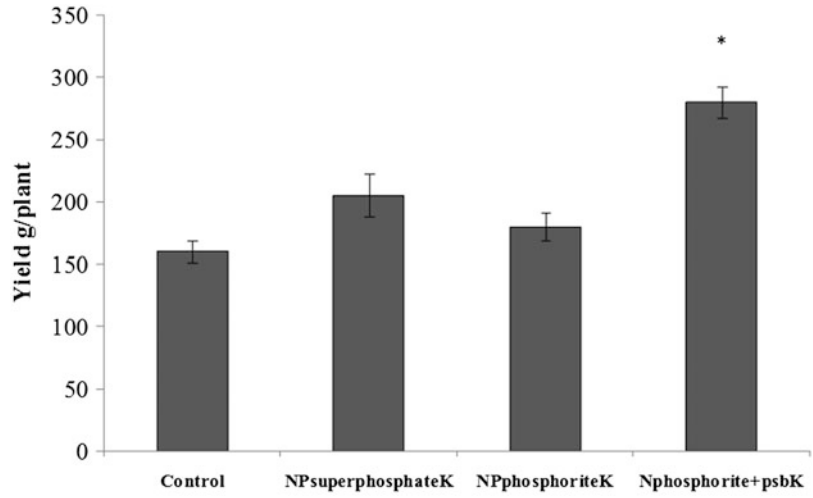

Fig. 11.2 The effect of phosphate solubilizing bacteria (PSB) *Rizobium meliloti* URM1 combined with phosphorite on cotton fiber yield under semi-arid salinated soil condition. Treatments were: Control – plants without treatments, $NP_{superphosphate}$ K, $NP_{phosphorite}$ K, $NP_{phosphorite+PSB}$ K, (PSB – phosphate-solubilizing bacteria, *Rizobium meliloti* URM1), *significantly different from control plants at $P < 0.05$

Table 11.2 The effect phosphate-solubilizing bacteria (PSB) *Rizobium meliloti* URM1 combined with phosphorite on N and P uptake of cotton (field experiments[a], N and P content in %)

	Leaves		Stem		Bud case		Cotton fibers	
Treatments	N	P	N	P	N	P	N	P
Control	1.45	0.51	0.68	0.21	0.78	0.19	1.78	0.81
$NP_{superp}K$	1.55	0.75*	0.75	0.24	0.83*	0.22	1.87	0.84
$NP_{phosphorite}K$	1.20	0.20	0.30	0.10	0.50	0.10	1.60	0.40
$NP_{PSB}K$	1.62*	0.8*	0.75	0.24	0.83*	0.25*	1.90*	0.89

[a]Recommended rates of phosphorus (140 kg P h^{-1}, as phosphorite and superphosphate), nitrogen (200 kg N ha^{-1}, as ammonium sulphate) and potassium (60 kg K h^{-1}, as potassium sulphate) were applied. Treatments were: Control – plants without treatments, $NP_{superphosphate}$ K, $NP_{phosphorite}$ K, $NP_{phosphorite+PSB}$ K, (PSB – phosphate-solubilizing bacteria, *Rhizobium meliloti* URM1), *significantly different from control plants at $P < 0.05$

increased up to 68 % (275.0 g^{-1} plant) (Poberejskaya et al. 2003).

According to the results obtained, PSB was able to mobilize phosphorus use efficiency in cotton. The phosphorus content was significantly increased in cotton plants when treated with PSB combined with phosphorite (Table 11.2). The standard treatment with fertilizer did not affect P uptake in plants. In another similar study, Shah et al. (2001) reported phosphorus uptake efficiency and yield increases with phosphorus application and with inoculation.

The positive influence of treatments on soil P content is marked (Table 11.3). Soil P content in the variant with PSB reaches 6.0 mg P_2O_5/100 g soil. It has been found that the application of phosphorite combined with PSB leads to the increase of P content in soil (tillering, flowering and maturity stages of plants). This result suggests that PSB combined with phosphorite were able to mobilize more P to the plants and improve plant growth. Yasmin et al. (2013) also observed improved yield of cotton under reduced fertilizer conditions by formulations based on *Pseudomonas aeruginosa* Z5 and *Bacillus fusiformis* S10. Similar results were observed for wheat when biofertilizer based on *Azotobacter* in combination with manure improved plant growth, stress tolerance and yield under water stress conditions (Singh et al. 2013).

The immobilized microbial inoculants *A. brasilense* and *Pantoea dispersa* in clay pellets combined with organic olive residue stimulated root dry weight (by 133 % with respect to control plants), shoot dry weight

Table 11.3 Phosphorus content in soil as affected by posphate solubilizing bacteria (PSB) *Rizobium meliloti* URM1 combined with phosphorite (field experiments[a], before sowing 1.8 mg P_2O_5/100 g soil)

Treatments	Tillering	Flowering	Maturity
Control	2.4	1.5	2.2
$NP_{superp}K$	2.8	6.0*	4.7*
$NP_{phosphorite}K$	2.0	1.8	1.9
$NP_{PSB}K$	5.4*	6.0*	4.0*

[a]Recommended rates of phosphorus (140 kg P h^{-g}, as phosphorite and superphosphate), nitrogen (200 kg N ha^{-1}, as ammonium sulphate) and potassium (60 kg K $h^{-1,}$ as potassium sulphate) were applied. Treatments were: Control – plants without treatments, $NP_{superphosphate}$ K, $NP_{phosphorite}$ K, $NP_{phosphorite+PSB}$ K, (PSB – posphate solubilizing bacteria, *Rizobium meliloti* URM1), *significantly different from control plants at $P < 0.05$

(by 106 % with respect to control plants), P and K content (by 100–70 % with respect to control plants). In addition microbial inoculants increased total C, total organic C and microbial biomass C content and enzyme activities (dehydrogenase, urease and protease) of the rhizosphere of *Cistus albidus* (Schoebitz et al. 2014). In another study, peat-based inoculants of *Rhizobium phaseoli* and *P. fluorescens* reduced adverse effects of salinity on relative water contents, photosynthetic rate, water use efficiency and chlorophyll content of mung beans over the un-inoculated control. In addition, combined inoculation improved the ionic balance and also increased the phosphorus and protein concentrations in grain of mung bean under salt-affected conditions (Ahmad et al. 2013). Peat-based inocula of *R. phaseoli* mitigated salt

stress in mung bean and improved growth and yield under saline conditions (Zahir et al. 2010). The plant height was improved by 28.2 %, number of nodules by 71.4 %, plant biomass by 61.2 % and grain yield by 65.3 % compared with untreated control.

11.5 Conclusion and Future Perspectives

In arid and semi-arid regions, soil fertility is diminishing gradually due to water stress, salinity, poor nutrients, erosion, high/abrupt changes in temperature and other climatic conditions. There is good evidence that fertilizers based on microbial inoculants could be very useful to improve crop yield and soil productivity. Several materials have been evaluated as carriers for bacterial inoculants, successfully protecting them from various stress factors and prolonging shelf life. Stress-tolerant microorganisms with plant-stimulating properties are being discovered, selected and tested under field conditions and the number of successful applications is increasing. The combination of inoculants has the potential to alleviate abiotic stress, promote plant growth, enhances the root system and nutrient availability that can assist the plant to absorb more nutrients. Those results imply that formulated microbial inoculants could be a useful approach for improving growth and yield of crop plants under hostile environmental conditions. Further research should be directed at optimizing methods for microbial formulations considering agro-ecological conditions and to determine the feasibility of using microbial fertilizers under severe environmental conditions. In addition, further research should be directed at studying the effectiveness of inoculants in integrated systems that examine cropping practices such as fertilizer inputs, tillage practices and crop rotation as they affect introduced microbial inoculants, their survival and plant beneficial properties.

Acknowledgment The research activity of Dilfuza Egamberdieva was supported by a Georg Forster Research Fellowship for experienced Researchers (HERMES), Alexander Von Humboldt Foundation.

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12 Use of Indigenous Cyanobacteria for Sustainable Improvement of Biogeochemical and Physical Fertility of Marginal Soils in Semiarid Tropics

Luigi Paolo D'Acqui

Contents

Abstract

This chapter describes a realistic and innovative approach for promoting a sustainable increase of overall soil fertility of marginal lands in representative semiarid environments in East and Southern Africa (South Africa, Tanzania and Zimbabwe). This realistic and innovative approach was based on the application of indigenous selected strains of nitrogen-fixing cyanobacteria and releasing organic compounds such as exopolysaccharides (EPS) to improve soil fertility and structural stability. To achieve these goals, 17 cyanobacteria strains (over 200 identified in soils of the African countries), able to fix atmospheric nitrogen and to release EPS in considerable amounts, were isolated, purified and grown. Using adequate techniques and procedures, large amounts of cyanobacterial biomasses were produced to be applied to poor soils to ameliorate their quality and to improve their productivity. Laboratory and greenhouse experiments showed that the application of these biomasses significantly improved overall soil fertility and crop yield. In spite of the need to confirm the relevance and persistence of the beneficial effects by further medium and long-term field experiments, the application of selected cyanobacteria strains to marginal lands appeared to be a very promising tool for a

L.P. D'Acqui (✉)
Istituto per lo Studio degli Ecosistemi, CNR, via Madonna del Piano n. 10, 50019 Sesto Fiorentino, Italy
e-mail: dacqui@ise.cnr.it

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_12

sustainable improvement of fertility and productivity of degraded soils in semiarid tropics.

12.1 Introduction

In many tropical countries, the scarcity of productive land and water are the major constraint for the improvement of the standard of living for inhabitants due to the increase of population and, consequently, of food demand. This leads to a progressive use of marginal lands with inherently poor, overall fertility. In these areas clearing and cultivation frequently lead to a rapid decline of natural biochemical fertility as a consequence of loss of nutrients, in particular nitrogen (N) and organic matter (OM), exacerbated by the removal of crop residues from soil used for feeding livestock or for other purposes. Cultivation and the associated loss of OM also promote degradation of physical properties and in particular crusting of the soil surface. This seriously affects soil erosion, effective use of water (whether under irrigation or rain-fed conditions), tillage, seedling emergence and general productivity of the land, especially in low-input agricultural systems of Southern African Tropics (SAT).

An appropriate management of soil and water resources is therefore needed, particularly in arid and semiarid regions. Hence, it is aimed to report how to establish a possible realistic, innovative approach for promoting a sustainable increase of overall soil fertility of marginal lands in representative semiarid environments in East and Southern Africa (South Africa, Tanzania and Zimbabwe) by application of indigenous selected strains of soil cyanobacteria fixing atmospheric N_2 and releasing organic compounds (EPS), able to improve soil structural stability.

The presented results mainly come from a study carried out within a project (CYANOSOILS) funded by the European Union. The idea was based on the application of local strains of cyanobacteria, widespread autotrophic soil and water microorganisms, as a low-input tool easily accessible to small-scale farmers in Southern African countries, to improve resilience and overall soil fertility. Many strains of cyanobacteria are able to fix significant amounts of atmospheric N_2 and to release EPS, well-known biological amendments to enhance soil physical properties.

Cyanobacteria (oxygenic, photosynthetic bacteria) are known to improve chemical and biological characteristics of the soils, increasing the content of N (by N_2-fixing activity) and C (by photosynthesis) and stimulating microbial community activities. The ability to fix N_2 (one of the macronutrients often limiting productivity in soils of tropical regions) is shared by many species belonging to the genera *Nostoc*, *Anabaena*, *Fischerella* and *Scytonema*.

The practice of soil inoculation with living N_2-fixing cyanobacteria (algalisation or more appropriately cyanobacterisation), has been largely used in Asian countries for many years for increasing soil organic N content mainly in tropical rice cultivation (Venkataraman 1979; Zimmerman 1993; Ghosh and Saha 1997). Data obtained in several experiments suggest an improvement of total N to around 10–30 % in alkaline soils and up to 120 % in neutral soils.

The photosynthetic activity of these organisms involves other beneficial effects such as the increase of soil OM, under favourable conditions of light, moisture and pH, due to the accumulation, as cyanobacterial biomass, up to 450 kg ha^{-1} of organic carbon (OC) and 60 kg ha^{-1} of N. As a consequence, in the long term, a large amount of OC arising from cyanobacterial biomass contributes to a stable C pool in soils. Moreover, the improvement of biological soil conditions by cyanobacterial inoculation results in: (1) a very large increase of total bacterial soil population and specifically of the genus *Azotobacter* with a consequent enhancement of the nitrogenase activity, (2) an increase in the number of actinomycetes and limited increase of fungi, (3) an increase in indigenous cyanobacterial population, (4) an improvement of seedling emergence, (5) biological control of plant pathogenic microbes and (6) a general increase of soil enzymatic activities (Hu et al. 2012).

The positive effects of cyanobacteria can be extended to soil physical properties. In particular,

it has been suggested that the role of these microorganisms in the formation of aggregates could be of considerable importance in arid and semiarid tropical soils (Barclay and Lewin 1985; Cogle et al. 1995). In well-aggregated soils, OM and iron oxides bind clay and other particles into water stable structures that resist to the destructive energy of the raindrop impact. Therefore, under conditions of arable agriculture, and particularly in those prevailing in SAT farming systems, soil degradation takes place over time as a result of the progressive loss of OM leading to a substantial weakening of aggregates that become more and more vulnerable to the droplet impact.

Cyanobacteria have been recognised to contribute to soil stabilisation through the production of EPS that have been demonstrated to be among the most effective organic substances for enhancing soil aggregate stability (Rao and Burns 1990; Rogers et al. 1991; Martens and Frankenberger 1992; Rogers and Burns 1994; Falchini et al. 1996; Malam Issa et al. 2007; Maqubela et al. 2012). However, experiments using these microorganisms as soil conditioners are rare, in spite of their potential effectiveness in controlling soil structure. Although growth and activity of cyanobacteria are related to the availability of a sufficient moisture, the importance of soil desiccation and soil water potential on the establishment and activity of inocula and on polysaccharides production have not been specifically investigated.

It can be expected that, if sound studies on the most appropriate conditions for field application in SAT are performed, the inoculation with living cyanobacteria may lead to the establishment and growth of this group of microorganisms in the soil with durable positive effects on overall fertility.

12.2 Site Selection, Sampling and Soil Characterisation

The study was conducted in three Southern African countries with semiarid environments: South Africa, Tanzania and Zimbabwe. In South Africa and Zimbabwe, a large number of small-scale farming enterprises were created, and many of these settled in marginal lands because of a massive land-reform programme. In the case of Tanzania, differently, the country had to face with a limited food supply, due to very little commercial agriculture activity and, as a consequence, the need to develop a small-scale agricultural sector to produce efficiently more food, consistently with a good environmental protection ethos. The study sites were selected in representative marginal land areas typical of each country. Seven relatively nutrient poor soils located in important agricultural areas were selected in the three African countries. In Tanzania the sites were Mkindo in Mvomero district and Mafiga located at Sokoine University Farm in Morogoro district. The soils of Mkindo, classified as Gleyic Cambisol, according to World Reference Base for Soil Resource (WRB) classification (IUSS 2014), had been cultivated for 15 years with rice being the crop usually grown. Mafiga site had been cultivated with maize for more than 35 years, with periodic 2-year fallows. This soil was classified as Rhodi-Profondic Lixisol (WRB). In South Africa two soils from the Eastern Cape Province were selected. They were Guquka, located below the escarpment of the Amatola Mountains and Hertzog in the Northern part of the Kat river basin. Guquka soil (Ferric Luvisol, WRB) had been cropped with maize under rain-fed conditions but was on fallow during the time of sampling. Hertzog site was a small-scale irrigation scheme that had also been cropped with maize and was on its second fallow year at the time of the sampling. This soil was defined as Haplic Luvisol in WRB classification. In Zimbabwe, the sampling sites were located in the Central region near Domboshawa village, Chikwaka village and Henderson research station in Mazowe district. The three Zimbabwean sites were under rain-fed maize and represented low-fertility sandy cropping environments occupied by small-scale poor households. These soils were classified in WRB classification as Haplic Lixisol, Chromic Luvisol and Haplic Lixisol, respectively.

Table 12.1 Main characteristics of the studied soils (average of three replicates) (Source: Pardo et al. 2010)

Characteristics	Units	Mkindo	Mafiga	Domboshawa	Chikwaka	Hertzog	Guquka
Clay	%	9	23	17	10	7	13
Silt	%	30	44	5	2	18	41
Sand	%	61	33	78	88	75	46
pH (H_2O)	1:2.5	6.8	6.4	6.1	6.1	7.3	5.4
Total N	g kg^{-1}	0.9	1.1	0.8	0.3	1.4	0.4
Organic C	g kg^{-1}	16.3	12.3	10.2	6.7	16.1	6.1
CEC	$mmol_c$ kg^{-1}	81	101	21	11	133	18
ESP	%	1.4	1.3	1.9	3.1	0.2	4.8

CEC Cation exchange capacity, *ESP* Exchangeable sodium percentage

Topsoil samples were collected at each site in agricultural areas where soils with chemical or physical constraints occurred. Twelve individual samples, in an area of around 1 ha, collected from each sites were used to prepare three replicate composite samples (D'Acqui et al. 2006; Pardo et al. 2007, 2010).

Soil particle-size distribution and pH measurements were carried out according to Società Italiana della Scienza del Suolo (SISS) methods (1985). Total OC and N content were determined by a Carlo Erba (Milan, Italy) NA 1500 CHNS Analyzer. Cation exchange capacity (CEC) was determined by using 1 M NH_4OAc (Juo et al. 1976). Main soil properties and analyses of the selected soils are reported in Table 12.1.

12.3 Isolation, Identification and Characterisation of Indigenous Cyanobacterial Strains Suitable for Soil Inoculation

12.3.1 Isolation and Identification of Soil Cyanobacteria

Traditional microbiological techniques were used for the isolation from soil samples of cyanobacteria suitable for soil inoculation with particular reference to N_2-fixing activity, EPS production (i.e. cyanobacteria strains with biofertilisation and bioconditioning potential, respectively).

The cyanobacteria strains were isolated from fresh soil samples using BG11 growth medium and strains, able to grow diazotrophically, and were isolated with medium $BG11_0$ (BG-11 without $NaNO_3$) as described by Rippka et al. (1979). Cultures were incubated at 28 °C in a growth chamber with a light of approximately 10–30 μmol photons m^{-2} s^{-1}. The enumeration of soil cyanobacteria was assessed after 2 weeks, through both the most probable number (MPN), by using McGrady's tables (McGrady 1915) and the colony-forming units (CFU) counting in Petri dishes. For both enumerations, the BG-11 and $BG\text{-}11_0$ media were used. A Harrison disc was used to randomly pick different colonies that had developed on the Petri dishes based on their diversity in morphology, colour of the colonies and micromorphology of the organisms as observed under a light microscope. The selected colonies were purified by repeatedly streaking on new growth media supplemented with cyclohexemide (eukaryote inhibitor). The isolation of cyanobacterial strains in monoculture and identification was carried out according to Castenholz (2001).

More than 200 strains of cyanobacteria and green microalgae (from all countries included in the study) were isolated and conserved in agar slants. After a careful observation under light microscope, around 150 strains were identified and maintained in culture for further studies.

12.3.2 Selection of Suitable Cyanobacteria

Selection of cyanobacterial strains with biofertilisation and bioconditioning potential was done

by determining the nitrogenase activity of diazotrophic strains and their ability to produce EPS. Nitrogenase activity was determined using acetylene-reduction assay. Liquid cultures were set in Erlenmeyer flasks containing 25 mL of the $BG11_0$ broth inoculated with the cyanobacterial strains and incubated at 30 °C under light (75 μmol photons m^{-2} s^{-1}) with continuous shaking at 80 r.p.m. After 10 days of incubation, cells were harvested by centrifugation, resuspended in 2 mL of fresh BG11 medium and incubated in air composed of 13.3 % acetylene in 17 mL Erlenmeyer flasks tightly sealed with rubber stoppers. The amount of ethylene produced after incubation was determined by gas chromatography and expressed as nmol of ethylene produced per g chlorophyll per hour. The strain *Anabaena sp*. PCC 7120, a filamentous, heterocyst-forming cyanobacterium with nitrogenase activity of 17.04 nmol C_2H_4 μg^{-1} chl h^{-1}, was used as a reference (Maqubela et al. 2010). EPS production was qualitatively assessed using the Indian ink staining technique. Cyanobacterial strains with large white zones around their cells were considered to have high EPS content compared with strains with smaller white zones around their cells (Maqubela et al. 2010). On the basis of N_2-fixing capability and EPS production, 17 strains were chosen, and only 2–3 strains from each country were used for the experiments (see Table 12.2).

The amount of N_2-fixation capacity and EPS production by cyanobacterial strains to be used as biofertilisers and bioconditioners is fundamental but not sufficient for selecting a suitable strain. It is also important to consider the capacity of soil colonisation by the strains in terms of biomass production. Selected strains exhibiting good performances of N_2 fixation and EPS production (when grown in the medium) could have problems when inoculated in soil. Therefore, an assessment of the interactions between soil and the strains (selected on the basis of high ability of N_2 fixation and EPS production) was carried out. The evaluation of the interactions between native soils and indigenous, inoculated, selected strains was carried out in the lab by measuring: microbial competition, water-stress resistance and growing attitude on soil. On the whole, 32 replicates for each soil were prepared, and half of them were previously sterilised before strains inoculation. Observations of the inoculated cyanobacterial growth and behaviour under different experimental conditions were carried out by visual, light microscope observation and image analyses. EPS production was also tested by light microscope observation after negative staining with Indian ink.

Table 12.2 Selected strains employed as inoculants for the experiments

Country	Soil	Strain
Tanzania	Mkindo	*Nostoc 9v*
	Mkindo	*Nostoc 9z*
	Mafiga	*Nostoc 11ii*
South Africa	Hertzog	*Nostoc 3g*
	Guquka	*Nostoc 7e*
Zimbabwe	Domboshawa	*Calothrix 16m*
	Chikwaka	*Scytonema 19c*
	Henderson	*Nostoc 3v*

The capacity of the selected strains to grow on soil was evaluated as percentage of soil surface coverage (image analysis) in inoculated Petri dishes on four replicates. Two different types of data were obtained: (*a*) qualitative data by visual analysis at each step of the experiment (15, 45 days) and (*b*) quantitative data by measuring the strain-biomass production by a combination of C/N elemental and image analysis for the same time intervals.

The determination of the optimal biomass inoculation rate (3 g m^{-2} of dry weight) was established in pilot experiments.

The experimental design was as follows:

Soil Treatments
- Inoculation with indigenous strains, 1 level (3 g m^{-2} of dw)
- Sterilisation plus inoculum
- Control

Tested on Two Water Conditions
- Continuously moist at 80 % of water-holding capacity (WHC)
- Wet and dry (WD) cycles

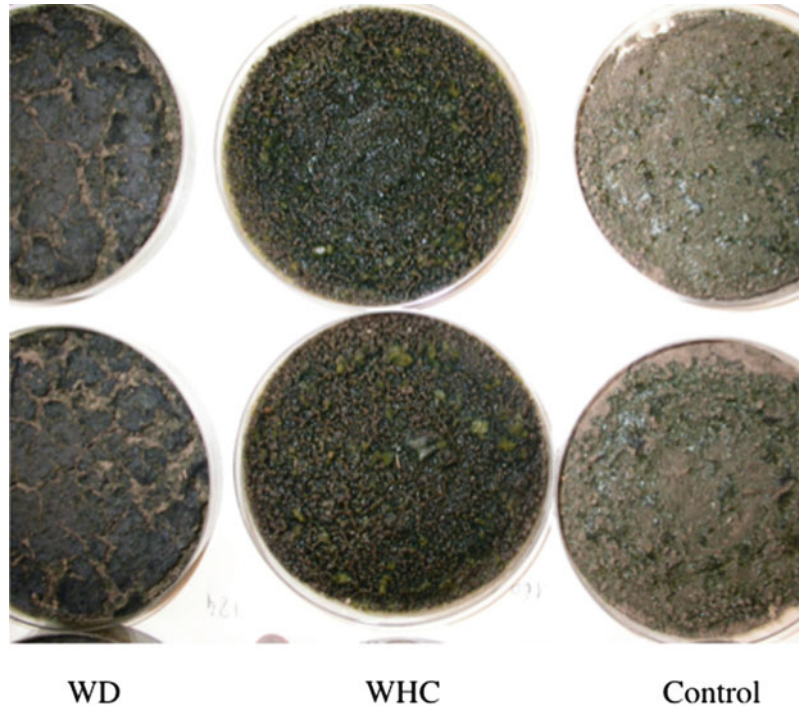

Fig. 12.1 Mkindo soil inoculated with *Nostoc* 9v after 45 days

Replications
– Four

Duration of Experiment
– 45 days

The soil samples were 2 mm sieved and distributed in Petri dishes in amounts of 40 g of dry soil per plate, and half of these Petri dishes were sterilised by twice autoclaving in order to assay the competitiveness of the inoculated strains. Non-inoculated plates were included as control. The strains were inoculated at the rate of 3 g of dry biomass per m^2 on the respective original soils. The incubation was performed under artificial lighting of 150 μmol photons m^{-2} s^{-1} with dark cycles (12:12) at about 28 °C for 45 days. The soils in the Petri dishes were maintained continuously moisted at 80 % of the water-holding capacity (WHC) by daily additions of water and monitoring of soil humidity or submitted to wetting and drying (WD) cycles (about every 2–3 days) for the whole period of the experiment, in order to evaluate the water-stress resistance (Fig. 12.1).

The indigenous selected strains *Nostoc* 9v and 9z isolated from Mkindo (Tanzania) and *Nostoc* 11ii isolated from Mafiga (Tanzania) were inoculated on their original soil. Guquka and Hertzog soils (South Africa) were inoculated with their indigenous *Nostoc* strains 7e and 3g, respectively. Domboshawa, Chikwaka and Henderson soils (Zimbabwe) were inoculated with their indigenous strains *Calothrix* 16m, *Scytonema* 19c and *Nostoc* 3v, respectively. Strain *Nostoc* 9v, found to be the best strain in the pilot experiment, was considered as a reference and inoculated not only on the soil of origin but also on the soils from South Africa (Hertzog) and Zimbabwe (Chikwaka) (Table 12.2). This strain possessed a good nitrogenase activity (about 14 nmol μg chl^{-1} h^{-1}), fairly comparable with the *Nostoc* PCC 7120 (17 nmol μg chl^{-1} h^{-1}) used as a reference.

Biomass production was measured by sampling a known surface area of cyanobacterial biomass developed on a Petri dish using a small metallic

cylinder with a cross section of 1 cm^2. Samples were dried at 105 °C and analysed by elemental C/N analyser. The obtained data were expressed on biomass basis by considering the relative C and N contents of the different freeze-dried strain biomasses separately measured.

Both isolated and selected strains were maintained in culture on agar slants and grown in liquid cultures under standard conditions in order to obtain sufficient biomass quantities to perform the planned laboratory experiments. The biomasses were grown in big flasks containing liquid $Bg11_0$ medium at 27 °C and cycle of light and dark, besides shaking and bubbling. About 50 % of the isolated cyanobacterial strains were re-plated in the presence of cycloheximide in order to obtain fungi-free cultures. Several techniques were applied to obtain many of the strains in axenic conditions.

12.4 In Situ Mass Production of Selected Indigenous Strains for Their Application in Greenhouse and Field Experiments

The massive biomass production of selected cyanobacterial strains was performed using a batch culturing technique in the laboratory and, successively, outdoors. Small samples of selected strains were first cultured in small flasks and then transferred to increasingly larger containers (50, 100, 250, 1000 and 2000 mL conical flasks, 25 L glass bottles and finally 200 L in outdoor ponds (motorised-paddled or masonry)) whenever the cyanobacteria dry matter (DM) concentration reached 1 g L^{-1}. Different solutions were adopted in different countries (Fig. 12.2a–d).

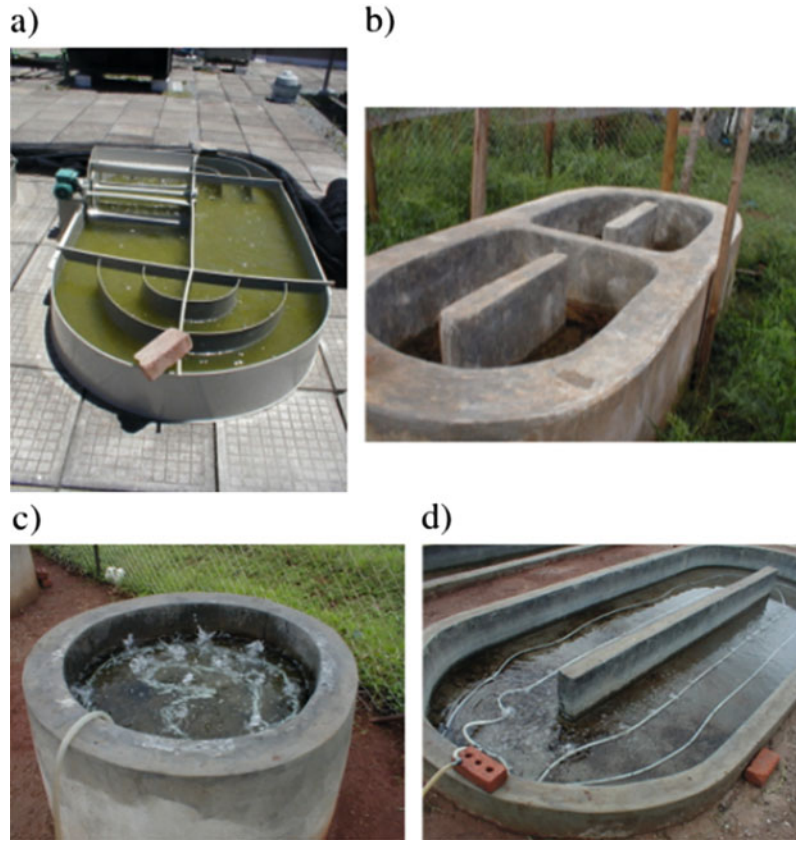

Fig. 12.2 Different solutions adopted in different countries: (**a**) South Africa, motor paddle; (**b**) Tanzania, masonry; (**c, d**) Zimbabwe, masonry, small and large pond

In South Africa it was possible to build a motorised-paddled pond to air the culture for speeding up and promoting the cyanobacteria growth.

In Tanzania and Zimbabwe, the outdoor ponds were built in masonry, and air to the culture was provided by a compressor where electricity was available; otherwise, air providing was performed manually. In Zimbabwe an intermediate small pond was used (Fig. 12.2c) to control and favour the conditions of culture transfer from the lab to outdoor.

12.5 Assessment of the Interactions Between Soils and Selected Strains

12.5.1 Lab Experiments

12.5.1.1 N_2 Fixation and EPS Production

A large morphotype diversity among the *Nostoc* population was observed and confirmed by other features like N_2-fixing activity and EPS secretion (Fig. 12.3a, b). *Nostoc* was found to be the best genus compared to others because it fixed a good amount of N_2 and produced large amount of EPSs. The nitrogenase activity reached values extraordinarily high in the *Nostoc* strains 7e and 9v (more than 16 and 14 nmol μg chl^{-1} h^{-1}, respectively) (Table 12.3). The EPS secreted by *Nostoc* diffused into the medium embedding the entire colony (Fig. 12.3a, b), whilst from *Scytonema* and *Calothrix* surrounded the trichome as a sleeve (Fig. 12.3c).

12.5.1.2 Light Microscope Observation of Cultures in Liquid Media

Qualitative composition of microbial population, determined by observing under light microscope (both the investigated soil samples and the soil enrichment cultures in liquid media) revealed an absolute prevalence of filamentous cyanobacteria, dominated, in order of abundance, by heterocystous (*Nostoc*, *Scytonema*, *Calothrix*, *Cylindrospermum*) and non-heterocystous (*Leptolyngbya*, *Microcoleus*, *Lyngbya*) genera. The enrichment of cultures illustrates the high occurrence of cyanobacteria and green microalgae; instead diatoms were scarcely represented. Some pictures by light microscopy of cyanobacteria in liquid culture enrichments are given in Fig. 12.4.

12.5.1.3 Enumeration of Cyanobacterial Soil Population

The enumeration of the cyanobacterial population present in the samples showed a remarkable abundance of these organisms. This aspect is important as it means that cyanobacteria are not only distributed forming association with biological crust (Belnap and Lange 2001; Garcia-Pichel et al. 2003), but they also can spread as free-living in top soils. Enumeration of cyanobacterial soil population estimated as MPN in liquid media, and those obtained from CFU counting in agarised media in Petri dishes, ranged from 10^2 to 10^6 and 10^2 to 10^5 per g of soil, respectively. The highest values were observed in the Tanzanian soil from Mkindo cultivated soil, where numerous heterocystous genera, mainly *Nostoc*, were present. Indeed, from Mkindo samples some strains of *Nostoc* were isolated. Strain 9v was characterised by a very good diazotrophic growth, nitrogenase activity (about 14 nmol μg chl^{-1} h^{-1} of ethylene produced) and by a large EPS secretion.

12.5.1.4 Light Microscope Observations of Inoculated Cyanobacteria

Observations of the inoculated cyanobacteria growth and behaviour under different experimental conditions were carried out in order to assess the characteristics of the strains and their interactions with the various soils. The remarks included visual, light microscopy observations of the growth on soil of the indigenous microflora and of the inoculated strains, microbial competition, behaviour under the two different water regimens and EPS production at each step of the experiment (after 15 and 45 days). The capacity of the selected strains to grow on soil was evaluated as percentage of soil surface covering on four replicates of inoculated Petri dishes.

The observation under light microscope of the Petri dishes allowed a deeper comprehension of

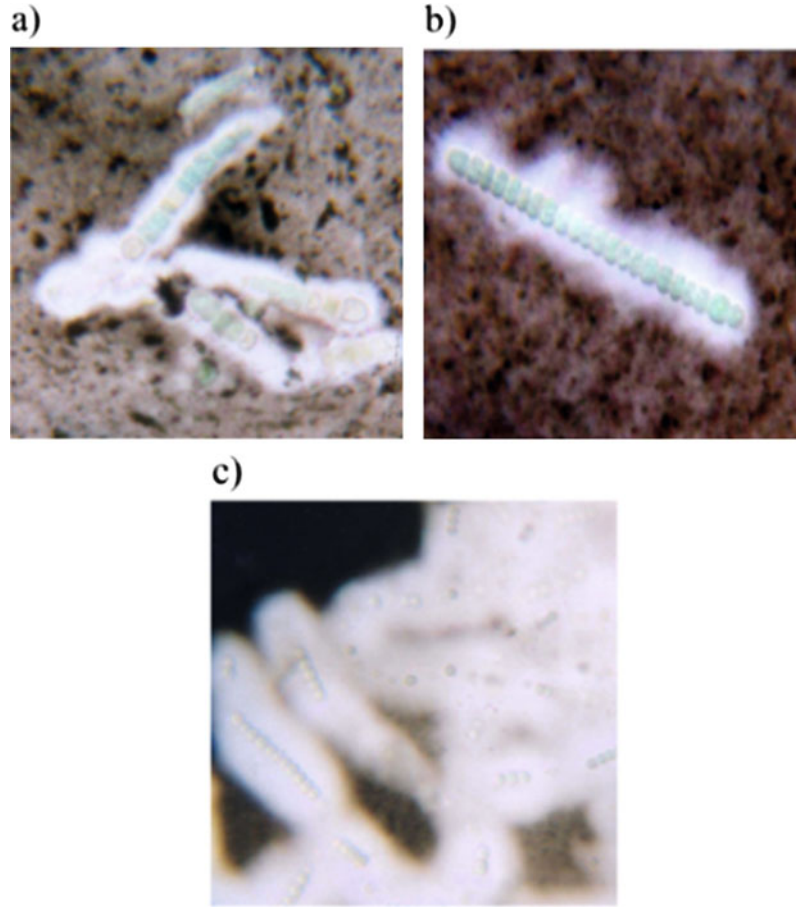

Fig. 12.3 Indian ink staining. White area represents EPS production; (**a**) *Nostoc* 3v from Guquka (South Africa); (**b**) *Nostoc* 9v from Mkindo (Tanzania); (**c**) *Calothrix* 16m from Domboshawa (Zimbabwe)

Table 12.3 Nitrogenase activity measured by the acetylene-reduction technique in $BG11_0$-grown cells (nmol μg chl^{-1} h^{-1})

Country	Strains	Fixed N_2 nmol μg chl^{-1} h^{-1}
Tanzania	*Nostoc* 9x	7.28
	Nostoc 9v	14.12
	Nostoc 9z	7.51
	Scytonema 11ii	4.95
South Africa	*Nostoc* 3v	1.33
	Nostoc 3g	4.71
	Nostoc 7e	16.08
Zimbabwe	*Calothrix* 16m	2.54
	Scytonema 19c	13.65

the interactions between strains and soil. The results are reported in succession for each country and inoculated strain (Table 12.2).

***Nostoc* 9v on Mkindo Soil (Tanzania)** Strain *Nostoc* 9v inoculated on the original Tanzanian soil (Mkindo) showed a massive and rapid development in both water regimen conditions (Fig. 12.5, Plate 5a). This would demonstrate a high resistance of 9v against water stress. The water regimen determined the aspect of the development on soil surface: formation of a thick, raised layer that appeared crusted under water-stress conditions (WD cycles) and mucous and shiny under continuously wet conditions. In WHC conditions *Nostoc* filaments appeared embedded in a large amount of EPS; cyanobacterial layer was continuous and entrapped bubbles of photosynthetically evolved oxygen. Under water-stress conditions, the EPS glued soil particles without discontinuity. *Nostoc* 9v in Mkindo showed a very good competitiveness against the indigenous

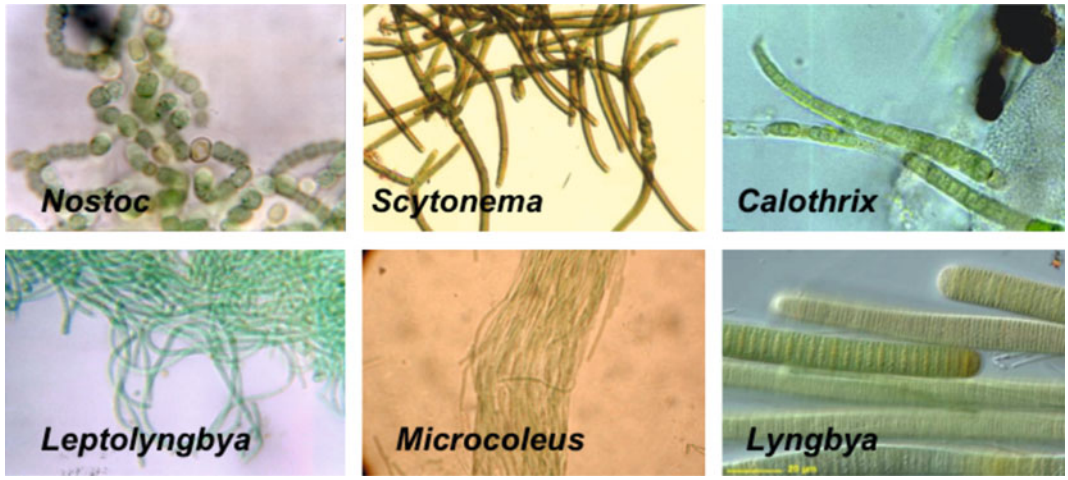

Fig. 12.4 Main strains occurring in the investigated soil samples (observed under light microscope)

microflora, which was relatively abundant and mainly represented by *Microcoleus*, *Leptolyngbya* and some green algae (*Chlamydomonas*, *Chlorococcum*) (Fig. 12.5, Plate 5b).

***Nostoc* 9z on Mkindo Soil (Tanzania)** *Nostoc* 9z displayed a very good and rapid development under continuously wet conditions, but a not equally good behaviour against water stress, and evidenced a good competitiveness against the indigenous microflora. The most common indigenous organisms were represented by green algae *Chlorococcum*, *Chlorosarcinopsis* and *Trebouxia* and by cyanobacteria (*Lyngbya* and *Microcoleus*). *Nostoc* 9z produced an abundant amount of EPS under WHC conditions forming a thick, raised layer. Under water-stress conditions, *Nostoc* 9z gave rise to a matted development, and the cyanobacterial layer was not uniform indicating a lesser degree of soil particle aggregation mainly due to filaments than to the limited EPS production.

***Nostoc* 11ii on Mafiga Soil (Tanzania)** *Nostoc* 11ii strain inoculated on Mafiga soil was characterised by small-sized cells and showed a limited development and a reduced presence of indigenous microflora. This strain formed a thin layer covering the soil surface better under WD than under WHC conditions and producing a large amount of EPS cementing soil particles. The mucous layer, formed under continuously wet conditions, often entrapped bubbles of photosynthetically evolved oxygen. Cyanobacteria (*Lyngbya*, *Leptolyngbya*, *Microcoleus* and *Nostoc*) and a few green algae (*Chlorococcum*, *Chlorosarcinopsis*) represented the most common indigenous organisms.

***Nostoc* 7e on Guquka Soil (South Africa)** Soil from Guquka inoculated by *Nostoc* 7e presented a scarce indigenous microflora under water-stress conditions and particularly relevant under WHC conditions leading to a reduced development of the inoculated 7e strain (Fig. 12.5, Plate 5c). Hence, 7e strain resulted to have a similar development under WD conditions especially on the sterilised soil. The biomass developed a thin layer that produced small amount of EPS uncapable to form aggregates with soil particles, and its distribution on Petri dishes was discontinuous. Among the indigenous microflora, a branched green alga (*Ulothrix*), *Chlamydomonas* and some cyanobacterial forms were particularly abundant. The reduction, after 45 days of incubation, of the development of the strain inoculated on sterilised soil, could have been due to a successive and massive growth of actinomycetes and fungi.

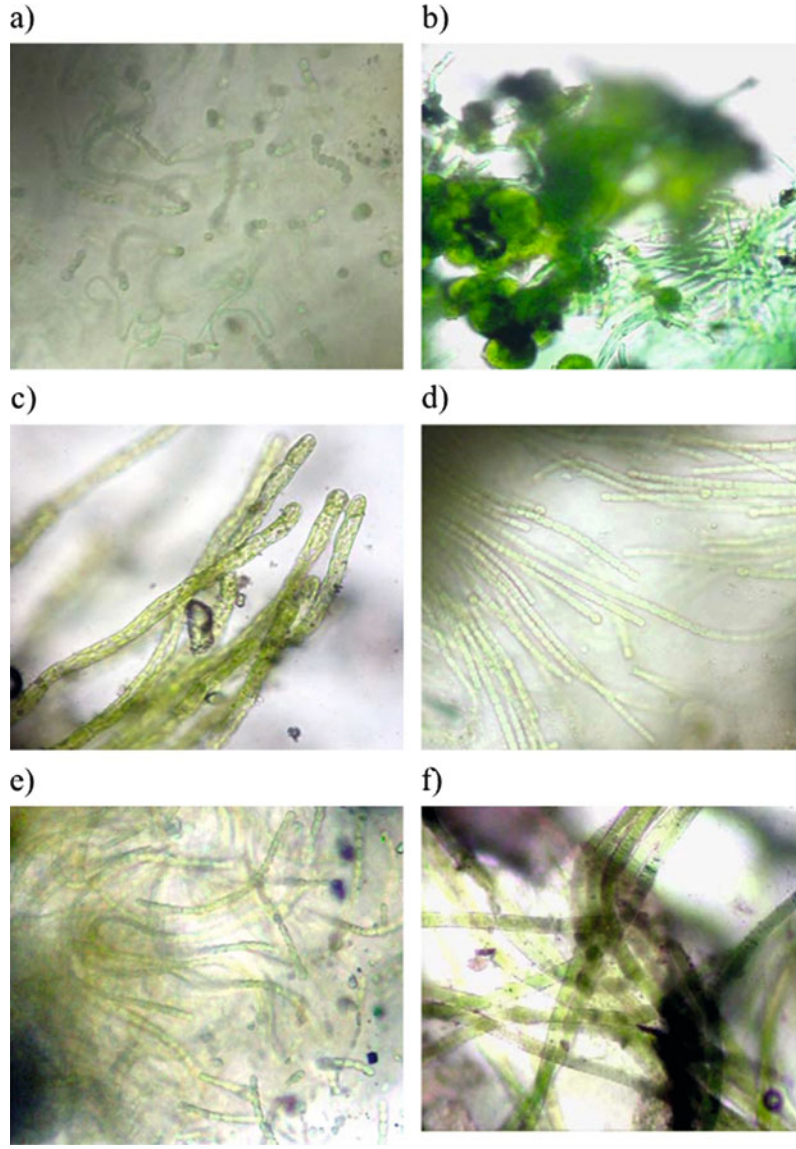

Fig. 12.5 *Plate* (**a**) *Nostoc* 9v inoculated on Mkindo unsterilised soil from Tanzania under WHC conditions. Note the presence of indigenous cyanobacteria (*Leptolyngbya*); *Plate* (**b**) indigenous microflora, green algae (*Chlorococcum*, *Trebouxia*) and cyanobacteria (*Leptolyngbya*, *Microcoleus*), developed on Mkindo soil from Tanzania under WD conditions; *Plate* (**c**) indigenous microflora represented by green algae (*Ulothrix*) developed on Guquka soil from South Africa under WHC conditions; *Plate* (**d**) *Nostoc* 9v inoculated on Hertzog unsterilised soil from South Africa under WHC conditions; *Plate* (**e**) *Nostoc* 9v inoculated on Chikwaka sterilised soil from Zimbabwe under WD conditions; *Plate* (**f**) indigenous microflora mainly represented by cyanobacteria (*Lyngbya*), developed on Chikwaka soil from Zimbabwe under WD conditions

***Nostoc* 9v on Hertzog Soil (South Africa)** *Nostoc* 9v strain inoculated on the Hertzog soil showed a behaviour similar to the one noticed in the soil of origin, Mkindo (Tanzania), notwithstanding the stark abundance of the indigenous microflora (Fig. 12.5, Plate 5d). *Nostoc* 9v grew very well and showed a good competitiveness and water-stress resistance, confirming the data obtained on the soil of origin. The strain also produced a big amount of EPS, mainly under WHC conditions, forming a thick, crusted, risen up and wrinkled layer sealing soil surface.

***Nostoc* 3g on Hertzog Soil (South Africa)** *Nostoc* 3g strain inoculated on its soil of origin (Hertzog) evidenced a good competitiveness against the indigenous microflora, displaying a massive and rapid development notwithstanding the high presence of the indigenous microflora, mainly represented by a *Nostoc* sp., morphologically different from the inoculated strain. Moreover, *Nostoc* 3g showed a rapid, dense and thick development of biomass in both conditions of water regimen, with a very massive production of EPS. It formed a continuous, mucous layer

under WHC and a crusted and discontinuous layer under WD conditions.

***Calothrix* 16m on Domboshawa Soil (Zimbabwe)** The growth of the *Calothrix* 16m strain on its soil of origin (Domboshawa) was scarce and markedly influenced by water conditions. Under WHC conditions the development of biomass was scarce probably due to a rapid development of green algae, being the majority of the indigenous microflora. The growing of the green algae was favoured by the high humidity and prevailed over the inoculated 16m strain. *Calothrix* 16m strain did not produce EPS.

***Nostoc* 9v on Chikwaka Soil (Zimbabwe)** The behaviour of *Nostoc* 9v strain isolated from Mkindo (Tanzania) was similar to that obtained on the original soil (Fig. 12.5, Plate 5e). The strain 9v showed a good development, competitiveness and high EPS production.

***Scytonema* 19c on Chikwaka Soil (Zimbabwe)** *Scytonema* 19c strain, isolated from Chikwaka soil, showed a high degree of soil colonisation under WD condition and efficiency and competitiveness against indigenous microflora mostly represented by filamentous cyanobacteria (*Lyngbya*, *Phormidium*, *Plectonema*) (Fig. 12.5, Plate 5f). *Scytonema* 19c formed (on Petri dishes) a thick mat with densely entangled, continuous and raised filaments. Moreover, 19c strain was characterised by high resistance to water-stress conditions.

***Nostoc* 3v on Henderson Soil (Zimbabwe)** *Nostoc* 3v strain, isolated from Henderson soil, evidenced a massive and rapid development notwithstanding the high presence of the indigenous microflora. The last was represented by a *Nostoc* sp., morphologically different from the inoculated 3v strain, and by *Microcoleus* and other filamentous cyanobacteria. *Nostoc* 3v showed, in both conditions of water regimen, a rapid, dense and thick development of biomass, with a very massive production of EPS with high capacity to bind soil particles. *Nostoc* 3v developed a continuous, mucous layer under WHC conditions and a crusted and discontinuous layer under WD conditions.

In regard to the adaptation capacity of the selected strains to growth on soil, *Nostoc* 9v strain confirmed to be the best among all tested soils, especially when inoculated on the soil of origin (Mkindo). A very high capacity of covering was also shown by the *Nostoc* 3v isolated from Henderson (Zimbabwe); on the contrary, strains *Nostoc* 3g from Hertzog (South Africa) and *Scytonema* 19c from Chikwaka (Zimbabwe) showed only a good colonising capacity. All these strains were also characterised by a very fast growth, being able to cover almost the whole soil surface in about 2 weeks.

The other two *Nostoc* strains, 9z from Mkindo (Tanzania) and 7e from Guquka (South Africa), showed a satisfactory colonising capacity, higher than that of *Calothrix* 16m from Domboshawa (Zimbabwe) and *Nostoc* 11ii from Mafiga (Tanzania).

The response of the strains to the experimental water regimens, i.e. WHC and WD, indicated, as expected, a better growth in high humidity conditions for all strains. However, *Nostoc* 3g from Hertzog (South Africa) showed a good water-stress resistance growing well both under WD and WHC conditions.

The water conditions markedly influenced the appearance and consistency of the cyanobacterial biomass development. When soil samples were subjected to WHC conditions, the development of cyanobacteria formed mucilaginous, thick, sometime raised, mucous and shiny layer, because of the abundance of secreted EPS with bubbles due to the entrapment of the produced oxygen. When soil samples were subjected to WD, cyanobacterial development assumed a crust-like aspect.

In regard to competitiveness degree of the selected strains, *Nostoc* 3g, 3v and 9v were the most effective.

The most frequent groups and genera of the indigenous microflora were represented by cyanobacteria as *Nostoc*, *Microcoleus*, *Lyngbya*, *Leptolyngbya*, and chlorophyta as

Chlamydomonas, *Chlorella*, *Chlorococcum*, *Chlorosarcinopsis* and *Trebouxia*. *Nostoc* and *Leptolyngbya* resulted to be more prevalent.

The highest amounts of EPS were secreted under WHC conditions, especially by the *Nostoc* strains 9v, 3v and 3g. The produced EPS promoted the soil particles aggregation. In the case of *Scytonema* 19c, the aggregation was essentially due to the dense entanglement of filaments; instead, for the other strains, the aggregation was due to the gluing effect of EPS.

12.5.1.5 Image Analysis: Quantitative Data

The quantitative analysis confirmed substantially the findings previously described. Data of biomass production, expressed in g m^{-2}, were obtained by image analysis combined with analytical C/N data. In Fig. 12.6a, b, the response of the inoculated strains to a water regimen of WHC and to WD cycles, simulating the stress conditions of the natural environment after 15 and 45 days, is shown. As expected, the larger production of biomass was measured in wet conditions for all strains. Only *Nostoc* 3g strain showed a comparable production also in WD conditions, especially in the first 15 days, in spite of the stress constraints. Domboshawa 16 m strain exhibited a very good resistance because the biomass yield did not improve by increased humidity and with time. However, its biomass production was low. In some cases the production of biomass after 45 days was less than after 15 days; this could be explained by the presence of heterotrophic microorganisms and mineralisation processes. In Fig. 12.6c, d the productions of microbial biomass of the different strains inoculated after sterilisation for different soils and different water regimens at the end of the experiment are shown. The biomass growth in sterilised conditions was considered as maximal production without any competition linked to the indigenous microbial population.

The interactions of indigenous microbial activity on the strain could also favour both the

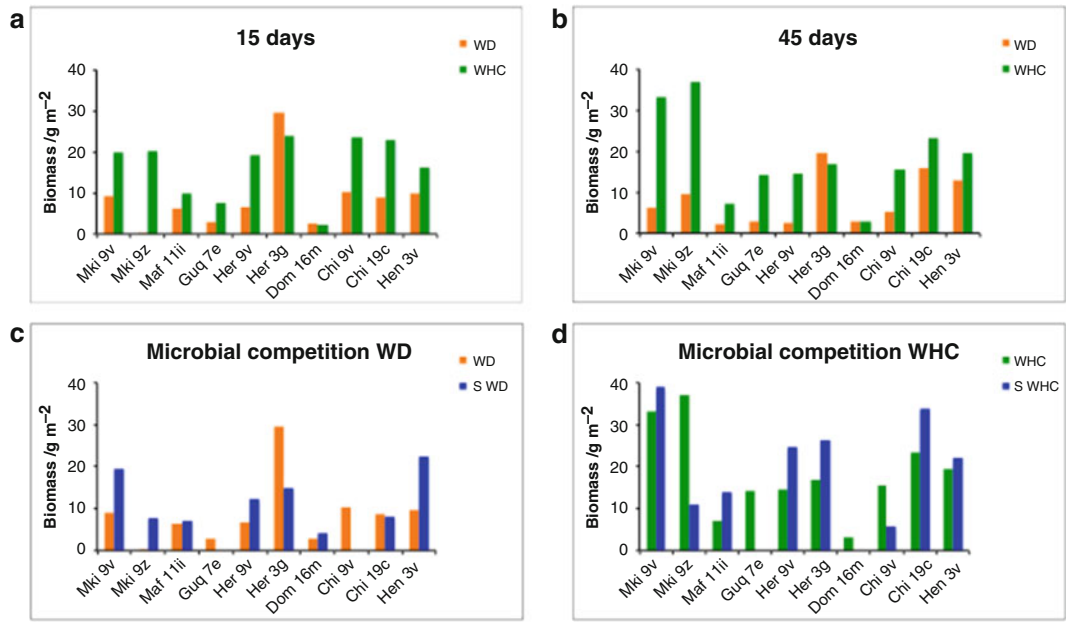

Fig. 12.6 Interactions between inoculated strains, at different soil humidity regimen, and soil local microbial populations measured in terms of microbial biomass production: (**a**, **b**) biomass production in inoculated soil samples after 15 and 45 days at WD and WHC regimen, respectively; (**c**, **d**) effect of microbial competition on biomass production at the end of experiment (after 45 days) in inoculated soil samples (WD and WHC) at WD and WHC regimen, respectively, and inoculated after sterilisation (S) soil samples (S WD and S WHC) at WD and WHC regimen, respectively

growth of the inoculated cyanobacteria and the local population promoting a synergic action. However, as confirmed by the optical microscope analysis, the presence of inoculated strain was predominant in all samples, also when the contamination of indigenous population was substantial (i.e. Guquka 7e in WHC conditions (Fig. 12.5, Plate c) etc.).

From Fig. 12.6c, it can be observed that Hertzog 3g's indigenous microbial population, in natural conditions (WD), had significant synergic effect in promoting the biomass growth. A similar effect, but to a lesser extent, was found for Chikwaka 9v and 19c. As regards the WHC conditions (Fig. 12.6d), Mkindo 9z's indigenous microbial population had a significant synergic effect in promoting the biomass growth. Also Chikwaka 9v exhibited this effect but to a lesser extent. Mkindo 9v strain displayed a high competiveness (similar biomass production of the respective sterilised sample) and a large amount of biomass production both at different humidity and time conditions, as well as Henderson 3v and Chikwaka 19c but to a lesser extent. When strain 9v was inoculated on other soils, as in the case of Hertzog 9v, a good competiveness was observed as for Mkindo 9v; differently, in the case of Chikwaka 9v, a synergic effect in promoting biomass growth for both humidity regimens was evidenced. However, their biomasses production was relatively lower.

The reference strain *Nostoc* 9v isolated from Mkindo soil (Tanzania) confirmed to possess a very good capacity to colonise all tested soil types. It also exhibited a high competitiveness against the indigenous microflora. The suitability of the genus *Nostoc* for soil inoculation was also evidenced by the performances of the other tested *Nostoc* strains. The property of development in soil, coupled to EPS production and N_2-fixing capacities, should ensure a successful introduction in soils of the cyanobacterial biomass both as soil fertiliser and conditioner.

12.5.1.6 Effects on Soil Physical Properties: Microstructure, Soil Structural Stability

The impact of inoculation of cyanobacteria *Nostoc* 9v strain on physical characteristics of poorly aggregated soil from Guquka was investigated in the lab by Malam Issa et al. (2007). In this study the improvement of the soil physical quality was demonstrated to occur few weeks after cyanobacteria inoculation. A dense superficial network of cyanobacterial filaments and extracellular polymer secretions (EPS) covered soil aggregate surface after 4–6 weeks of incubation, and organo-mineral aggregates comprising cyanobacterial filaments and EPS were observed (Fig. 12.7a, b).

The results of aggregate breakdown tests showed no significant difference between non-inoculated samples after 6 weeks, whilst they revealed improvement of aggregate stability for inoculated samples. An increase in aggregate stability by two to four times compared to that of non-inoculated samples (6 weeks following inoculation) was reported (Malam et al. 2007). The improvement of aggregate stability appeared after 2 weeks following inoculation and increased gradually with time and cyanobacterial

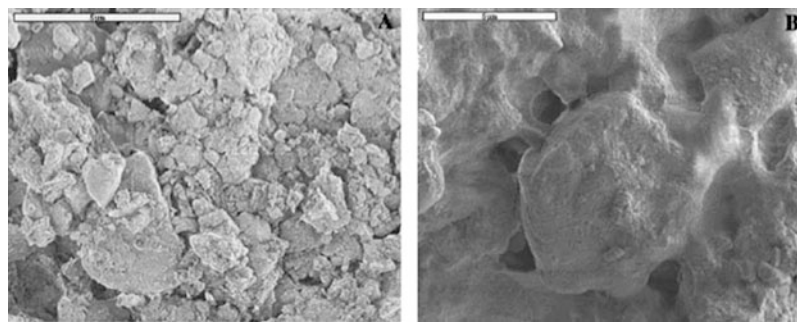

Fig. 12.7 SEM images. Microstructure and soil aggregate stability of Guquka soil aggregates inoculated by *Nostoc* 9v; (**a**) surface of non-inoculated sample; (**b**) surface of an inoculated sample. *Scale bar*, 5 μm (Source: Malam Issa et al. 2007)

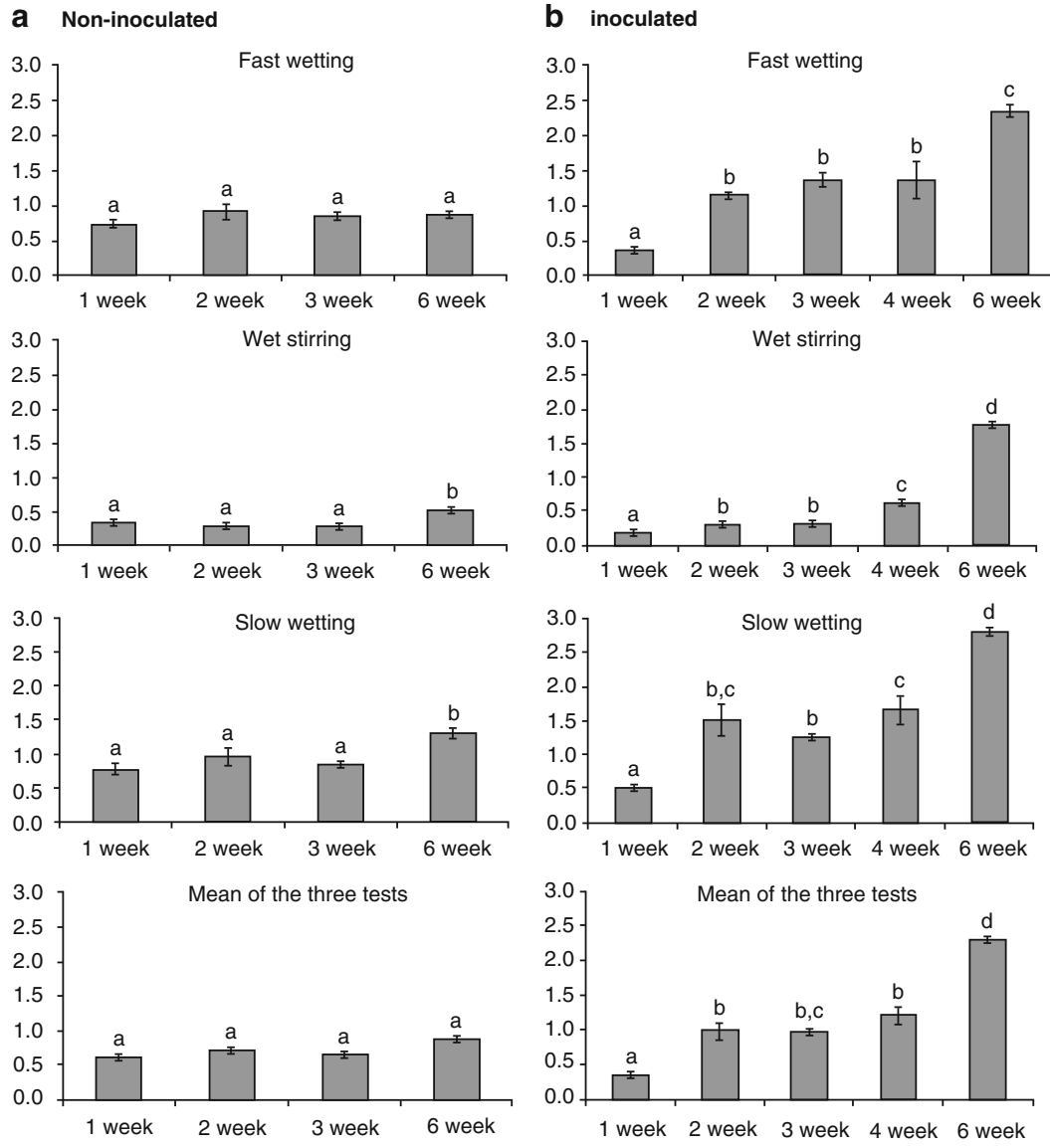

Fig. 12.8 Soil aggregate stability test. Mean weight diameter of (**a**) non-inoculated samples incubated for 1, 2, 3 and 6 weeks and (**b**) inoculated samples incubated for 1, 2, 3 and 6 weeks (Source: Malam Issa et al. 2007)

growth (Fig. 12.8). The increase of aggregate stability was related to the changes induced in micromorphological characteristics by cyanobacterial filaments and EPS that promoted the coating, enmeshment, binding and gluing of aggregates and isolated mineral particles.

12.5.1.7 Effect on Chemical Properties: Soil Organic Matter Content and Quality

The effect of cyanobacteria *Nostoc* 9v strain on the concentration and composition of soil OM of the degraded soils was studied by Pardo

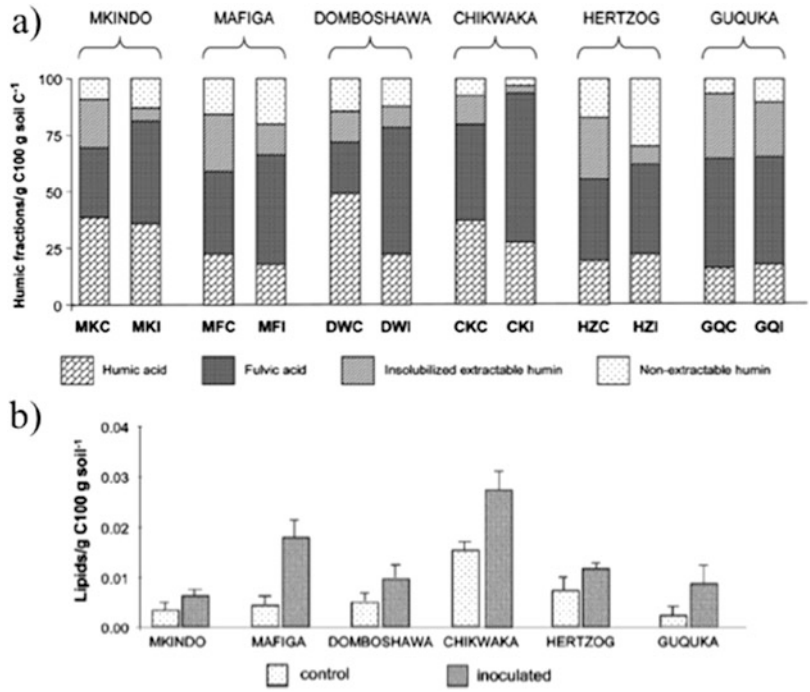

Fig. 12.9 Effects of cyanobacteria inoculation on soil OM: (**a**) changes in the humic fractions of the control (C) and cyanobacteria inoculated (I) soils; (**b**) lipid content in the control (C) and cyanobacteria inoculated soils (I) (Source Pardo et al. 2010)

et al. (2010). They studied, under laboratory conditions, the effects of *Nostoc* 9v on the soil OM content and quality of different soils from Tanzania, Zimbabwe and South Africa. Soils were inoculated with *Nostoc* 9v and incubated for 3 months, and moisture content was maintained at 60 % of field capacity. *Nostoc* 9v proliferated and colonised the surfaces of all soils very quickly. The results obtained showed significant changes in the quantitative and qualitative characteristics of the soil OM due to cyanobacterial growth (Fig. 12.9a). Cyanobacterial growth resulted in an increase of OC from 0.4 g C kg^{-1} soil to 9.0 kg^{-1} and also affected soil OM characteristics through the incorporation of free, extractable or particulate biomass with a predominantly aliphatic characteristics. Important descriptors of the extent to which cyanobacterial metabolism modified the characteristics of the native soil OM were the changes in the observed amounts of the two humin types (extractable and non-extractable), the increase in lipid concentration (Fig. 12.9b) and the changes in the optical density of the HAs. These results proved the feasibility of improving soil OM content and quality by cyanobacteria-based remediation practices. This fact would be important especially in developing countries where inorganic fertilisers are not commonly used or available.

12.5.2 Greenhouse Experiments

In this and the next subchapter, some examples of application of biomasses to soil at different scales (greenhouse and field plots) are reported in order to describe the effects of cyanobacteria inoculation on overall soil fertility.

A study to assess the potential use of cyanobacteria for the enhancement of the structure and fertility of the two degraded arable Hertzog and Guquka soils was carried out by Maqubela et al. (2009) under tunnel house conditions. The effect of inoculation of *Nostoc* 9v strain, on soil C, soil N, mineral N and EPS content and on soil structural stability, and the interactive effect of inoculation of these strains and maize cropping on the above-mentioned soil characteristics were investigated.

The *Nostoc* inoculum, uniformly applied over potted Guquka and Hertzog soils soon after

maize germination, established well and improved significantly C, N and EPS contents of the two soils. The highest contents of soil C, soil N and mineral N, however, were found in non-cropped *Nostoc* inoculated soils. This suggests that the cyanobacteria-fixed N in soil was uptaken by maize to improve growth. The *Nostoc* inoculation increased maize dry matter yields by 49 % in Hertzog and 40 % in Guquka soils. Corresponding increases in maize tissue N were 23 % and 14 %, respectively. The aggregate stability of the two soils was also improved as a result of increased production of EPS and soil C in the *Nostoc* inoculated soils. Inoculated soils cropped with maize displayed a lower proportion of stable aggregates presumably due to their low soil C and EPS contents compared to non-cropped soils. Scanning electron microscopy revealed that soil particles and fragments of non-cropped inoculated soils exhibited coatings of extracellular polymeric substances (EPS) with other particles enmeshed in networks of filaments, whilst, by contrast, little or no EPS and/or filaments were observed on cropped and/or non-inoculated soils. Another greenhouse study by Maqubela et al. (2010) on biofertilisation and/or bioconditioning effects in potted crop (maize) soils, as a result of cyanobacteria ability to fix N_2 or produce EPS, was carried out on samples from South African soils of Guquka, Hertzog and Qunu villages and Fort Cox College. The results showed the ability of selected strains (3g, 3v and 7e) to influence maize DM and soil C and N contents. Inoculation with cyanobacteria strains increased maize DM and N uptake significantly, on par with the reference strain. These increases were consistent with increases in nitrate-N observed at harvest time in inoculated cropped and non-cropped soils. Strain 7e exhibited larger increases in soil nitrate-N, tissue N and uptake than strain 3g, possibly because of its higher ability to fix N_2. Cropping with maize reduced soil total C and N, possibly owing to its negative effects on cyanobacteria establishment.

A trial pot study in a greenhouse in Tanzania (D'Acqui et al. 2004, 2006) was carried out at Sokoine University of Agriculture (SUA), Morogoro, to assess the effect of cyanobacteria

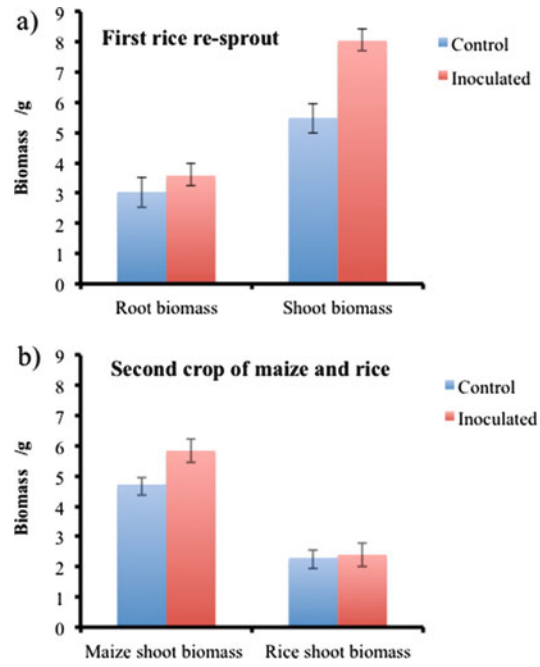

Fig. 12.10 Effects of soil cyanobacteria inoculation on crop growth: (**a**) effect of inoculation on first rice resprout and (**b**) residual effect of inoculation on shoot biomass of second crop of maize and rice

inoculation on maize and rice crop biomass production. The Mkindo 9v strain was inoculated on soil samples (packed into 4 L pots) from Mkindo and Mafiga soils. The results showed little effect of the cyanobacteria inoculation on biomass production of first maize and rice crops. Instead, in a second maize crop (planted after the first has been harvested without any additional inoculation), the shoot biomass production was significantly higher ($p < 0.005$) for inoculated soil samples with respect to non-inoculated ones. Similarly the rice resprout crop, i.e. the regrowth of first rice crop (rootstocks, remaining after the harvesting of the shoot, were allowed to sprout and grow for 6 weeks) exhibited a significant increase of the biomass (Fig. 12.10a, b). These findings indicated a residual effect in soil of the biofertilisation, as evidenced by an increase in crop biomass production.

This effect disappeared for the third maize crop and second rice resprout (D'Acqui et al. 2004, 2006). Greenhouse experiments with rice and maize indicated that inoculation

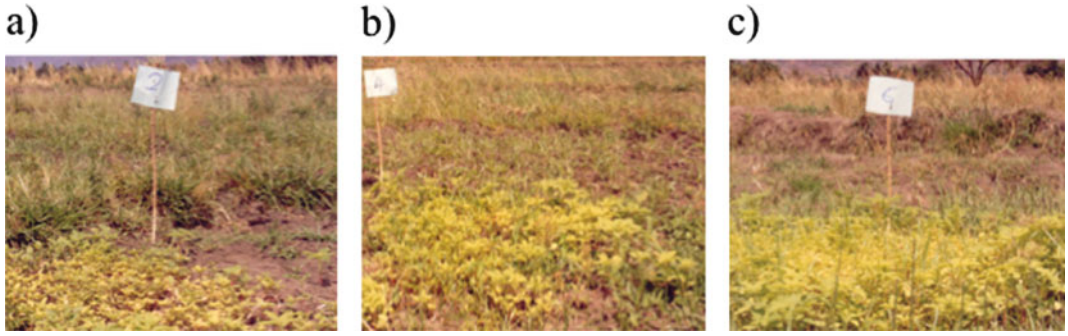

Fig. 12.11 Field experiment carried out on *Amaranthus* spp.: (**a**) cropped without inoculation, (**b**) cropped plus inoculation 3 g^{-2} cyanobacteria (**c**) cropped plus inoculation 6 g m^{-2} cyanobacteria (Photo S. Maliondo)

with cyanobacteria had limited effect on the first crop, but the yield of the subsequent crop was significantly increased. The effect of cyanobacteria on crop growth varied with soil types, and this was related to different extents of cyanobacteria establishment. In fact, soils differed in the ability to support biomass production in the pots with the Mafiga soil producing significantly more biomass (9.96 ± 1.30) than Mkindo soil (5.04 ± 1.97).

12.5.3 Field Experiments

A field experiment was performed in Mkindo area to evaluate the residual effect of cyanobacteria inoculation on crop (*Amaranthus* spp.) in the field at small-scale (5 × 5 m^2) plots in complete randomised block design with four replications (D'Acqui et al. 2004, 2006).

The small plots were initially inoculated with two different rate of indigenous *Nostoc* 9v (3 g m^{-2} and 6 g m^{-2}) and cropped with rice. After harvesting, plots were cleared of vegetation and *Amaranthus* spp. crop replanted. The production of biomass observed in the different plots was higher in the plots earlier inoculated than in the not-inoculated ones, and the highest biomass production was related to the highest level (6 g m^{-2}) of inoculation (Fig. 12.11). Unfortunately, results of this study were only indicative because of different problems encountered during the experiment (i.e. flooding, illegal harvesting, etc.), some data were missing and as a consequence the statistic treatment of available data was not possible.

Field experiments, in spite of the serious problems encountered, as previously explained, confirmed the positive results of greenhouse tests. In fact, this field experiment carried out for *Amaranthus* spp., a vegetable commonly eaten in these countries, showed the beneficial effect of soil cyanobacteria inoculation on improving crop yield. Moreover, field experiments confirmed that main favourable effects on the crops are transferred to a successive crop as found for trials in greenhouse as well.

12.6 Conclusions

Study revealed that a huge number of different indigenous cyanobacteria strains already adapted to their environment could be identified in soils of semiarid environments in East and Southern Africa. This meant that cyanobacteria were not only distributed forming association with biological crust, but they also could spread as free-living cyanobacteria in top soils. A large number of these strains exhibited high capacity of N_2 fixation and produced considerable amounts of EPS, thus being able to improve soil fertility.

A number of the identified strains were found to compete favourably against the other microorganisms in soil, to maintain their high capability to fix N_2 and to produce EPS under drought conditions.

In the scientific community, there was only a very general awareness of cyanobacteria presence in soils of these African countries, but no attempts, up to now, were made to isolate and purify, by appropriate procedures and techniques, the most productive strains (able to fix N_2 and produce EPS) for reducing soil degradation and for a sustainable improvement of overall soil fertility.

Results of the laboratory, greenhouse and field experiments demonstrated that cyanobacteria were capable to grow well even under limiting conditions (soil with low nutrients availability and low pH). In spite of the cyanobacteria activity which decreased the mineral N concentration in soils (as they used these forms of N to grow), the overall soil C and N content increased. Cyanobacteria inoculation modified the soil OM characteristics and increased the more stable humic fractions. Moreover, an important increase in lipid contents was observed. Cyanobacteria inoculation also increased the aggregate's stability of poorly structured soils. The improvement of aggregate's stability appeared shortly after inoculation and increased gradually with time and cyanobacteria growth. The improvement of stability was accompanied by changes of soils surface microstructure and formation of organo-mineral aggregates. The reference strain *Nostoc* 9v isolated from Mkindo soil (Tanzania) confirmed to possess a very good capacity to colonise all tested soil types. It also showed a high competitiveness against the indigenous microflora and capacity of soil particles aggregation owing to the copious EPS secretion. Moreover, in conditions of humidity, Mkindo 9v resulted to have the highest competitiveness. In natural conditions, simulated by wetting and dry cycles, Hertzog 3g, Chikwaka 19c and Henderson 3v strains showed good yield, demonstrating resistance to stress conditions.

The suitability of the genus *Nostoc* for soil inoculation was also evidenced by the performances of the other tested *Nostoc* strains. The property of development in soil, coupled to EPS production and N_2-fixing capacities, ensures a successful introduction in soils of the cyanobacterial biomass, both as soil fertiliser and conditioner.

In spite of the need of further medium and long-term experiments for finally confirming the relevance and persistence of the beneficial effects, on the basis of the achieved results, the application of selected cyanobacterial strains seemed to be a very promising tool for a sustainable improvement of fertility and productivity of degraded soils in semiarid tropics. Moreover, taking into account the possible synergic effects of indigenous microbial population on the inoculated strain, the effects of an inoculum constituted not only by one but by more strains suitable to grow together for obtaining a possibly more efficient biofertiliser should be investigated.

These results suggest that by using cyanobacterial soil conditioners, the yield and nutritional value of food crops could be enhanced without using costly fertilisers. Therefore, development of low-cost techniques for screening and culturing of suitable cyanobacteria to be used in countries where crop yields are low in marginal lands and fertilisers are cost-prohibitive is necessary.

Acknowledgements This work is based on results from CYANOSOILS project funded by European Union, contract no. ICA4-CT-2001-10058. The author gratefully acknowledges Dr L. Tomaselli for optical microscopy observations, Prof. L. Calamai and Dr B. Pushparaj for the revision of the manuscript and all Partners who took part in the CYANOSOILS project.

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Part III

Present Scenario and Future

The Contribution of Secondary Metabolites in the Success of Bioformulations

13

María A. Morel, Célica Cagide, and Susana Castro-Sowinski

Contents

Abstract

Bioformulations are biologically active products containing one or more beneficial microbial strains, commonly used to increase plant growth, soil fertility and suppression of phytopathogens. Many reports support that the use of a combination of beneficial microorganisms has additive or synergistic effects on plant growth and yield. However, the addition of microbial- or plant-produced secondary metabolites to bioformulations may increase agricultural productivity, improving the performance of the inoculation. This chapter focuses on the use of some secondary metabolites (flavonoids, lipochitooligosaccharides, phytohormones, etc.) in bioformulations, focusing mainly on formulations that improve leguminous crop yields. The information supports that the addition of these molecules may contribute to the sustainable development of new agronomic products.

13.1 Introduction: Use of Beneficial Microbes as Bioformulations

Bioformulations are biologically active products containing one or more beneficial microbial strains, embedded in an economical carrier material, that are used to increase plant growth, soil

M.A. Morel • C. Cagide
Unidad Microbiología Molecular, Instituto de Investigaciones Biológicas Clemente Estable, Av. Italia 3318, Montevideo, Uruguay

S. Castro-Sowinski (✉)
Unidad Microbiología Molecular, Instituto de Investigaciones Biológicas Clemente Estable, Av. Italia 3318, Montevideo, Uruguay

Sección Bioquímica y Biología Molecular, Facultad de Ciencias, Universidad de la República, Igua 4225, Montevideo, Uruguay
e-mail: s.castro.sow@gmail.com; scs@fcien.edu.uy

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_13

fertility, and suppression of phytopathogens (Arora et al. 2010).

The carrier is an abiotic substrate that should deliver viable cells in good physiological conditions and must provide them a protective niche. Different materials can be used as carriers, for example, soils (peat, coal, clays, among others), waste plant materials, inert materials (polymers, vermiculite, perlite), or liquids (Bashan et al. 2014). Most of the investigation done in the field of improving bioformulations has focused on: 1) the improvement of the carrier properties; 2) the search of microbes with better performance on plant yield and; 3) the improvement of the metabolic state of these cells and their capability to use intercellular storage material for survival within the carrier itself (Kadouri et al. 2005).

Due to their conditions of production and storage, bioformulations are stressful environments where bacterial cells must survive many situations, such as desiccation and possibly hot conditions (Kadouri et al. 2005). Bacteria should maintain high survival rates and the ability to improve plant growth during a long period. In order to survive, bacteria use different strategies, such as the production and accumulation of osmolytes or polyhydroxyl alkanoates (PHA). Osmo-adapted microbes that accumulate osmolytes, like trehalose or glycine–betaine, show a higher tolerance to desiccation than non-osmo-adapted cells and significantly improve their plant growth performance (Bonaterra et al. 2005). Cells with higher PHA content can survive longer than those with lower amounts, because PHA provide the cells with the ability to endure a variety of harmful physical and chemical stresses (Kadouri et al. 2005; Morel et al. 2012).

The best-known microbe–plant mutualistic interaction is the diazotrophic microbial association with plants. Diazotrophs are free-living or symbiotic bacteria that fix and reduce atmospheric nitrogen to ammonia (a process known as biological nitrogen fixation; BNF). Examples of diazotrophs are *Azotobacter* (free-living diazotroph), *Azospirillum* (associative symbiont), *Azoarcus* and *Gluconacetobacter diazotrophicus* (endophytic non-nodular symbionts), and rhizobia (nodular symbionts). Some diazotrophs, and other plant growth-promoting bacteria (PGPB), also produce phytohormones, iron-sequestering siderophores, phosphate-solubilizing molecules, and/or 1-aminocyclopropane-1-carboxylate (ACC) deaminase, among other compounds. Examples of non-diazotrophic PGPB are *Pseudomonas* and *Bacillus* (Morel and Castro-Sowinski 2013).

The legume–rhizobia association is the most studied symbiotic microbe–plant interaction and, together with plant–mycorrhizal fungi interactions, is recognized for its importance in sustaining agricultural ecosystems and productivity. During the plant–rhizobia interaction, a complex network of molecular events leads to the nodule formation and finally BNF. The morphological and physiological alterations that conclude in the formation of root nodules involve many molecules such as plant flavonoids and bacterial nodulation factors, chemically identified as lipochitooligosaccharides (LCOs), among others (Morel and Castro-Sowinski 2013).

Much evidence supports that the use of a combination of beneficial microorganisms, showing different mechanisms of plant growth promotion, has additive or synergistic effects on plant growth and yield (Morel et al. 2012). However, new evidence also support that the addition of microbial- or plant-produced secondary metabolites to bioformulations may increase agricultural productivity, improving the performance of the inoculation (Morel et al. 2015). This chapter collects information about the use of some secondary metabolites in both bioformulations and formulations (that do not contain microorganisms), focusing our work mainly on bioformulations that improve leguminous crop yield.

13.2 Developing Improved Bioformulations: Future Design and Considerations

The application of PGPB in agricultural systems enhances plant growth, development, and yield of many crops. Their effect has been studied in several field crops (cereals and legumes) in both

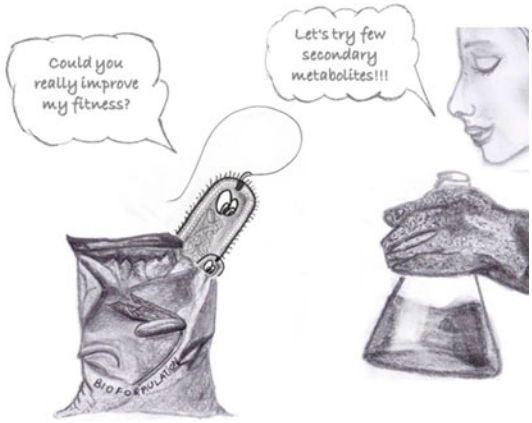

Fig. 13.1 How to improve the inoculation efficiency? Industrial and academic collaboration

extensive and intensive farming conditions for many years. For example, bacteria of the genus *Azospirillum* and rhizobia have been studied for their ability to promote plant growth via the production of phytohormones (as indole-3-acetic acid (IAA) and cytokinins) and/or to fix atmospheric nitrogen (Morel et al. 2012).

During the establishment of the legume–rhizobia association, root colonization by rhizobia is accompanied by important changes in root architecture and plant gene expression, which lead to the nitrogen-fixing phenotype. Even if the compatible combination of rhizobia and plants (each rhizobial strain establishes a symbiosis with only a limited set of host plants and *vice versa*) is used in the field, different reasons may lead to nodulation failure. Thereby, it is extremely important to improve the performance of the inoculation efficiency in different ways, other than the use of some agricultural practices that have economic and environmental negative effects on soils (Bajsa et al. 2013) (Fig. 13.1). This section summarizes the current knowledge about the addition of secondary metabolites in the performance of formulations and bioformulations.

13.2.1 Effect of Flavonoids

Flavonoids are important plant-to-bacteria signal molecules involved in the legume–rhizobia symbiotic behavior. They have been proposed as the first molecular signals exchanged between both host plant and symbiont. These root-released molecules interact with the rhizobial NodD protein, triggering the coordinated expression of a series of bacterial nodulation (*nod*) genes. The *nod* genes are responsible for the biosynthesis of LCOs known as Nod factors, which elicit multiple responses required for the nodulation of the appropriate host plants (Morel and Castro-Sowinski 2013). In addition to flavonoids, other root-exuded compounds (such as betaine and aldonic acids) may act as signal molecules that influence the ability of rhizobia to colonize the roots, thus affecting the symbiotic interactions with the plant (Janczarek et al. 2015). Thereby, the potential use of these *nod* gene-inducing compounds (flavonoids and non-flavonoids) could be exploited when designing new bioformulations.

Several studies have documented the addition of flavonoids for enhancing nodulation in legumes. For example, the application of luteolin and naringenin into the soil enhances the nodulation of alfalfa plants (*Medicago sativa*) by *Sinorhizobium meliloti* (Kapulnik et al. 1987; Jain et al. 1990). The direct application of flavonoids into the rhizosphere of inoculated soybean (*Glycine max*) plants increases the grain yield (Zhang and Smith 1996), the nitrogen content of shoots, and the size, number, and weight of nodules (Pan et al. 1998) and also increases the root colonization by mycorrhizas (Xie et al. 1995; Vierheilig et al. 1998). In pea (*Pisum sativum*), the addition of naringenin to the rhizosphere of plants inoculated with *Rhizobium leguminosarum*, grown in vitro, increases the root–shoot ratio, plant biomass, and nodule number (Bandyopadhyay et al. 1996; Novák et al. 2002). It has also been shown that the inoculation of legumes with flavonoid-induced rhizobial cells (inoculation with rhizobia grown in the presence of flavonoids) enhances nodulation, nitrogen fixation, and plant growth, compared with rhizobial cells obtained by fermentation without the addition of flavonoids (Table 13.1).

The addition of flavonoids to inoculated crops enhances the nitrogen fixation (Dashti et al. 2000), improves the rhizobial competitiveness (Pan and

Table 13.1 The effect of the addition of flavonoids to rhizobial inoculants

Flavonoid	Legume and rhizobia	Results	References
Genistein	Soybean (*Glycine max*) – *Bradyrhizobium japonicum*	Increase in the number of nodules and nitrogen fixation upon co-inoculation with *Serratia liquefaciens* or *S. proteamaculans*	Dashti et al. (2000)
Genistein	Soybean – *B. japonicum*	Increase in the number of nodules and dry matter, under saline/osmotic stress conditions	Muñoz et al. (2014)
Genistein	Soybean – *B. japonicum*	Increase in the number and size of nodules, nitrogen fixation, and plant yield, under suboptimal growth temperature	Zhang and Smith (1995)
Genistein	Soybean – *B. japonicum*	Increase of protein and grain yield	Zhang and Smith (1996)
Genistein	Soybean – *B. japonicum*	Increase in the nodule size, number and weight, shoot weight, and yield, at three levels of nitrogen fertilization	Pan and Smith (2000)
Genistein	Soybean – *B. japonicum*	Increase in nodulation and plant yield, under stressful soil salinity and acidity	Miransari and Smith (2007, 2009a)
Genistein	Soybean – *B. japonicum*	Increase in nodulation, nitrogen fixation, root and shoot growth, photochemical efficiency, photosynthetic rate, stomatal conductance, and transpiration under salinity stress	Dolatabadian et al. (2012, 2013)
Genistein	Soybean – *B. japonicum*	Improvement of nodulation, under water deficit	Nápoles et al. (2009)
Genistein	Common bean (*Phaseolus vulgaris* L.) – *R. leguminosarum*	Increase of nodule number, nitrogen plant content, and dry matter	Poustini et al. (2010)
Hesperitin and naringenin	Pea (*Pisum sativum*) and lentil (*Lens culinaris*) – *R. leguminosarum*	Increase of nodule number and plant dry matter, under two growth temperature	Begum et al. (2001)
Hesperetin and apigenin	Fenugreek (*Trigonella foenum-graecum* L.) – *R. tibeticum*	Increase in total number and fresh weight of nodules, nitrogenase activity, and plant biomass, under salinity stress or in cobalt-contaminated soil	Abd-Alla et al. (2014a or 2014b), respectively

Smith 2000), and nodulation (Paau et al. 1990; Zhang and Smith 1995; Pan and Smith 2000). The plant inoculation with rhizobial cells that were previously induced with flavonoids during growth significantly alleviates the adverse effects of salinity (Abd-Alla et al. 2014a; Muñoz et al. 2014), acidity (Miransari and Smith 2007, 2009a), low temperature (Zhang and Smith 1995; Begum et al. 2001; Broughton et al. 2003), water stress (Nápoles et al. 2009), and heavy metal toxicity (Abd-Alla et al. 2014b) of soils. In addition, the single application of flavonoids (Bandyopadhyay et al. 1996) into the soil or the inoculation of plants with flavonoid-induced rhizobial cells reduces the inhibitory effect of mineral nitrogen on nodulation (Pan and Smith 2000).

Although flavonoids are expensive, they act at very low concentrations, and their addition during the industrial fermentation of inoculant strains would be economically justified if the performance of the final bioformulation is much improved. Among the molecules involved in the communication between legumes and rhizobia, flavonoids were the first ones produced on an industrial scale for agronomic purposes (Broughton et al. 2003). The first methods for enhancing soybean grown in the field, using genistein/daidzein-induced rhizobial cells were patented by Smith and Zhang (1999) (US5922316A) and commercialized as SoyaSignal™ (Leibovitch et al. 2001).

13.2.2 Effect of LCOs

An increasing number of studies demonstrated the influence of the exogenous application of LCOs in the legume–rhizobia symbiosis. These molecules elicit the development of nodules at specific positions in the plant root, and their exogenous addition to rhizobial-inoculated plants promotes nodulation, photosynthesis, plant growth, and seed germination, compared with single rhizobial inoculation (Table 13.2).

The exogenous application of LCOs also affects the growth of non-leguminous plants, mainly accelerating germination and seedling growth (Zhang and Smith 2002). They also enhance corn growth (Souleimanov et al. 2002; Khan et al. 2008) and the yield of tomato fruit (Chen et al. 2007), conifer (*Picea abies*) (Dyachok et al. 2000), and carrot (De Jong et al. 1993). Other examples are the improvement of root growth of *Arabidopsis thaliana* (Khan et al. 2011) and the germination of a variety of economically important plants belonging to diverse families (Prithiviraj et al. 2003).

The data support that the addition of low concentrations of LCOs to bioformulations may increase their performance. However, LCOs have low stability in the soil, probably due to their degradation by plant or bacterial chitinases (Broughton et al. 2003), questioning, in our opinion, the effectiveness of their use in the field. Even though, some companies declare the production of successful bioformulations containing rhizobia and LCOs. For example, the Novozymes patented "LCO promoter technology" product known as a seed treatment (for more information see Sect. 13.3).

13.2.3 Effect of Phytohormones

Phytohormones are chemical messengers produced by plants that participate in multiple functions at low concentrations. In general, they are classified into five major classes: auxins, cytokinins (CKs), gibberellins (GAs), abscisic acid (ABA), and ethylene (ET). These phytohormones are involved in plant growth and development mainly due to their ability of inducing cell proliferation and expansion (Perrot-Rechenmann 2010; Davière and Achard 2013). Other phytohormones with relevant functions in plant growth are brassinosteroids, salicylic acid (SA), jasmonates (JAs), polyamines, nitric oxide, and strigolactones, among others (Morel and Castro-Sowinski 2013). In addition to plants, diverse microbial species produce phytohormones (Spaepen et al. 2007; Cohen et al. 2009; Spaepen and Vanderleyden 2011; Morel et al. 2011; Spaepen 2015). The inoculation of plants with these microorganisms has positive effects on plant development and yield, possibly due to their phytohormone production.

The positive effect of phytohormones (from bacterial or plant origin) in plant growth has been described earlier (Ketring and Schubert 1981; Hedin and McCarty 1991, 1994; among others). Then, the following question arises: will the addition of phytohormones increase the performance of bioformulations? The answer is "yes." Many works support the idea that the addition of phytohormones to bioformulations increases plant development and yield when compared with single bioformulations (containing only bacteria). In this section we provide an overview of the effects that phytohormones have on plant growth, briefly exploring reports of their single application in plants (formulations) or in combination with PGPB (bioformulations) (Table 13.3).

13.2.3.1 Effect of Auxins

Bacterial auxins have been deeply studied (Spaepen et al. 2007), IAA being the best characterized one. Examples of auxin-producing microorganisms are *Azospirillum* (Khalid et al. 2011), *Pseudomonas* (Khakipour et al. 2008), *Rhizobium* (Etesami et al. 2009), *Bacillus* (Lim and Kim 2009), and *Delftia* (Morel et al. 2011; Ubalde et al. 2012). IAA promotes plant growth, mainly because this phytohormone increases the root volume, thereby increasing the active area for the absorption of mineral nutrients and water (Morel et al. 2012). IAA also triggers the expression of bacterial genes involved in adhesion, adaptation, and virulence, thus leading to a better plant colonization (Spaepen et al. 2007). Auxins are also involved

Table 13.2 The effects of the addition of LCOs on legume plant growth and nodulation. *non-host plant or non-nodulate rhizobia; Nod⁻, mutant cells of rhizobia that failed to form nodules

Source of LCOs	Plant-rhizobia system	Place of LCOs application	Results	References
R. leguminosarum bv *viciae*	Vetch (*Vicia sativa*) (uninoculated plants)	Rhizosphere	Production of preinfection structures	van Brussel et al. (1992)
R. leguminosarum bv *viciae*	Vetch – *R. leguminosarum*	Rhizosphere	Reduction of auxin transport that preceded the root nodule primordium formation	Boot et al. (1999)
Rhizobium sp.	*Macroptilium atropurpureum* – wild type or mutant (Nod⁻) – *Rhizobium* sp.	Rhizosphere	Deformation and curling of the root hairs; colonization of roots by mutants cells	Relić et al. (1993)
Rhizobium sp.	Soybean – *B. japonicum* (Nod⁻)	Rhizosphere	Formation of pseudonodules in the absence of rhizobia; colonization, nodulation,, and nitrogen fixation by the combinations of LCO with mutant cells	Relić et al. (1994)
	Calopogonium caeruleum – *R. freedi**			
	Vigna unguiculata wild type or mutant (Nod⁻) – *Rhizobium* sp.			
S. meliloti	*M. truncatula* – *S. meliloti*	Rhizosphere or seeds (by soaking)	Increase in number of nodules per plant	Macchiavelli and Brelles-Mariño (2004)
R. leguminosarum bv. *viciae*	Pea (*Pisum sativum*) (uninoculated plants)	Soaked seeds	Enhanced nitrogenase activity and total plant nitrogen content	Siczek et al. (2013, 2014)
R. leguminosarum bv. *viciae*	Pea and vetch (*Vicia villosa*) (uninoculated plants)	Soaked seeds	Increase in shoot and root fresh and dry weights and nodule number	Kidaj et al. (2012)
R. leguminosarum bv. *trifolii*	Clover (*Trifolium pratense*) – *R. leguminosarum* bv. *trifolii*	Soaked seeds	Enhanced clover nodulation and growth of plants	Maj et al. (2009)
R. leguminosarum bv. *viciae*	Pea (uninoculated plants)	Soaked seeds or foliar application	Increase in number and weight of nodules, chlorophyll content in leaves, and pea yield	Podleśny et al. (2014)
B. japonicum	Soybean – *B. japonicum* or corn (*Zea mays*) (uninoculated plants)	Rhizosphere or stem injection	Increase in total length and surface area of the roots	Souleimanov et al. (2002)
B. japonicum	Soybean – *B. japonicum*	Foliar application	Increase in photosynthesis and plant dry weight	Almaraz et al. (2007) and Khan et al. (2008)
B. japonicum	Soybean (uninoculated plants)	Foliar application	Increase in flower and pod numbers, under water stress	Atti et al. (2005)
B. japonicum	Soybean (uninoculated plants)	Foliar application	Changes in the global plant gene expression, under suboptimal temperature	Wang et al. (2012)
B. japonicum	Soybean – Genistein-induced *B. japonicum* cells	Rhizosphere	Increase in calcium uptake into leaves	Supanjani et al. (2006)

(continued)

Table 13.2 (continued)

Source of LCOs	Plant-rhizobia system	Place of LCOs application	Results	References
LCO analogue produced by *Serratia proteamaculans*	Soybean – *B. japonicum*	Root or foliar application	Enhancement in nodulation, growth, and seed germination	Bai et al. (2002)
B. japonicum	Soybean (uninoculated plants)	Rhizosphere	Increase dry matter of aerial and radical parts	Muñoz et al. (2014)
B. japonicum	Soybean (uninoculated plants)	Rhizosphere	Changes in frequency of root-hair deformations	Duzan et al. (2004)
B. japonicum	Soybean (uninoculated plants)	Rhizosphere	Induce plant resistance to powdery mildew caused by *Microsphaera diffusa*	Duzan et al. (2005)
Azorhizobium caulinodans	*Sesbania rostrate* – *A. caulinodans* (cell defectives in LCOs production)	Rhizosphere	Formation of preinfection structures	D'Haeze et al. (1998)
Rhizobium sp.	Soybean – *Glomus mosseae*	Watering	Enhance mycorrhizal colonization	Xie et al. (1995)

in the establishment of the symbiosis between microorganisms and non-leguminous plants, increasing the nitrogen fixation capability (Benson and Silvester 1993; Péret et al. 2008).

Many reports show that the exogenous application of phytohormones (alone or in combination with their biosynthetic precursors) or a mixture of phytohormones increases nodulation rate, number of nodules, dry shoot weight, and/or grain yield of many crops (Table 13.3).

Other reports show that seed priming with phytohormones (IAA, GAs, ABA, and ET) increased the speed and synchronicity of germination and increased crop yields. Possibly, the increase in the rate of amino acids and amide biosynthesis may explain the improvement in germination performance, seedling growth, and grain yield, as shown in maize (*Zea mays* L.) (Tian et al. 2014). The overall results strongly suggest that the application of these phytohormones to seeds might improve germination and finally the crop yield (Table 13.3).

13.2.3.2 Effect of Gibberellins

Gibberellic acid (GA3) is commonly used either alone or with fertilizer to stimulate pasture production (Matthew et al. 2009) and legume crops (Table 13.3). Among commercial products, Nufarm Ltd has developed a water-soluble formulation with GA3 as active constituent (www.nufarm.com.au). However, this phytohormone is sensitive to heat and light and can be quickly degraded in soil, resulting in its low utilization. Thus, farmers repeatedly apply conventional formulations containing GA3, increasing the cost of crop production. A solution to this problem may be the use of controlled release formulations. Liu et al. (2013) successfully prepared a novel gibberellic acid–chitosan (GA3–CS) conjugate and evaluated the controlled release of GA3 in vitro, showing the potential of this strategy for the good use of GA3.

13.2.3.3 Effect of Cytokinins

CKs affect many aspects of growth and plant development, including cell division, stem initiation and growth, leaf senescence, apical dominance, nutrient absorption, gametophyte and embryo development, and response to biotic and abiotic factors. They also play a critical role in nodule formation, triggering the cell division that initiates the nodule development (Tirichine et al. 2007; Argüeso et al. 2012). The efficient use of formulations containing CKs has been shown in some plants as cotton (*Gossypium hirsutum*) (Hedin and McCarty 1991, 1994),

Table 13.3 The effect of the addition of phytohormones (*IAA* indole-3-acetic acid, *CKs* cytokinins, *GAs* gibberellins, *ABA* abscisic acid, *SA* salicylic acid, *JAs* jasmonates, *ET* ethylene) to crops

Crop	Phytohormone	Plant effect	References
Wheat (*Triticum aestivum* L.)	IAA+GA+ABA	Increase the speed and synchronicity of germination and grain yield by restructuring tillers and weight in wheat grain	Cai et al. (2014)
	ABA – SA	Decrease osmotic stress effects	Marcińska et al. (2013)
	CKs	Stimulate amino acid root exudation	Kudoyarova et al. (2014)
	JAs	Improve tolerance to salt stress	Qiu et al. (2014)
Soybean (*Glycine max*.)	IAA	Increase nodule number and dry weight of roots and higher yield	Sudadi and Suryono (2015)
	GAs	Ameliorate the adverse effects of salt stress and restores normal growth and development	Hamayun et al. (2010)
	JAs	Alleviate the harmful effects of saline stress	Yoon et al. (2009)
	IAA+GAs+ ABA+ET	Increase the speed and synchronicity of germination and crop yield	Tian et al. (2014)
Barley (*Hordeum vulgare* L.)	GAs	Increase the rate of germination and suppress inhibitors acting during dormancy	Miransari and Smith (2009b)
Rice (*Oryza sativa*)	JAs	Improve tolerance to saline stress; increase ABA endogenous production on plant	Seo et al. (2001)
Cotton (*Gossypium hirsutum*)	IAA+GAs	Increase the number of fibers per ovule	Seagull and Giavalis (2004)
Grape (*Vitis vinifera*)	ABA	Inhibit the anthocyanin accumulation; improve color	Roberto et al. (2012)
Pearl millet (*Pennisetum americanum* L.)	GAs	Increase production of lateral roots	Tien et al. (1979)
	CKs	Increase production of lateral roots	
	IAA+GAs+CKs	Changes in root morphology; stimulation of root-hair formation, reduction of lateral root production, and main root elongation	
Bedding plants	ABA	Reduce the loss of water and extend the shelf life of several plants	Waterland et al. (2010)
Muskmelon (*Cucumis melo* L.)	ABA	Excess levels delay post-stress growth and produce chlorosis, during water stress	Agehara and Leskovar (2012)
Potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.)	JAs	Protect against the infection with *Phytophthora infestans*	Cohen et al. (1993)
Ornamental plants	JAs	Protect against the attack of pathogenic fungi	Meir et al. 1998
Arabidopsis	JAs	Reduce development of the disease caused by fungi (*Alternaria brassicicola*, *Botrytis cinerea*, and *Plectosphaerella cucumerina*	Tomma et al. (2000)

peanut (*Arachis hypogaea*) (Ketring and Schubert 1981), and *Sophora tonkinensis* (Jana et al. 2013), among others (Table 13.3). In wheat, the application of synthetic trans-zeatin or CKs-producing bacteria increased rhizodeposition of amino acids (Kudoyarova et al. 2014).

13.2.3.4 Effect of Ethylene

ET, jasmonic acid (JA), and ABA, usually known as immunity hormones, have a regulatory function during the plant infection with pathogens (Pieterse et al. 2009). ET is also produced in response to biotic and abiotic stresses. High levels of ET inhibit the normal plant growth

(Saleem et al. 2007) and the nodulation process in numerous plant species, as it was shown by Shaharoona et al. (2011). In addition, ET precursors (as ACC) also inhibited the rhizobial infection and decreased the number of nodules (Peters and Crist-Estes 1989; Nukui et al. 2000). Thus, the reduction of endogenous ET level (by the ACC deaminase activities, in roots) suppresses the negative effects of ET (Shaharoona et al. 2011). Some rhizobial strains produce inhibitors of the ET biosynthesis, thereby improving nodulation in legumes, even in presence of ET (Glick 2005; Glick et al. 2007). Up to know, we did not find reports about the exogenous application of molecules that inhibit ET synthesis in inoculated plant.

13.2.3.5 Effect of Abscisic Acid

ABA is involved in seed dormancy and senescence and in the control of stomatal opening in response to environmental changes such as water deficiency. They have negative effects in nodule formation (Liang et al. 2007) and can promote disease or increase plant resistance depending on the plant–pathogen system (Ulferts et al. 2015). Among ABA-producing plant growth-promoting rhizobacteria (PGPR), it has been reported that *A. brasilense* ameliorated the response of *A. thaliana* to drought and stimulated growth and seed yield, among other, mainly by the increase of plants' ABA content (Cohen et al. 2008).

Among other effects (Table 13.3), the exogenous application of ABA reduced the water loss and extended shelf life of several plants, including bedding plants before shipping, thus maintaining their marketability even under severe drought stress conditions (Waterland et al. 2010). Other interesting agronomic use of ABA is its exogenous application to grape berries with patchy skins that improved the fruit color by increasing anthocyanin accumulation (Roberto et al. 2012). In 2013, a patent for the methodology involved in the modification of the sensory characteristics of white grapes and white wine (US8349768 B2) by the application of ABA was registered (Venburg et al. 2013).

The exogenous application of ABA, or SA, also minimizes the negative effect of osmotic stress on wheat seedlings, probably due to an increase in proline and carbohydrate content as well as in antioxidant compounds of plants (Marcińska et al. 2013). In addition to the positive effects of adding ABA, several side effects have been reported on growth and physiology of muskmelon (*Cucumis melo* L.) seedlings. Agehara and Leskovar (2012) also showed that the excess levels of ABA delay post-stress growth and produce chlorosis, during water stress. The authors suggested that the concentration of ABA should be minimized in the formulation.

13.2.3.6 Effect of Jasmonates

The chemical group of JAs includes: JA; JA biosynthetic precursor, the 12-oxophytodenoic acid; JA derivative molecules, such as the methyl jasmonate (MeJA); and the conjugate jasmonate-isoleucine (Ile-Ja). They are molecular signals involved in the plant response to abiotic and biotic stresses, as well as in plant growth and development (Moons et al. 1997; Pedranzani et al. 2003; Wasternack 2007).

The evidence supports that the exogenous application of JAs protects plants from the attack of some fungi (Table 13.3). Briefly, the exogenous application of JAs has important effects in the control of pathogenic fungi and alleviates the detrimental effects caused by some environmental stresses. Among many examples, the foliar application of JAs to potato and tomato plants protects them against the infection with *Phytophthora infestans* (Cohen et al. 1993). Also the postharvest application of JAs to ornamental plants protects against the attack of pathogenic fungi (Meir et al. 1998). Tomma et al. (2000) showed that by spraying JAs to *Arabidopsis* plants, the development of the disease caused by fungi (*Alternaria brassicicola*, *Botrytis cinerea*, and *Plectosphaerella cucumerina*) is reduced. Also the exogenous application of JAs is useful for alleviating the harmful effects of salinity stress in soybean (Yoon et al. 2009) and wheat (Qiu et al. 2014). JAs increase the endogenous level of ABA in rice crops (Seo et al. 2001) and act as inducers of *nod* genes of *B. japonicum* (Mabood and Smith 2005; Mabood et al. 2006).

Many patents documented several methodologies for suppressing diseases and inducing plant defense by the use of JAs in single formulations or in combination with other agri-products (herbicides, pesticides) (such as US5436226A or US20120077674A1, Lulai et al. 1995 and Cargeeg and Seevers 2011, respectively).

13.2.4 Addition of Pooled Metabolites

Certainly, the application of phytohormones, flavonoids, or LCOs, increases plant growth and yield (Tables 13.1, 13.2, and 13.3). So, what effect would have the application of a mixture of molecules? This idea was recently developed by Marks et al. (2013) and Morel et al. (2015). Marks et al. (2013) reported that the addition of rhizobial metabolites (produced during the growth of rhizobial cells) enhances grain yields of soybean and maize when inoculated with *Bradyrhizobium* and *Azospirillum* spp., respectively. These enhancements might be explained by the combined effect of the diverse rhizobial secondary compounds (exopolysaccharides, phytohormones, and LCOs). Molla et al. (2001) also found that the addition of a cell-free supernatant of *A. brasilense* Sp7, or IAA, stimulates root growth of soybean, in greenhouse conditions. It has also been shown that the watering of alfalfa plants with the hydroponic cell-free solution obtained during the co-inoculation of alfalfa with *Sinorhizobium* and *Delftia* strains increased plant growth (Morel et al. 2015). The hydroponic solution contained the bacterial- and root-secreted molecules, including flavonoids, phytohormones, and LCOs.

In summary, the inoculation of some plants in combination with the addition of a set of secondary metabolites (plant and bacterial metabolites) enhances plant growth and yield. But the use of commercial bioformulations containing a pool of plant and microbial metabolites is still incipient.

13.3 Conclusion

The gathered information shows the huge potential of including secondary metabolites in bioformulations (Fig. 13.2). Although it is

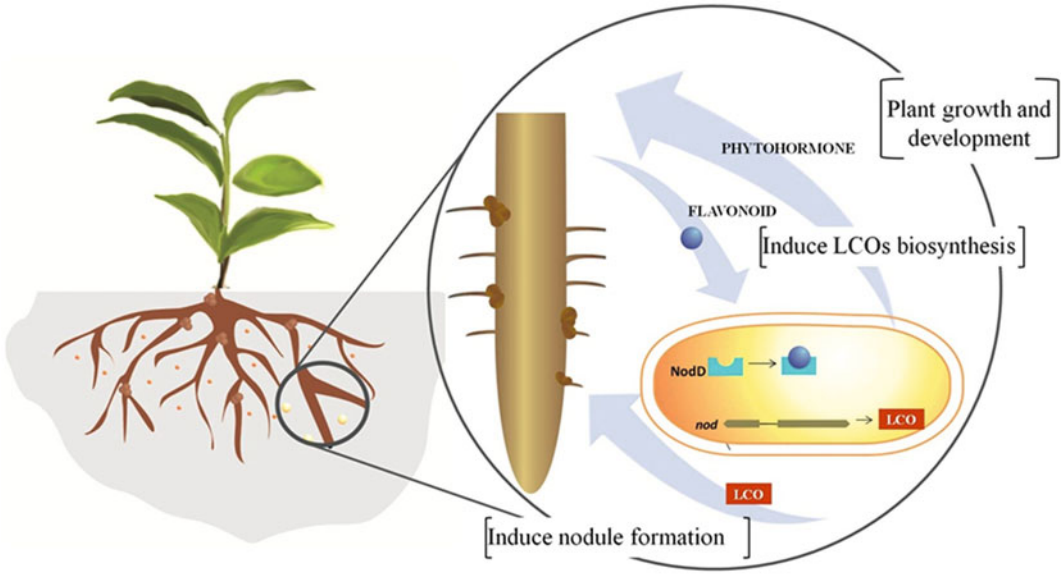

Fig. 13.2 The effect of secondary metabolites on plant fitness: how to improve bioformulations? The information supports that the addition of LCO, flavonoids, and/or phytohormones improves the performance of bioformulations, rendering better commercial products

Table 13.4 Commercial formulations containing secondary metabolites. *LCO* lipo-chitooligosaccharides, *GAs* gibberellins, *IAA* indol-3-acetic acid

Metabolite	Other active components	Crop	Product name	Company	Web page	Country	References
LCO	*B. japonicum*	Soybean	Optimize II	Nitragin	www.nitragin.com.ar	Argentina	Torres (2015)
Bioinductors	*B. japonicum*	Soybean	Signum	Rizobacter	http://www.rizobacter.com	Argentina	Rizobacter.com (2015)
LCO	*B. japonicum* + *Penicillium bilaiae*	Soybean	Nitragin Triple	Nitragin	www.nitragin.com.ar	Argentina	Torres (2015)
LCO	Aqueous carrier	Corn, Soybean, alfalfa	Ratchet	Novozymes	www.bioag.novozymes.co	USA	Bioag.novozymes.com (2015)
LCO	Aqueous carrier	Corn	Torque	Novozymes	www.bioag.novozymes.co	USA	Bioag.novozymes.com (2015)
LCO	*Bradyrhizobium* sp.	Peanuts	Optimize	Novozymes	www.bioag.novozymes.co	USA	Bioag.novozymes.com (2015)
LCO	*Penicillium bilaiae* + *Rhizobium leguminosarum*	Pea lentil	TagTeamLCO	Novozymes	www.bioag.novozymes.co	USA	Bioag.novozymes.com (2015)
LCO	*P. bilaiae*	Wheat, Corn	JumpStart LCO	Novozymes	www.bioag.novozymes.com	USA	Bioag.novozymes.com (2015)
LCO	*B. japonicum*	Soybean, Peanuts	Dyna-StartMax	Loveland	www.lovelandproducts.com	USA	Lovelandproducts.com (2015)
Isoflavonoids	Aqueous carrier	Cotton	Revv	Novozymes	www.bioag.novozymes.com	USA	Bioag.novozymes.com (2015)
GAs	N/D	Blueberries, Cherries, Citrus, Cranberries, Grapes	Falgro 20SP	Fine Americas INC	www.fine-americas.com	USA	Fine Americas (2014)
IAA	Mineral nutrients	many crops	Bayfolan	Bayer CropScience AG	www.bayercropscience.com.mx	Mexico	Bayercropscience.com.mx (2015)
Thidiazuron	N/D	Grapes	Splendor 5 % SC	Bayer CropScience AG	http://www.cropscience.bayer.cl/	Chile	Cropscience.bayer.cl (2015)
Ethephon	N/D	Grapes, tomato	Ethrel 48 SL	Bayer CropScience AG	http://www.cropscience.bayer.cl/	Chile	Cropscience.bayer.cl (2015)

N/D no data

important to conduct more tests, the addition of flavonoids, phytohormones, and/or LCOs increases plant growth and development and finally crop yield, when they were applied alone or in combination with PGPB. This information has led to the generation and use of patents, demonstrating the pivotal importance of the industry–academic collaboration. However, only few manufacturers produce bioformulations containing these secondary metabolites (Table 13.4). Some of these novel products are commercially available, and most of them declare to contain LCOs in single formulations, such as Ratchet® and Torque®, or in bioformulations (contain PGPB also), such as Optimize II®, Signum®, and DynaStartMax®, among others.

Three main groups of molecules involved in the plant–microbe communication have been analyzed in this chapter, but still remains to gather the information about the effect of many other molecules, as extracellular polysaccharides, osmo-protecting molecules, etc.

Acknowledgments We thank PEDECIBA-Uruguay (Programa de Desarrollo de las Ciencias Básicas), LAGE y Cia SA (www.lageycia.com), and ANII (Agencia Nacional de Investigación e Innovación; FMV-3-2011-6089) for partial financial support. The work of M.A. Morel and C. Cagide was also supported by ANII. We are especially grateful for Cecilia Hermann's assistance during the search of commercial products. The cartoon or Fig. 13.1 was drawn by Juan José Marizcurrena.

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Encapsulation Techniques for Plant Growth-Promoting Rhizobacteria

14

Mauricio Schoebitz and María Dolores López Belchí

Contents

Abstract

Application of microbial inoculants into the soil can enhance soil properties and plant nutrient acquisition and increase the efficiency of mineral fertilizers and manures. The potential use of microbial inoculants in sustainable agriculture and soil restoration has been gaining increasing interest. Encapsulation of beneficial soil microorganisms has been applied and used in the agricultural industry, particularly in processes such as spray drying, interfacial polymerization, or cross-linking. This chapter presents different techniques for microbial inoculants and their benefits for agricultural and environmental purposes. Techniques include fluidized bed, coacervation, and ionic or inverse gelation. The major topics discussed are conventional inoculants, formulation of microbial inoculants, encapsulation techniques, and application trends. In addition, the use of biochar as inoculant carrier is proposed in order to develop new formulations. This innovative microbial inoculant has many advantages, such as increased water-holding capacity, high internal porosity, and large surface area, while it also provides a suitable habitat for microorganisms to enhance colonization and bacteria protection in the soil.

M. Schoebitz (✉)
Departamento de Suelos y Recursos Naturales, Facultad de Agronomía, Universidad de Concepción, P.O. Box 160-CConcepción, Chile
e-mail: mschoebitz@udec.cl

M.D. López Belchí
Departamento de Producción Vegetal, Facultad de Agronomía, Universidad de Concepción, Av. Vicente Méndez 595, Chillán, Chile

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_14

14.1 Introduction

The development of microbial inoculants has been increasing worldwide due to the recognition of the deleterious effects on the environment generated by the excessive and improper application of chemical fertilizers, as well as the improved knowledge about plant-microorganism interactions in the soil (Malusá and Vassilev 2014). In this context, the reduced use of chemical fertilizers with increased applications of plant growth-promoting rhizobacteria (PGPR) is considered as essential to decrease the pressure on the environment derived from current agronomical practices (Malusá et al. 2012).

Sustainable agriculture production and reclamation of degraded soils can be achieved by emphasizing the use of PGPR as microbial inoculants (Schoebitz et al. 2013a; Mengual et al. 2014b). In general, microorganisms promote plant growth in three different ways: synthesizing growth-promoting hormones for the plants, facilitating the uptake of nutrients from the soil, and lessening or preventing plant diseases (Martínez-Viveros et al. 2010). The mechanisms involved in plant growth by PGPR are (1) solubilization of mineral phosphate; (2) biological nitrogen fixation; (3) ability to produce hormones like auxin, i.e., indoleacetic acid (IAA), abscisic acid (ABA), gibberellic acid, and cytokinins; (4) ability to produce 1-aminocyclopropane-1-carboxylate (ACC) deaminase to reduce the level of ethylene in roots of developing plants and thereby increasing root length and growth; (5) antagonism against phytopathogenic bacteria by producing siderophores and antibiotics; and (6) mediated resistance to drought and oxidative stress (Hayat et al. 2012).

Encapsulation of PGPR has been widely used in agriculture to obtain a surrounding structure that allows for protection, release, and functionalization of microorganisms (John et al. 2011). In fact, encapsulation tends to stabilize cells, providing less exposure to abiotic and biotic stresses and potentially enhancing their viability and stability in the production, storage, and handling, while it also confers additional protection during rehydration (Kim et al. 1996).

Numerous studies have focused on developing different encapsulation techniques to improve the survival of microbial inoculants (Herrmann and Lesueur 2013; Schoebitz et al. 2013a; Bashan et al. 2014; Campos et al. 2014). Encapsulation of microorganisms is one of the newest and most efficient techniques. For instance, encapsulation via dripping technique has been employed for the protection of cells to allow for better survival in soil after inoculation (Schoebitz et al. 2012). Encapsulated cells could be released into the target soil in a slow and controlled manner, providing greater long-term effectiveness (John et al. 2011). Nevertheless, a literature survey by Xavier et al. (2004) showed that since the 1980s, most of the studies on beneficial soil microorganisms focused on bacterial genetics and physiology and that research on inoculant formulations represented less than 1 % of the scientific articles on microorganisms. The formulation, storage, and application methods are critical to the success of the product (Jha and Prasad 2006). Some constraints in their widespread use are short shelf life, lack of suitable carrier material, susceptibility to high temperature, problems in transportation, and storage (Ghomarde et al. 2011). However, there is a real need for improving formulations, also developing and commercializing new biofertilizers that could be more effective and stable over time (Herrmann and Lesueur 2013).

Beneficial microorganisms isolated from agricultural lands and crop plants (Bashan and de-Bashan 2005; Park et al. 2005; Lugtenberg and Kamilova 2009; Schoebitz et al. 2009) can decompose organic residues, suppress plant disease and soilborne pathogens, and enhance nutrient cycling and bioactive compounds, such as vitamins, hormones, and enzymes (Singh et al. 2011). Nevertheless, colonization of plant roots by direct inoculation of free microorganisms into the soil is not easy because it is susceptible to environmental variations, such as soil conditions, fluctuation of pH and temperature, humidity, protozoa predation, and salt stress (Wu et al. 2012). This unpredictability in the success of microbial inoculation is mainly

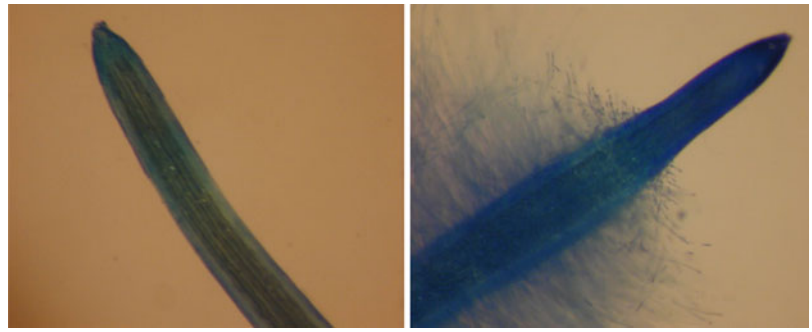

Fig. 14.1 Effects of rhizobacteria on root growth of tomatoes. Representative pictures of seedlings growing in control plants (*left*) and plant inoculated by *Enterobacter ludwigii* (*right*)

related to the quality of formulations containing an effective bacterial strain and determines the success or failure of a biological agent. Cell immobilization through biodegradable carriers can enhance cell survival so that formulation turns to be the industrial "art" of converting a promising microorganism strain into a commercial inoculant (Bashan 1998). The use of microbial inoculants in agriculture has greatly increased during the past years (Bashan et al. 2014). Microbial inoculants are biological products containing living microorganisms that, when applied to seeds, plant surfaces, or soil, promote growth by several mechanisms, such as nitrogen fixation, phosphate solubilization, and production of various phytohormones, which improve root growth (Fig. 14.1), water absorption, and nutrient uptake (Calvo et al. 2014). In this regard, microbial inoculants have a potential role in developing sustainable agriculture for crop production (Bashan et al. 2004; Rivera-Cruz et al. 2008) and reclamation of degraded soils (Mengual et al. 2014a). Inoculant carriers have been used to improve effectiveness by supplying nutrients, protecting from desiccation, and slowing cell release. Information about the beneficial effects of rhizobacteria on growth promotion is well documented under laboratory and greenhouse conditions. However, reports of rhizobacteria-immobilized inoculants evaluated under field conditions are scarce (Schoebitz et al. 2014). The success of using microbial inoculants introduced into soil requires the survival of an adequate number of cells that can reach suitable habitats where they can thrive (Heijnen and Van Veen 1991). Based on this, formulation is a key factor in the success of microbial inoculants.

14.2 Traditional Microbial Inoculants

Peats are the most commonly used carriers to inoculate seed legumes with rhizobia (Denton et al. 2009). Peat is a complex organic material with a high variability. As microbiological contamination decreases the shelf life of the inoculants, this particular situation affects the quality of the final product, its stability during storage, and the survival of microorganisms in the final product (Bashan 1998). For example, Fallik and Okon (1996) found that cell concentration decreased from 10^{10} to 10^{5} CFU g^{-1} after 6 months of storage with peat inoculated with *Azospirillum brasilense*.

Liquid formulations are inoculants that use broth cultures mainly in water, but also in mineral or organic oils. The seeds are dipped into the inoculant before sowing, or an applicator evenly sprays the liquid inoculant on the seeds (Bashan et al. 2014). Liquid formulations are easier to produce, can be more easily applied by farmers, and present some advantages based on the fact that they use low-cost materials and are easily attainable by small producers (Singleton et al. 2002; Albareda et al. 2008). Liquid inoculants allow direct contact of seeds and microorganisms and, consequently, increase the survival of bacteria on plant roots. However, bacterial survival rates on liquid formulations decrease because this technique does not provide

a protective environment for microorganisms and the number of bacteria distributed in each seed is quite heterogeneous. In addition, microorganisms are not sufficiently protected against environmental conditions and contamination during storage, transport, and application into the soil (Bashan et al. 2002). The use of liquid inoculants mainly requires cold conditions for long-term storage to maintain their efficiency and cell viability. Liquid inoculants containing concentrations of 2×10^9 CFU mL^{-1} and a stable population of microorganisms can be stored for 3 months (Albareda et al. 2008). *Bacillus subtilis* and *Pseudomonas corrugata* in liquid formulation showed a decrease from 10^{10} CFU mL^{-1} to 10^7 CFU mL^{-1} after 6 months of storage at room temperature (Trivedi et al. 2005).

Clays are widely used as support for microbial inoculants and have a long history of usage in various agricultural formulations applied as granules, suspensions, and powder. Clays can act as a desiccant, providing excellent storage for dried inoculants due to large surface area, pore size distribution, and total porosity. Besides, water can be controlled to provide moisture for biologically active formulations (Goss et al. 2003). In addition, clays absorb or distribute dispersing and suspending agents. Some authors have found that clay-based inoculant carriers increased the survival of rhizobia in the soil for 60 days, reporting that these beneficial effects result from the creation of protective microhabitats, which are accessible to bacteria, but inaccessible to predators (Heijnen and Van Veen 1991; Heijnen et al. 1992).

14.3 Principle of Microbial Inoculant Formulation

The principle of rhizobacterial inoculants is to protect the microorganisms introduced into the soil and to ensure a gradual and prolonged release (Kim et al. 2012). The degradation rate of the encapsulation matrix is directly related to the biological activity of the microorganisms. In fact, the quicker the degradation of the matrix, the lower the protection and biological activity of the microorganisms, which allows for encapsulation and storage at room temperature for a long period, resulting in a favorable environment for bacteria and reducing the risk of decreased survival. These inoculants can be improved by incorporating essential nutrients for bacterial growth, transforming the capsules in bioreactors, which are capable of increasing the number of encapsulated bacteria and inoculated into the soil. These bacterial inoculants have solved many problems associated with traditional peat inoculants, which originate great variability in peat quality (Deaker et al. 2004). In fact, the encapsulation of rhizobacteria presents numerous advantages, such as the controlled release of bacteria into the soil and the protection of soil microorganisms against biotic and abiotic stresses.

Sodium alginate is the most commonly used material for the encapsulation of cells, enzymes, and biological control agents. Alginate is a natural polymer found in different marine macroalgae. The preparation of beads containing microorganisms involves a multistep procedure conducted at room temperature and with the use of minimal amounts of additional chemicals or equipment (Bashan et al. 2014).

Sodium alginate has been extensively used for the encapsulation of microbial inoculants (Fig. 14.2) due to its simplicity of handling, viscosity, and gel-enhancing properties. Although this is a simple and quick way to obtain beads or capsules, the method presents a major disadvantage referred

Fig. 14.2 Immobilization of microbial inoculants in calcium alginate beads by ionic gelation technique (beads 2–3 mm in diameter)

to the cell loss that occurs during drying process (Schoebitz et al. 2012). In fact, the main difficulty with encapsulation techniques until now is cell survival during the dehydration process and storage since these techniques exhibit more than 90 % mortality of the initial population of microorganisms. Dehydration is one of the most critical and stressful phases for the bacteria during the encapsulation process. This is a severe problem for non-spore-forming, Gram-negative bacteria, which correspond to most species among the PGPR (Bashan et al. 2014). Besides, the presence of pores in the alginate matrix facilitates the diffusion of hydrophilic molecules (Wu et al. 2014). The incorporation of a filler material into an alginate matrix is a good strategy to solve the above mentioned limitation. Common additives are starch or clays which improve survival, mostly of phosphate solubilizing bacteria and other PGPR (Wu et al. 2012). Besides, adding trehalose and fructose to the culture medium preserves the viability of bacterial cells in a liquid medium and protects cells in the drying process (Schoebitz et al. 2012).

14.4 Innovative Techniques for Encapsulation of PGPR

Before describing the encapsulation techniques, it is important to note that three steps are needed to carry out encapsulation of PGPR. Firstly, the nature of the active ingredient that will be incorporated into a matrix (liquid or solid) needs to be identified. Secondly, a mechanical operation needs to be conducted by dispersing or spraying a solution onto solid particles under mechanical stirring. Thirdly, stabilization should be conducted either by a chemical process of polymerization, a physical-chemical process (gelation, coacervation), or a physical process (evaporation, solidification) on a droplet or pellet formed during the second step.

It is well known that encapsulation of living microorganisms is important for the production of special chemicals for industrial and agricultural use. The methods used to immobilize bacterial cells may be classified as physical, chemical, and chemical processes. Physical processes include spray drying, spray chilling/cooling, and fluidized bed; chemical processes include co-crystallization or interfacial polymerization; and chemical processes include coacervation and gelation/inverse (Table 14.1) (Madene et al. 2006). New encapsulation techniques continue to emerge for developing formulations and processes to allow for the improvement of capsule, material properties, and characteristics.

14.4.1 Spray Drying

Spray drying is a well-known method of producing a dry powder from a liquid or slurry, which is

Table 14.1 Comparison of different encapsulation processes

	Spray drying	Fluidized bed	Interfacial polymerization	Coacervation	Ionic gelation	Inverse gelation
Size <500 μ	Yes	No	Yes	No	No	No
Water soluble	Yes	Yes	No	Without cross-linking	No	No
Time required	10–30 s	10 min–1 h	10 min–2 h	12–16 h	1–2 h	1–2 h
Relative cost	Very low	Low	Low-medium	High	Medium	Medium
Advantage	Fast, inexpensive	Flexible, inexpensive	Well-developed process, large batches	Insoluble wall, wall impermeable to hydrophobic molecules	Biocompatible	Biocompatible
Disadvantages	Possible dust	Coating fine particles	Core wettability, few wall materials	Aggregation, core wettability, few wall materials, process control	Few wall materials, process control	Few wall materials, process control

dried by a hot gas. It is a process that involves the dispersion of the bacterial cells in a carrier material forming an emulsion or dispersion, with homogenization of the liquid followed by atomization and spraying of the mixture into a hot chamber (Watanabe et al. 2002). Spray drying, which is a dehydration process, is one of the immobilization techniques used for the production of microbial inoculants that can be incorporated into agricultural and nonagricultural systems. The use of spray drying as a standard production technique is challenging due to its detrimental impact on the cellular integrity of microorganisms during drying (if not correctly optimized) and resultant reduction in the survival of immobilized bacteria (Campos et al. 2014). It has been demonstrated that heat-induced cellular damage is primarily associated with changes in the physical state of the membranes (Behboudi-Jobbehdar et al. 2013). In addition to the carrier-microorganism interaction, the impact of the spray-drying process is associated with process parameters (inlet and outlet air temperature, feed flow rate, residence time at the drying chamber, design parameters of the drying chamber, and temperature of the drying medium) and the biology of the bacteria to be immobilized (species and strain type, adaptation of the bacteria to heat or osmotic stress conditions, growth state of the culture media) (Golowczyc et al. 2010; Schutyser et al. 2012).

One important aspect to consider for this technique is optimal wall material, selecting substances with high solubility in water, low viscosity at high concentration, effective emulsification film-forming characteristics, and efficient drying properties (Reineccius 1988). Some of the advantages of spray-drying techniques are that the process works on a continuous basis, low operating costs, high quality of capsules in good yield, rapid solubility of the capsules, small size, and high stability capsules. However, spray drying also presents some drawbacks, including low uniformity of microcapsule size, limitations in the selection of wall material, and the high temperature required in the process, which may not be suitable for encapsulating bacterial cultures. In addition, this process produces very fine powder, which needs further processing and is inadequate for heat-sensitive material (Risch 1995). Nevertheless, appropriate modification and control of the processing conditions (inlet and the outlet temperatures) achieve viable encapsulated cultures of the particle size distribution required.

As mentioned before, temperature is a key parameter. Some reports have indicated that the survival of bacteria during spray drying decreased with increasing inlet temperature (Mauriello et al. 1999). Studies that dealt with outlet temperatures above 60 °C resulted in poor drying and the humid product often accumulated in the cyclone. Other studies have confirmed these observations, also reporting that the lowest air temperature was associated with the highest survival rate for the microorganisms during the drying process (Gardiner et al. 2000; Golowczyc et al. 2010). In fact, Campos et al. (2014) reached a bacterial survival of 91 % for *Enterobacter* sp. immobilized using sodium alginate and maltodextrin as wall materials (2:13 w/w), feed flow rate of 73 m^3 h^{-1}, inlet temperature of 100 °C, and outlet temperature of 65 °C. In contrast, Schoebitz et al. (2013b) found lower cell survival after the encapsulation of microorganisms such as *Serratia* sp., using only maltodextrin as wall material, inlet temperature of 145 °C, and outlet temperature of 90 °C. In this sense, the type of strain, temperature of the drying process, and formulations of the polymer mixture used as vehicles are important parameters for the encapsulation of beneficial microorganism in order to obtain a successful microbial inoculant. However, despite the potential of this encapsulation technology in the microbial inoculant industry, the use and application of beneficial soil microorganisms to improve soil properties and plant performance is still scarce in agriculture and environmental restoration.

14.4.2 Fluidized Bed

The fluid bed technique involves drying, cooling, or coating of particulate materials for a wide

range of heat-sensitive products. In fact, fluid bed spray coating is a process used to avoid some problems of the spray-drying technique. In fluidized bed drying, particles to be coated are fluidized by hot air. After that, the coating material is sprayed through a nozzle onto the particles; film formation is started, followed by a sequence of wetting and drying stages. The small droplets of the sprayed liquid reach onto the particle surface and join together, and the solvent evaporates by hot air, while the coating material remains on the particles (Jacquot and Pernetti 2003).

This technology offers a number of advantages. It allows specificity in particle size distribution and yields low porosities of the granules (Uhlemann and Mörl 2000), while it also presents high drying rates, smaller flow area, high thermal efficiency, and simple operation, with lower capital and maintenance costs.

This process of coating operates on particle size less than 100–150 μ. Therefore, uncommon conditions should be considered in order to avoid aggregation of the lower quantity of coated particles (Sparks and Jacobs 1999).

An open cylinder, which is hanging above the fluidization plate and where the fluidization air fed goes to the center region of the fluidization plate, carrying the particles upward through the cylinder was a change introduced by Wurster (1966). The particles are not fluidized in this region, but are simply being sent out while they desiccate. This variation gives superior control of the recirculation action of the particles (Fig. 14.3).

Fluidized bed drying performs better than spray drying because it is considered less stressful for drying microbial cells than spray-drying technology. In addition, it also involves less extreme water loss and temperature gradients (Larena et al. 2003; Morgan et al. 2006).

Herridge and Roughley (1974) compared seed pelleting using a fluidized bed with the conventional rotating drum technique. They observed that fluidized bed produced a firm pellet, but the survival of the inoculum was low probably because the air temperature occasionally reached 35 °C. For this reason, the conditions required to obtain capsules of high integrity may compromise viability of the microbial inoculant (Deaker et al. 2004). This explains why, in spite of being more advantageous, the application of fluidized bed technology to coating some inoculants is less common.

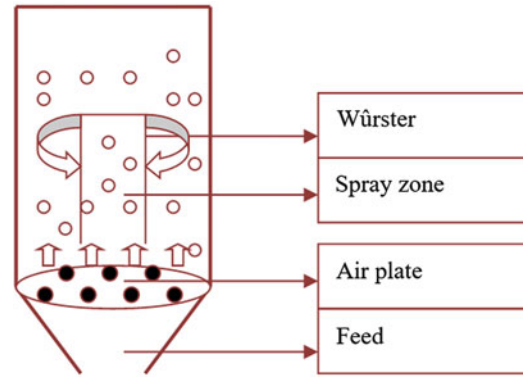

Fig. 14.3 Fluid bed drying process

14.4.3 Interfacial Polymerization

Interfacial polymerization is typically known as a condensation reaction between a diamine or diol and a diacid, where the intermediates are dissolved in a pair of immiscible liquids, one of which is preferably water. To sum up, this technique involves the formation of an emulsion with an aqueous suspension of the cells as the discontinuous phase and organic solvent as the continuous phase. The droplet containing the cells and the reaction is set off when a biocompatible reagent, soluble in the continuous organic phase, is added to the emulsion. This process allows obtaining a high active loading (up to 90 %), but it involves high pH and toxic chemicals, such as sebacoyl chloride (Yeo et al. 2001). Another example of this technique would be microcapsules produced by dripping an alginate suspension (polyanion) in a chitosan solution (polycation), in which the blend of alginate and cells is dripped in a solution of chitosan (acetic acid 1 % at pH 4) with continuous stirring. Chitosan, a water-soluble polymer (pH < 6), has been used to microencapsulate

some bacteria (Groboillot et al. 1994): a cross-linked chitosan membrane is formed by emulsification/interfacial polymerization using biocompatible reagents with oil-soluble cross-linking agents at low concentrations to minimize cell contact. Nevertheless, the antibacterial property of chitosan could limit its use as a coating material in encapsulation (Sudarshan et al. 1992).

For live cell encapsulation, some synthetic polymers such as nylon or cross-linked polyethylenimine membranes are incompatible due to toxicity of the reagents and hard conditions of encapsulation (Larisch et al. 1994).

Reports using monomers that are nontoxic for microorganisms have been carried out in order to increase their productivity in fermentations with relative success (Groboillot et al. 1993; Hyndman et al. 1993).

14.4.4 Coacervation

Coacervation is a widely used method for reversible gelification and microencapsulation of biological material. Coacervation is a phenomenon occurring in colloidal solutions. It consists of the separation from solution of colloid particles, which then agglomerate into a separate, liquid phase, called coacervate (Korus 2001). In general, the core material used in the coacervation must be compatible with the polymer and insoluble in the coacervation medium. In this technique, a core material, such as an oil phase, is dispersed in an aqueous solution of one or more polymers, while pH, ionic strength, temperature, and other parameters can change in the aqueous phase to induce the formation of a second polymer that becomes the wall material. Coacervation can be simple or complex. Simple coacervation involves only one type of polymer with the addition of strongly hydrophilic agents to the colloidal solution, and it is obtained by adding water-miscible non-solvent for the dissolved polymer (e.g., ethanol) or an electrolyte. On the other hand, complex coacervation involves two or more types of polymers and is achieved by lowering the pH of a solution containing two polymers, one of which increases positive charges as a result of the change (high-isoelectric-point gelatin), while the other has only negative charges (e.g., gum arabic) (Bungenberg de Jong 1949). Amiet-Charpentier et al. (1998) showed that due to the complex coacervation, it is possible to obtain a polymer microparticle containing rhizobacteria for inoculation of plants.

As concentration requires the formation of a fine emulsion, which may be different from that needed to increase the yield of microcapsules, optimization of wall material concentration in the emulsification and coacervation process is usually complicated (Nakagawa et al. 2004). Other limitations are evaporation, dissolution of active compound into the processing solvent, and oxidation of product (Flores et al. 1992). Hence, the coacervation method is effective but expensive, and it has some limitations.

14.4.5 Ionic Gelation

Ionic gelation produces microparticles through a drop of aqueous solution or suspension that contains the active material and sodium alginate, which is dropped into a solution of calcium chloride to form the capsules (Lim and Sun 1980). When the drop reaches the calcium chloride solution, a membrane of calcium alginate forms instantaneously, maintaining the drop shape in this aqueous/aqueous system. Calcium diffuses in, gelling the entire drop. The drop is then placed in a solution of a polycation that displaces the calcium from the outer surface, forming a permanent membrane. This capsule is then placed in sodium citrate, which slowly solubilizes the calcium through formation of the soluble citrate complex, ungelling the internal portion of the drop. By controlling the molecular weights of the reactants and the times of the reaction, the thickness and size selectivity of the permanent wall can be controlled over a wide range.

The established method produces calcium alginate beads through ionic gelation by dropping an alginate solution into a calcium chloride solution. The main advantage of gel

encapsulation is the biocompatibility, although scaling up is difficult and the beads are often porous to cells (Lacroix et al. 1990). A variation of this method is to add another material, such as starch, to improve the process to encapsulate rhizobacteria, as described by Schoebitz et al. (2012). In this method, matrix solution is prepared by mixing alginate and starch (or clay) to improve the survival of rhizobacteria species by encapsulating in alginate beads, which allows the stable production of dried beads that contain a high cellular concentration.

Drying is the most critical step for cell survival in encapsulation methods. The results obtained by Schoebitz et al. (2012) confirm this statement as these authors demonstrated that a large proportion of cells are destroyed during capsule dehydration. In fact, cell mortality during the drying of encapsulated cells has been recognized as a critical point of the encapsulation process by various authors (Bashan et al. 2002; Campos et al. 2014). The survival yield after drying did not exceed 1 % of the initial cell count for various *Azospirillum* and *Pseudomonas* species. Drying involves great physical stress for living cells, including an increase in osmotic pressure that causes a water outflow leading to plasmolysis. Cell death is mainly attributed to the disruption of the plasma membrane (Mille et al. 2002). Nevertheless, the survival of dehydrated cells depends on various factors such as drying conditions, microorganism species, adjuvant used, bacterial strain, and culture conditions (Morgan et al. 2006). It has been determined that drying kinetics is of particular importance for cell survival, showing a detrimental effect on fast drying (Poirier et al. 1997). Beads made of pure alginate contain about 97–98 % water, and such a matrix failed to protect cells during drying as there was a two-log decrease in *A. brasilense* cell number. Using starch in the encapsulation matrix allowed the reduction of water content to 65 % and resulted in a significant increase in cell survival (Schoebitz et al. 2012). This could be due to a decrease in bead drying speed resulting from the lower water content. There are wide range of materials for encapsulation. Clay, skim milk powder, humic acid, and starch have all been used in living cell carrier formulations (Schoebitz et al. 2013a). Starch is an inexpensive material for capsule formation as it is one of the most abundant natural biopolymers (Hickman 1999). It can be used as a nutrient by several soil bacteria, which could help cell release and development in the soil. Previous research carried out on probiotic carriers has found a protective effect of starch due to cell adhesion to granules (Wang et al. 1999; Crittenden et al. 2001). Apart from slowing the drying rate, the ability of granular starch to protect the rhizobacteria from drying stress may be due to cell adhesion to starch. Cell adhesion to starch may depend on the strain encapsulated since there is a relation between adhesion to starch and its use as a carbon substrate by cells (Crittenden et al. 2001). As the best survival rates were obtained with the alginate-starch matrix, various strategies were tested to improve cell survival during drying by using this formulation base. It is generally admitted that stationary-phase cells are more resistant to physical stresses, such as dehydration, than cells harvested in the exponential growth phase (Vriezen et al. 2006). In fact, it has been determined that the optimal growth phase for desiccation survival is largely dependent on microrganisms (Schoebitz et al. 2012). Nevertheless, stationary-phase cells are generally more resistant to physical stresses, such as drying (Vriezen et al. 2006). This is due to the stress response triggered by carbon starvation and exhaustion of available food sources (Morgan et al. 2006).

Protective agents can be added either during the growth of microorganisms or prior to drying. Some molecules are known to work as osmoprotectants for many living cell species. These are mainly reducing sugars (fructose, glucose) and nonreducing sugars (trehalose) (Morgan et al. 2006). The addition of trehalose to the growth medium increased the survival of *Raoultella terrigena* during the drying process. Moreover, adding trehalose to the growth medium presented a much more effective protection against desiccation than adding it to the matrix solution just prior to drying. This may

indicate that trehalose must be accumulated by cells to exercise its protective effect (Schoebitz et al. 2012). This result is consistent with the work conducted by Streeter (2003) on the survival of *Bradyrhizobium japonicum* during desiccation. In this study, the accumulation of trehalose within the cell cytoplasm contributed to membrane stabilization during desiccation. However, the accumulation of intracellular trehalose is only possible if the microorganisms cannot use it as a carbon source. Supplementation with fructose did not protect *Azospirillum brasilense* against drying, when added either to the growth medium or to the matrix prior to bead formation. This could be explained by the fact that *A. brasilense* can degrade fructose, using it as a carbon source.

Improving cell survival during encapsulation is not a simple procedure. It depends on various factors: growth media composition, strain and physiological state of cells, and process parameters. Each factor should be optimized to ensure the best inoculant activity after drying and during inoculant storage. By combining these factors, a biodegradable and dried inoculant with a high living cell concentration can be obtained (Schoebitz et al. 2012).

14.4.6 Inverse Gelation Microcapsules by Using Alginate

Inverse gelation occurs in a different form. It deals with dropping a calcium suspension in an alginate solution. The conventional method to produce calcium alginate beads through ionic gelation is by dropping an alginate solution into a calcium chloride solution. If the procedure is inversed, that is to say, calcium chloride solution dropped into an alginate solution, aqueous-core calcium alginate capsules are produced (Koyama and Seki 2004; Sasaki et al. 2008). By diffusion in the alginate solution, calcium will be gelifying the alginate and forming a membrane around the droplets. The calcium suspension consists of calcium chloride solution dispersing bacterial cells, and this emulsion finally drips into alginate solution (Abang et al. 2012; López et al. 2012). Same as recommended in ionic gelation, a variation of inverse gelation is performed by using modified starch, which is added in calcium chloride solution or even starch with alginate solution to increase solid content in the membrane.

Abang et al. (2012) studied the effects of process variables on the physical properties of capsules produced by inverse gelation. In this study, alginate was used to form the capsule membrane, and three different methods of incorporating the calcium source in oil were tested. The process variables examined were sodium alginate concentration, calcium chloride concentration, and curing time, while membrane thickness and elastic modulus were the physical properties of the capsules studied.

14.5 Reclamation of Degraded Soil by Immobilized PGPR

In recent years, research has shown that microbial inoculants can also play an indirect role on soil degradation and soil fertility. Bioremediation is recognized as an important tool to restore degraded ecosystems. In this sense, plants are essential in the recuperation of degraded soils, and the application of microbial inoculants in reforestation or afforestation of degraded lands is a promising research area (Calvo et al. 2014).

In degraded areas, the establishment of shrubs and trees is difficult due to low soil fertility and climatic conditions characterized by low precipitation and frequent drought periods. Therefore, it is necessary to apply methods to improve soil quality and resistance of planted species to degraded environmental conditions (Mengual et al. 2014b). The establishment of native plant species is widely used for reclaiming degraded lands and constitutes the most effective strategy in degraded areas (Mengual et al. 2014b). Recent studies on reclamation of degraded soils have determined the beneficial effects of the application of organic amendments, for example, alperujo (Schoebitz et al. 2014), sugar beet residue (Mengual et al. 2014a), sheep manure compost, and sewage sludge (Hueso-Gonzalez et al. 2014). Organic amendments have reported

beneficial results in the proliferation and development of natural populations of soil microbiota, since the organic residues can be used by soil microorganisms, as substrates and as carbon and energy sources (Medina and Azcón 2010). This effect could be extended to the enhancement of soil enzyme activities, which are key factors that contribute to soilborne microorganism activity and soil fertility (Caravaca et al. 2005). The use of organic waste materials not only increases organic matter and fertility of soils, but it also contributes to the palliation of environmental and economic situations related to waste disposal (Rincón et al. 2006).

Organic amendments could be useful for improving soil quality and when developing afforestation programs in degraded areas. The beneficial effects of the addition of organic amendments in horticultural and revegetation practices have been reported (Alburquerque et al. 2006; Medina et al. 2010). Additionally, the application of organic amendments interacts positively with soil microorganisms. Microbial inoculation increases microbial biomass, soil respiration, and enzyme activities (dehydrogenase, urease, and protease). Increases in enzymatic activities are properties, which are sufficiently sensitive to indicate changes caused by microbial inoculations (Schoebitz et al. 2014). Among the components of the soil microbiota, PGPR are free-living bacteria, which can colonize the rhizosphere and improve root system establishment (Antoun and Kloepper 2001). In this regard, PGPR have a potential role in the establishment of plant cover in degraded environmental conditions (Puente et al. 2004), where they can promote plant growth and improve both water and nutrient uptake (Bashan et al. 2004).

The beneficial effects of PGPR on the growth of plants under laboratory conditions have been well documented. Nevertheless, there is little information regarding the introduction of immobilized PGPR into the soil under field conditions, especially in nonagricultural systems. It has been demonstrated that the use of PGPR improves plant health and growth performance in degraded soils, while it also enhances their tolerance to drought and salinity (de-Bashan et al. 2012).

However, recent studies have indicated that inoculation with immobilized PGPR in field conditions is a useful strategy for the establishment of native shrub and tree species in degraded soils (Mengual et al. 2014b; Schoebitz et al. 2014). Microbial inoculants helped plants to compensate for deficiencies of immobile nutrients, such as phosphate. Therefore, the inoculation with immobilized PGPR can be considered as an effective biotechnological tool for the development of biofertilizers that could partially substitute chemical fertilization. In this sense, the introduction of beneficial microorganisms can improve nutrient availability and thereby increase the efficiency of applied organic amendments (Adesemoye and Kloepper 2009).

14.6 Conclusions and Application Trends

Advances in microbial inoculant formulations have been presented, featuring encapsulation materials and techniques used for introducing beneficial microorganisms into the soil. Nevertheless, conventional microbial inoculants are not able to ensure high cell viability during the formulation process. Conventional inoculants need to be stored at room temperatures, avoiding extreme temperature oscillation because shelf life of liquid inoculants in storage conditions is very short and their viability decreases by one or two logs. The use of liquid inoculants does not offer protection to PGPR against soil stresses (nutrient and water deficiency, salinity, pH, predation). Instead, encapsulation provides a niche where PGPR are protected from soil stresses. Furthermore, the liquid inoculum has an instantaneous and very fast release after being introduced into the soil, and the rhizobacteria are delivered only in the initial phase of plant growth.

Alternatively, immobilized inoculants confer a gradual microorganism release that achieves long-term fertilizing effects.

The development of new formulation procedures and carriers will be determined by the demand of the industry, so that the focus of

study will probably switch from laboratory scale to industrial production. In recent years, biochar has positioned itself as a beneficial soil material. The mechanisms are multiple and still a subject of intensive research, such as carbon sequestration, soil fertility, nutrient retention, and alteration of soil acidity (Novak et al. 2009; Joseph et al. 2010; Major et al. 2012). For instance, its adsorption and desorption properties are used to support an exchange platform between inorganic and organic compounds into the soil (De Luca et al. 2009). In addition, water-holding capacity and biological properties of the soil are also important (Downie et al. 2009). A favorable structure and morphology of biochar provide a suitable habitat for microorganisms to enhance colonization, growth and multiplication of bacteria, actinomycetes, and arbuscular mycorrhizal fungi (Thies and Rillig 2009). At the same time, the presence of hydrophilic sites on the biochar surface and macropores is relevant to improve soil physical properties, such as drainage and aeration and retention of water (Thies and Rillig 2009). This will produce consequent beneficial interactions with microorganisms of different sizes and adsorption affinities (Muñoz et al. 2014). Indeed, when biochar is sterilized during the pyrolysis process, it offers a great potential as an inoculum carrier. The incorporation of biochar into soils offers the opportunity for simultaneous application of plant beneficial microorganisms (Hale et al. 2014). Biochar properties vary depending on feedstock and production methods, but many biochars have characteristics that are also conducive for use as inoculum carriers, including high internal porosity, large specific surface area, and the ability to adsorb organic compounds and microorganisms (Abit et al. 2012).

The use of PGPR in agriculture is often limited by a low percentage of survival after microorganisms are introduced into the soil. Besides, direct microorganism inoculation of liquid inoculant decreases cell survival, which greatly reduces their vertical transport and the ability to colonize plant roots. For these reasons, the use of carrier materials to improve cell survival and protection could be one of the key factors for successful inoculation of PGPR into the soil. However, even though there are different encapsulation techniques, no formulation of PGPR has been developed by the microbial inoculant industry by this method (Bashan et al. 2014).

Encapsulation represents a wide area of research for the food, pharmaceutical, aquaculture, and cosmetics industries. In fact, different and efficient encapsulation methods have been developed to serve different purposes. Nevertheless, almost no method has been evaluated for the production of microbial inoculants. Many of these promising technologies, which are used in other fields, are worth to be evaluated in greenhouse and field conditions in order to improve the development of microbial inoculant formulations as well as quality of the bioformulations for successful inoculation.

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An Overview of Globally Available Bioformulations

15

Samina Mehnaz

Contents

Abstract

As the awareness about the environment is increasing among the public, the use of chemicals for the food production is scaring them. They are becoming more interested in organic food. Chemical fertilizers and pesticides are in regular use throughout the world, to increase the yield and to keep the plants and their products safe from diseases.

S. Mehnaz (✉)
Department of Biological Sciences, Forman Christian College (A Chartered University), Ferozepur Road, Lahore 54600, Pakistan
e-mail: saminamehnaz@fccollege.edu.pk

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_15

These chemicals have their advantages and disadvantages. However, nowadays, environmentalists, researchers, and the public are looking more critically at disadvantages. Fortunately, now we have options for replacing or at least minimizing the use of these chemicals. These alternatives are the bioformulations containing living microbes such as bacteria and fungi. These are commercially available all over the world as biofertilizers and biopesticides/biofungicides. These can be used alone or in combination of chemical fertilizers. This article is a review of globally available bioformulations which are "in use" in different parts of the world as well as those which are under the process of commercialization.

15.1 Introduction

Since green revolution, the use of chemical fertilizers has increased tremendously. The use of these chemicals is very important to get higher yield from smaller area, but on the other hand, excessive use of these chemicals is contaminating ground water and soil, decreasing soil fertility and ruining the atmosphere as well. It is becoming a major problem for the public, environmentalist, and industry. As these chemicals are becoming a risk to human health, advanced countries are implementing strong rules and regulations for their use. For Third World countries, cost, preparation, and availability of fertilizers are big issues. Biofertilizer is the most feasible solution to these issues as it is an eco-friendly option and maintains the soil and crop health with increased efficiency. The term "biofertilizers" has been used for several kinds of formulations. In general, anything which is not chemically synthesized, biodegradable, and can be used as a fertilizer, is known as biofertilizer, i.e., compost, humus, animal and human waste, organic matter, etc. However, scientifically, biofertilizer means a fertilizer which contains living organisms and can be broadly classified as nitrogen-fixing and phosphate-solubilizing biofertilizers which contain bacteria or fungi. Recently, zinc and sulfur solubilizers and potash mobilizers have also been identified as biofertilizers.

When hazardous effects of chemical fertilizers are discussed, the contribution of chemical pesticides cannot be ignored as these synthetic formulations are also equally contributing in destroying the soil, water, environment, and human health. When the use of chemical fertilizers increased the vigor and yield of crops, it also increased the diseases and pest attack on the plants. These factors became responsible for losses of billions of dollars, worldwide. As a next step, chemical pesticides, fungicides, herbicides, bacteriocides, etc. were introduced in the market to protect the crop. These chemicals are mostly applied as foliar sprays and as seed treatment and sometimes on fruits as well to protect them against postharvest diseases. Now, biopesticides have been introduced in the market to reduce the use of chemicals. These bioformulations also contain bacteria and fungi, individually or in combination. These can be used in the same way as the chemicals such as foliar spray, as seed dressing, or as soil treatment.

This chapter is focused on all types of bioformulations including biofertilizers and biopesticides, containing bacteria and fungi as main components. Those which do not contain any living organism have not been included. The brief introduction and information about biofertilizers and biopesticides available in different regions of the world, with their mode of action, wherever disclosed, are provided in the following sections.

15.2 Novozymes (www.novozymes.com)

It is a very well-known European company, based in Denmark which sells several bio-products to increase fertility and yield of crops as well as control the diseases. Their products are available with and without lipo-chitooligosaccharide (LCO) Promoter

Technology. LCO Promoter Technology is a unique molecule that, when present "at the time of planting," enhances growth processes such as root and shoot development, immediately and independently of variety, soil, and environmental conditions. It provides a healthier start for plants, translating into higher yields and better returns at the end of the season.

The products of Novozymes are available in three categories, i.e., biofertilizers, bio-yield enhancers, and biocontrol products. First two products are commercially available and most of the biocontrol products are under registration process. Only those products which contain microbes are listed below, and detailed information is available on the company's website.

15.2.1 Biofertilizers

JumpStart contains the fungus *Penicillium bilaii* that makes phosphate available to the plant. Fungus colonizes the plant roots, releases organic compounds into the soil that break the bonds between phosphate and other elements. The plant gets access to more phosphate, and the fungus gets its nutrition from plant, forming a symbiotic relationship. It is available for canola, wheat, and legume crops.

JumpStart LCO is a combination of *Penicillium bilaii* and LCO Promoter Technology. *Penicillium bilaii* unlocks bound phosphate, and LCO Promoter Technology increases root and shoot development in the early growth stages.

TagTeam is a multi-action inoculant, specifically for legumes. It makes better use of phosphate and provides more fixed nitrogen. It is a combination of rhizobia strains with *Penicillium bilaii*. This product is available in granular, peat, and liquid formulations for use on pea, lentil, chickpea, soybean, and dry bean.

TagTeam LCO is a multi-action inoculant that combines the LCO Promoter Technology® and TagTeam, available in liquid and granular formulation, for use on pea, lentil, and soybean.

Cell-Tech (N-Prove) contains "nitrogen-fixing bacteria" (name of organism is not disclosed), making nitrogen available for the plant to use.

Optimize® with LCO Promoter Technology® combines a nitrogen inoculant with LCO Promoter Technology, available in a liquid formulation for soybean.

Nitragin Gold® contains rhizobia strains with patented slow-drying system, assures a high number of bacteria on the seed, and results in high levels of nitrogen fixation for maximum yield, available for alfalfa and sweet clover.

Apron® XL/Allegiance® FL compatible Nitragin Gold compatibility with Apron XL and Allegiance FL ensures the combined benefits of an inoculant and a fungicide where required. Both products have high bacterial count for excellent longevity (18 months on alfalfa and sweet clover) and treat alfalfa and sweet clovers (white, yellow, hubam, madrid, bitter, and sour clover).

RhizoMyco contains 18 species of endo- and ectomycorrhizae and growth-promoting substances. It is available in a soluble/injectable form to provide broad-spectrum application for increased nutrient update and enhances root systems.

RhizoPlex is uniquely formulated with Novozymes proprietary blend of patented bacterial cultures and stress reducing ingredients, plus 18 species of endo- and ectomycorrhiza.

RhizoMyx is an endomycorrhiza inoculant designed to improve the plant performance by increasing root development, making nutrients more available.

Legume inoculants **Glycimax®**, **Rhizomax®**, and **Legumax®** effectively supply nitrogen to a wide range of legume crops.

15.2.2 Bio-yield Enhancers

RhizoBio® is composed of 18 species of endo- and ectomycorrhiza and a complex biostimulant for increased nutrient and water uptake and tolerance to stress.

RhizoMyx® is uniquely formulated with nine species of endomycorrhiza and a biostimulant to provide broad-spectrum application for increased nutrient and water uptake and tolerance to stress.

15.2.3 Biocontrol Products

Met52 is a bioinsecticide, containing spores of the soil fungus *Metarhizium anisopliae*. Suspended spores of *Metarhizium* attach to the surface of the target insect, germinate, penetrate the exoskeleton, and grow inside the pest, and death of target pest takes place in few days.

Taegro is a bacterial-based biofungicide/bactericide used for suppressing selected soilborne and foliar diseases.

15.2.3.1 Under Registration Process

TrichoderMax® contains the fungus *Trichoderma asperellum* for the effective control of a number of significant soilborne crop diseases.

BoveMax® is a bioinsecticide containing *Beauveria bassiana* for the control of Broca (*Hedypates betulinus*) infestations in Erva-mate plantations.

MethaMax® is a bioinsecticide containing *Metarhizium anisopliae* for the control of Cigarrinha (*Mahanarva fimbriolata*) infestations in sugarcane.

15.3 BioAgri (http://www.bioagri.se)

Lantmännen BioAgri AB is established since 1996 in Sweden. The main focus of the company is to control the seed-borne diseases of grains. The company developed and marketed three biopesticides and a biofertilizer. Biopesticides are based on *Pseudomonas chlororaphis* strains. Their products are available all over the Europe for 10 years. Brief information about their products is given below:

15.3.1 Cedomon®

Cedomon is a biopesticide that contains *P. chlororaphis* as an active ingredient. However, it also contains other ingredients, such as rapeseed oil, which supports its application. This product is available since 1997, and it is effective against several types of seed-borne diseases on barley and oats except barley loose smut caused by *Ustilago nuda*. In some countries, treated seed has also been approved for use as feed.

Cedomon®-treated seed can be stored, transported, and handled in the same manner as normal seed. The product can be used in most treatment equipment. The recommended dosage is 7.5 L/ton of seed. Shelf life of the product at 4–8 °C is up to 8 weeks after delivery and 3 weeks at room temperature. Treated seeds can be stored for up to 1 year. Cedomon® is currently approved for use in Sweden, Finland, Norway, Denmark, Poland, Lithuania, and Italy. A large portion of Swedish grain seed is treated with Cedomon®. Since its launch, a total of approximately two million hectares (in several countries) have been sown with Cedomon®-treated seed.

15.3.2 Cerall®

Cerall® is also a biopesticide with *P. chlororaphis* and water. It has been developed

so as to be specially adapted to bare seeds such as wheat. It is effective against the seed-borne diseases of wheat such as common wheat bunt (*Tilletia caries*), wheat leaf spot (*Septoria nodorum*), and *Fusarium* (*Fusarium* spp.) in wheat. It is also effective against the seed-borne diseases caused by *Ascochyta* spp. The recommended dosage is 10 L/ton of seed. Its shelf life is same as for Cedomon. Cerall® is currently approved for use in Sweden, Finland, Switzerland, Lithuania, and Austria.

15.3.3 Cedress®

This product also contains the same ingredient as Cerall (*P. chlororaphis* and water), but it has been developed specially for the treatment of pea seeds. Recommended dosage and shelf life are same as for Cerall. Cedress® is currently approved for use in Sweden.

15.3.4 Amase®

It is a biofertilizer, based on *Pseudomonas azotoformans* and organic and inorganic nutrients. It is a dry product for growth stimulation of plants and can be mixed in peat-based substrates or in irrigation water. It is easily absorbed by the roots to provide long-term growth-promoting effects. It helps plants to quickly produce a large and strong root system, more resistant to stress and grow faster. It is recommended to be used for potted plants and nursery plants. Pine, spruce seedlings, cucumber, lettuce, tomato, peppers, eggplant, cabbage, and broccoli have shown good results with this product.

15.4 Fertibio (www.fertibio.com)

Fertibio is a Spanish company which makes biofertilizers and biopesticides as well as organic fertilizers and pesticides which are biodegradable. Their bio-products are described below.

Fig. 15.1 Bioformulations of Fertibio, Spain; biopesticide Bioscrop BT16 and biofertilizer Rhizosum N

15.4.1 Bioscrop BT16

It is a biopesticide based on *Bacillus thuringiensis* var. *kurstaki* (16 million IU/g) (Fig. 15.1). It is active against lepidopteran larvae and beetles. δ-Endotoxin synthesized by this bacterium is ingested by insect and causes injuries and paralysis of the digestive tract. Insect dies within 24–48 h. It is applicable to cotton, citrus, cauliflower, deciduous fruit trees, horticultural brassicas, olives, pepper, banana, and tomato. It is dissolved in water and applied as foliar spray (0.5–1.5 kg dissolved in 800–1,000 L and applied on 1 ha).

15.4.2 Rhizosum® N

The biofertilizer product is named as Rhizosum N (Fig. 15.1). It contains nitrogen-fixing bacteria but genera are not disclosed. The advancement of technology allows the crops to get nitrogen from the atmosphere. Recommended doze supplies 30–50 units of nitrogen per hectare, depending on soil moisture and season.

15.5 Symborg (www.symborg.com)

Symborg SL is a Spanish company which sells its product all over Europe and USA. The company has launched three products (Fig. 15.2) described below.

Fig. 15.2 Bioformulations of Symborg, Spain

15.5.1 MycoUp

It is a biological inoculant based on *Glomus iranicum* var. *tenuihypharum*, a mycorrhizal fungus (1.2×10^4 propagules/100 ml substrate). It is a root colonizer that boosts plant growth by more efficient water and nutrient absorption, improving plant vigor. It is recommended for vegetables, fruits, and woody crops at the rate of 2 kg/ha.

15.5.2 MycoUp Attack

This biological inoculant is a combination of MycoUp (1.2×10^4 propagules/100 ml substrate) and "Attack." Attack is an organic complex, with MycoUp; it promotes the microbiotic activity of the soil, stimulates mycorrhizal growth, and activates the plant's defense system against nematodes and other phytopathogens. It is recommended for vegetables and woody crops at the rate of 2–3 kg/ha. Diluted solution of the product should be applied close to root system by injection or drip irrigation.

15.5.3 Resid

It is a biological product which contains *G. iranicum* var. *tenuihypharum* as its active ingredient with two mineral clay substrates, bentonite and smectite with a concentration of 0.5×10^3 propagules in 100 ml substrate. It is recommended for grains as seed coatings at the rate of 5 kg/100 kg seeds. Resid increases agricultural yields by optimizing fertilizer, water and soil inputs, tolerance to drought and salinity, and protection against fungal root diseases; contributes to soil regeneration through mycelium network; restricts the loss of CO_2 by recapturing it and converting it into fungal biomass; and produces no harmful residues.

15.6 Biagro (http://www.biagrosa.com.ar)

Biagro S.A. was established in 1984 in Argentina. Legume inoculants are their main products.

15.6.1 Nodulest 10

The soybean inoculant "Nodulest 10" is prepared by mixing a pure culture with sterilized *Sphagnum* peat moss. The product contains two different strains of *Bradyrhizobium japonicum* (USDA 138 for USA, 532C for Canada) depending on the country where it is used. The product has very high concentration of *B. japonicum* ($>2 \times 10^{10}$/ml). Therefore, a small quantity of carrier (peat moss) is used per bushel (60 lbs) of soybean seed. This characteristic allows the inoculant with

lesser risk of blockage of seeders. Biagro S.A. is actively engaged in the search and selection of more efficient rhizobial genotypes to be used under suboptimal soil and climatic conditions.

15.6.2 Liquid PSA

It is a commercial formulation that contains *Pseudomonas aurantiaca* strain SR1. It is registered with Argentina's National Service for Agricultural Health (SENASA) for wheat growth promotion.

15.7 Labiofam (www.labiofam.cu)

It is a scientific institution of Cuba producing pharmaceutical products, biopesticides, and biofertilizers as well. Biopesticides are for killing mosquito larvae and rats. Biofertilizers under the name of Nitrofix and Bioenraiz are available. Nitrofix contains *Azospirillum brasilense*, and Bioenraiz contains phytohormones extracted from *Rhizobium*. Information about application of these biofertilizers or any detail about these products is not shared on the website.

15.8 Flozyme (http://www.flozyme.com/agriculture)

Flozyme Inc. was founded in 2012 in Atlanta, USA, and offers the solution for cleaning wastewater and agriculture. One of their biofertilizer products is described below.

15.8.1 Inogro

It is a cocktail of more than 30 microbes, selected for their abilities to rehabilitate soil and make it more productive. The product is organic, sustainable, environment friendly, and highly compatible with organic farming practices. It is suspended in 12 % humic acid carrier and stabilized at pH 7, due to which it effectively works in varied climates and soil conditions. These humates are high in organic matter and micronutrients, including Ca, Mg, Zn, Mn, etc. and also act as carbon sources for the microbes. This microbial formulation unlocks bound nitrogen in the soil and absorbs nitrogen from the air, solubilizes bound phosphate, and makes it available to plants for optimal growth and development. It is highly competent in soil and also promotes a healthy soil pH. It captures additional water and available nutrients from naturally occurring organic matter in the soil that enables plants to better sustain adverse environmental conditions. This product is claimed to inhibit plant pathogens, enhance the plant's natural defense mechanisms by *Trichoderma* strains (present in product), and increase the plant's resistance to pests.

Inogro is shown to increase crop yields by 20–400 % and decrease the use of chemical fertilizer from 50 % to 100 %. The product also reduces water requirements (~20 %) and pesticide usage. Crops have shown higher nutrient content, faster and earlier germination, earlier maturation, and ability to withstand stresses. During greenhouse trials, increased yields of 301 % for rice, 400 % for tomatoes, 127 % for soybeans, 86 % for peas, 258 % for okra, 234 % for peanuts, and over 100 % for garden beans and wonder bush beans have been shown.

15.9 AgriBiotics Product, Inc. (www.agribioticproducts.com)

The product is developed by Michigan State University (MSU) and manufactured by BioSoil Enhancers, Inc. Mississippi. Product brand is described below.

15.9.1 AgriBiotic Microbics with SumaGrow

SumaGrow is a combination of several microbes including bacteria and fungi (Fig. 15.3). It is a polymicrobial inoculant, a cocktail of more than 30 microbes with multiple functions. These microbes include nitrogen-fixing microbes isolated from leguminous and nonleguminous crops. Liquid humate is used as a carrier for the microbes. These microbes work under aerobic,

Fig. 15.3 A bioformulation developed by Michigan State University, USA

non-aerobic, acidic, and alkaline conditions, therefore useful for multiple crops under multiple conditions. The SumaGrow microbial formulations improve nutrient and water uptake, enhance root and plant growth and crop yield, improve plant efficiency to use solar energy, reduce stresses of transplanting and drought, increase cation exchange capacity, improve soil health and water retention, help release micronutrients and trace elements, stimulate germination, increase healthy decomposition of organic matter, and reduce putrifaction.

The company claims innovation as compared to competitors due to four reasons, First, it contains multiple groups of organisms (up to eight), second, the product has six functions vs. two functions, third, it retains viability over a long period of time at ambient temperature, and fourth, it is concentrated at 10^{17} vs. 10^{8}–10^{10} CFU/ml. The product has demonstrated the ability to increase crop yields by 20–200 plus % while decreasing fertilizer usage from 50 to 100 % and reduces water (estimated 20 plus %) and pesticide usage. Experiments performed by MSU showed a 301 % increase in rice yield, 400 % increase in tomato yield, 127 % in soybeans, 86 % in peas, 258 % in okra, 234 % in peanuts, and over 100 % in garden beans and wonder bush beans in greenhouse.

Recommended application dose is one gallon per acre, diluted in the desired amount of water. For second application, foliar spray is recommended. It can be applied with irrigation water and with liquid nitrogen and herbicides but not with fungicides. Product should be applied without any fertilizer or with half dose of fertilizer.

15.10 Mapleton Agri Biotec Pty Limited (mabiotec.com)

It is an Australian company with three major products. This company is distributing its products in Australia, USA, UK, Europe, North and South Africa, Turkey, South America, and several other countries.

15.10.1 TwinN

It is a freeze-dried microbial inoculum packed under vacuum which provides it a longer shelf life. For application, initially, it is dissolved in small amount of water and later in large amount. It contains a consortium of microbes which includes nitrogen fixers, phosphate solubilizers, and growth hormone producers. These microbes can live in rhizosphere, root, shoot, and leaves as endophytes. It is an inoculant for crops, pastures, and trees. This product can be applied through irrigation system, sprinkler, spray, etc., depending on the crops.

15.10.2 CataPult

It contains the vesicular-arbuscular mycorrhizal (VAM) fungus *Glomus intraradices* and two species of *Bacillus* with its unique release and catch technology. It is available in granular form. It is easily applied at planting stage at the rate of 1.75 kg/ha. The P-solubilizing *Bacillus* species in CataPult colonize the roots and rhizosphere and solubilize P that is unavailable to the plant. In many soils, large amount of applied P ends up in bound form that can be accessed by CataPult "release and catch" technology. The *Bacillus* species stimulate a more vigorous root system and root hairs and suppress a number of root pathogens. VAM colonizes the roots and a network of hyphae extend beyond the root hair zone.

These hyphae collect P, N, Ca, Mg, and micronutrients and deliver them to the plant.

15.10.3 Nitroguard

Information about this product is not released.

15.11 EM Pro-agriculture

During 1980, EM technology was developed in Japan. EM is a naturally fermented liquid probiotic solution which contains "effective microorganisms" including lactic acid bacteria, yeast, and phototrophic bacteria. These are non-pathogenic and not genetically modified organisms (non-GMO). It is a cost-effective technology and beneficial for agriculture. EM works by improving the soil ecology to assist in plant growth and health.

EM microbes secrete vitamins, organic acids, minerals, and antioxidants, increase humus content of the soil, and provide better environment for other microorganisms and plants for better growth. Photosynthetic microbes, another constituent of EM, have powerful detoxifying, antioxidative, and antientropic properties which improve the poor soil. Plants inoculated with EM had shown higher yields. It improves soil structure and nutrient availability, lowers disease pressure, and improves the quality of produce and storage life. EM can be applied on seeds, at the time of transplantation, as foliar spray and for soil treatment.

15.12 Bio Power Lanka (www.biopowerlanka.com)

It is a company based in Sri Lanka. Among their agri-related bio-products, three of them contain microbes.

15.12.1 Bio Vaccine

It is a biofungicide that contains *Trichoderma viride* (Fig. 15.4). It protects the plant from rot and wilt diseases. It destroys the pathogenic fungi including *Pythium*, *Rhizoctonia*, and *Fusarium* spp. that cause root rot, stem rot, seed rot, fruit rot, and wilt diseases. *T. viride* grows like coils around the pathogen and degrades the cell wall of the pathogenic fungi by secreting a wide variety of enzymes including celluloses and chitinases. This process is known as mycoparasitism – one fungus killing the other fungus by limiting its growth and metabolic activity. *T. viride* also induces systemic resistance and prepares the plants to destroy the pathogens. The product helps the root system to

Fig. 15.4 A biofungicide and a liquid biofertilizer launched by BioPower, Sri Lanka

increase uptake level of nutrients and moisture which will improve the tolerance to stressful growth conditions. The product contains 2×10^8 spores/ml. It is recommended to dilute and apply at nursery and transplantation stage to control soilborne diseases.

15.12.2 Bio Gold

Bio Gold is a liquid formulation that contains native isolates of compatible microorganisms such as *Azotobacter chroococcum* and *Pseudomonas fluorescens* (Fig. 15.4). *A. chroococcum* is a nitrogen-fixing soil bacterium which improves the soil properties by secreting polysaccharides and provides nitrogen to the crop in a balanced way. This bacterium also contributes to drought and disease resistance by improving soil's physical properties and secreting various growth-promoting substances. *P. fluorescens* is a potential bionematicide and used for cardamom, potato, and other vegetable crops, fruits, and cereal crops. Secondary metabolites produced by this bacterium are highly effective to control rot and wilt diseases of plants and nurseries. This bacterium also secretes various organic acids that help in the solubilization of insoluble phosphorus, and hence, the phosphorus availability to crops is increased. The product can be applied to all agricultural and horticultural crops by spraying around the root zone, as a foliar spray, by drip irrigation, and as a seed inoculant.

15.12.3 Bio Phos®

It is a liquid formulation which contains *Bacillus megaterium* – a phosphate-solubilizing bacterium. The use of Bio Phos® and Eppawala rock phosphate (ERP) mixture for plantation crops can reduce the recommended ERP doze by 50–75 % to the fact that available phosphorus content is increased. ERP+ Bio Phos® mixture could be used for annual crops to supply the recommended phosphorus requirement, as a substitute of Triple Super Phosphate (TSP).

15.13 AgriLife (www.agrilife.in)

The company is based in Hyderabad, India, and their products include biofertilizers, biopesticides, biostimulants, and other agri-related products. Among biopesticides, three types of products are available based on botanicals, microbes, and nanoparticles. Those which contain microbes have been discussed in this chapter.

15.13.1 Biofertilizers

AgriLife has launched 15 biofertilizers (Fig. 15.5), based on nitrogen fixing, phosphate solubilizing, potassium, ferrous, sulfur, silica and zinc mobilizing bacteria, manganese-solubilizing fungus, and vesicular arbuscular mycorrhizae (VAM). Each biofertilizer has a

Fig. 15.5 Biofertilizer formulations of AgriLife, India

single bacterial strain; consortium is not used in any biofertilizer. For each nutrient, a specific biofertilizer is available.

15.13.1.1 Agrilife Nitrofix

These are nitrogen-fixing biofertilizers that contain *A. chroococcum* (MTCC 3853), *A. vinelandii* (NCIM 2821), *Acetobacter diazotrophicus* (MTCC 1226), *Azospirillum lipoferum* (NCIM 2908), and *Rhizobium japonicum* (NCIM 2743). Each biofertilizer is recommended for a different crop and with a different mode of application depending on crop. However, all of these are available as carrier-based powders containing 5×10^7 CFU/g or in liquid form 1×10^8 CFU/ml.

15.13.1.2 P Sol B

These are phosphate-solubilizing biofertilizers and contain *Pseudomonas striata* (*NCIM 2847*), *Bacillus polymyxa* (NCIM 2188), and *Bacillus megaterium* (NCIM 2087). All of these are available as carrier-based powder containing 5×10^7 CFU/g or in liquid form 1×10^8 CFU/ml. These biofertilizers can be applied on seed, seedling, and soil or through drip irrigation.

15.13.1.3 Agri Life AgriVAM

It is based on spores and fragments of VAM (*Glomus* species) with vermiculite as carrier. It has 100 infective propagules/g. It helps in water absorption, phosphorus solubilization, and macro- and micronutrient availability and improves drought tolerance and resistance to soilborne fungal pathogen. It can be applied to soil, seed, and nursery bed and at planting stage.

15.13.1.4 Fe Sol B

It contains an autotrophic, acidophilic *Acidithiobacillus ferrooxidans* that releases iron oxidase which metabolizes ferrous. It is available as carrier-based powder or in liquid form. It can be applied to seedlings and soil or by drip irrigation.

15.13.1.5 K Sol B

It contains *Frateuria aurantia* which produces organic acids and enzymes which mobilize potassium ions. It is available as carrier-based powder containing 5×10^7 CFU/g or in liquid form 1×10^8 CFU/ml and can be applied to seedlings and soil or through drip irrigation.

15.13.1.6 Mn Sol B

It contains an aerobic fungus *Penicillium citrinum* which produces citric acid and oxalic acid and solubilizes manganese. It is available as wettable powder and contains 5×10^7 CFU/g, applicable to seedlings and soil and through drip irrigation as well.

15.13.1.7 Si Sol B

It contains spores of *Bacillus* species releasing organic acids which play role in silicate weathering. Silica helps the plant to tolerate biotic and abiotic stresses and pest and disease attack. This product is available as wettable powder containing 1×10^8 CFU/g, applicable to seed, seedling, and soil and by drip irrigation.

15.13.1.8 S Sol B

This product contains autotrophic, acidophilic bacterium, *Thiobacillus thiooxidans*, that oxidizes sulfur and secretes organic acids, bringing down the pH of soil and helpful in reclaiming alkaline soil. It is applicable to seedling and soil and by irrigation.

15.13.1.9 Zn Sol B

This biofertilizer contains another strain of *T. thiooxidans* that oxidizes zinc and makes it available to the plant. This product can be applied in the same way as S Sol B.

15.13.2 Biopesticides

Twenty-two biopesticides have been launched by the company (Fig. 15.6). One of them is for mosquitoes; however, the rest of them are to treat agriculture-related problems. Few of them are consortium based and their active ingredient and/or details of microbes are not shared. Some of them are based on individual bacterial or fungal strains to kill nematodes and insects or treat fungal diseases. A brief introduction is given below.

Fig. 15.6 Biofungicides formulated and marketed by AgriLife, India

15.13.2.1 BioKuprum

This product contains the fungus *Chaetomium cupreum* and protects the plants from diseases like rusts, blights, rots, and leaf spots. It is formulated as wettable powder with 2×10^6 CFU/g. It can be applied on seeds and tubers or as a foliar spray.

15.13.2.2 Biotilis

This biofungicide also works as biofertilizer as it contains *Bacillus subtilis*, plant growth-promoting rhizobacteria (PGPR). As a fungicide, it targets rots, blights, wilt, leaf spot, and mildews. This product is available in powder form containing 1×10^8 CFU/g or 1×10^9 CFU/g.

15.13.2.3 Downycare

This product is specifically formulated to treat downy mildew disease of plants caused by different fungal pathogens. It contains spores and mycelium fragments of fungus *Fusarium proliferatum*. Powder formulation containing 1×10^8 CFU/g is recommended to be used as foliar spray.

15.13.2.4 Ecosom TV

Trichoderma viride containing bioformulation protects the plants from soilborne and seed-borne fungal pathogens. With 2×10^6 CFU/g, it can be applied as seed dressing, in the soil, or by drip irrigation.

15.13.2.5 Ecosom TH

Trichoderma harzianum containing formulation is a biofungicide and a bionematicide. It protects the plants from fruit rot and pathogenic nematodes. Formulation is available in powder form containing 2×10^6 CFU/g. It can be applied as foliar spray, by drip irrigation, by soil drenching, by root dipping, and at nursery stage.

15.13.2.6 Powderycare

Ampelomyces quisqualis is a fungus that protects plants from powdery mildew causing plant pathogens. Powdery formulation contains 2×10^6 CFU/g and it is applied as foliar spray.

15.13.2.7 Sheathguard

This product is a biofungicide, a bionematicide, and a biofertilizer as it contains the PGPR *P. fluorescens*. It kills pathogenic nematodes and protects plants from sheath blights and other fungal diseases. Formulation is used as a seed dressing or applied on nursery beds before transplanting the crops.

15.13.2.8 Biofit

It is a microbial consortium with an antifungal and PGP activity. It targets the fungi which cause rots, blights, and mildews.

15.13.2.9 Biorub

This product is specific for pink disease of rubber plant caused by a fungus. Formulation contains

consortium of antagonistic microbes with 1×10^8 CFU/g. It can be applied as foliar spray, by soil application, and by stem swabbing.

15.13.2.10 Diebackcare

It is a consortium-based biofungicide for dieback disease of orchards and should be applied on soil.

15.13.2.11 Seedguard

It comprises PGPR and microbial cultures with antifungal properties which encourage seed germination and control seed-borne fungal diseases. It is a microbial concentrate developed for seed coating.

15.13.2.12 Insecticides and Nematocides

Other than biofungicides, several nematocides and insecticides have also been launched (Fig. 15.7). They include: Bionemagon (nematocide) which contains *Bacillus firmus*; Paecilo (nematocide) which contains *Paecilomyces lilacinus*; BorerGuard (insecticide) which contains microbial consortium and protects plants from borer attacks; Lipel which contains *B. thuringiensis* var. *kurstaki* and protects crops from flying insect pests; Mealikil plus (insecticide) which contains *Verticillium lecanii* that kills mealybugs and sucking insects; Pacer (insecticide) which contains *Metarhizium anisopliae* and kills soil insects; Paecilomite (insecticide) which contains *Paecilomyces fumosoroseus* and kills many types of insects and mites; and Racer (insecticide) which contains *B. bassiana* and kills insects pests.

Fig. 15.7 Bioinsecticide and bionematicides products by AgriLife, India

15.14 Commercially Available Biofertilizers in Pakistan

15.14.1 Fertibio

It is a biofertilizer launched by a private company "Microbial Biotechnologies." It contains a mixture of nitrogen-fixing, phosphate-solubilizing, and growth hormone-producing bacteria. Formulation is available in powder form, and it can be applied as a seed dressing and through irrigation as well as by sprinkling. It is recommended for rice, wheat, corn, cotton, sugarcane, and vegetables.

15.14.2 BioPower

A government research institute has launched a biofertilizer with the commercial name of "BioPower" in 1996. It is being produced and marketed for various leguminous and nonleguminous crops, like cotton, maize, rice, sugarcane, and wheat (Fig. 15.8). For every crop, a different consortium of PGPR has been used. It can be applied in powder form as well as in solution form, at the time of transplantation and after transplantation depending on the type and requirement of crops.

15.14.3 Auriga Group (www.aurigagroup.com)

Biofertilizer is one of the products manufactured by Auriga Group of industries. This product contains gram-positive phosphate-solubilizing bacteria which increase fertilizer use efficiency of added phosphate fertilizer and ensures phosphorus availability till crop matures. Plant growth hormone-producing bacteria are also included in this product that enhance the plant growth and enable the high yield. This company

Fig. 15.8 A biofertilizer produced by a research institute of Pakistan

is currently working on formulation of biopesticides.

15.15 Future of Bioformulations

Among bioformulations, biofertilizers can be divided into two major categories: nitrogen fixers and nutrient solubilizers/mobilizers. Most of the nitrogen-fixing biofertilizers contain *Azospirillum*, *Azotobacter*, and *Rhizobium*, and phosphate solubilizing have *Bacillus* and VAM. Recently, zinc, sulfur, and potash mobilizers/solubilizers have also been launched in the form of biofertilizers. Biofungicides are based on *Trichoderma*, *Bacillus*, and *Pseudomonas* strains of different species. These bacterial and fungal strains are common residents of plants rhizosphere.

For more than 100 years, biofertilizers are in use, in developed part of the world. However, it could not get the same level of popularity and usage as the chemical fertilizers still do. Among advanced countries, the major reason is inconsistency in the results of field application of biofertilizers. In the developing world, lack of awareness among farmers is the major hurdle in the growth of biofertilizer market. Looking at the current situation of fossil fuels, other alternative energy resources, high prices of chemical fertilizers, pesticides, herbicides, fungicides, and insecticides, on-time availability of these chemicals; bioformulations seem the only answer and the most effective solutions of these problems.

North America is promoting the use of bioformulations over chemical ones, due to their concern about human health- and environment-related issues. European union is promoting not only the use of bioformulations but organic farming as well through "common agriculture policy" by providing up to 30 % of payment as direct green payment. Among Asian countries, the government of China is promoting the production of bioformulations indirectly by tax holidays, exemption of VAT, and excise and agriculture tax to manufacturing industries of these products. Promoting the use of biofertilizers is part of the 5-year plan of the Indian government. The first biofertilizer, "BioPower," in Pakistan was launched by a government organization in 1996, and since then, they are selling it. Later on, several private companies started its manufacturing. Currently, at private level, Auriga is the biggest group of companies manufacturing as well as promoting the awareness and use of bioformulations among farmers.

A statistical review on the global scale of biofertilizers was published in January 2014. (http://www.grandviewresearch.com/industry-analysis/biofertilizers-industry). According to this, North America is the most dominant market of biofertilizers as it is valued at USD 420 million in 2012, followed by Europe. It reflects the fact that biofertilizers were first commercialized in North America and Europe; however, now, demand is increasing in South America and Asia Pacific as well, making it third in the row, in the biofertilizers market. Among the global revenues from biofertilizers, nitrogen fixers make the major contribution of 77 %, phosphate solubilizers are at 15 %, and the rest of the biofertilizers are responsible for 8 %. The global biofertilizers market is projected to generate USD 1650 million by 2019.

Looking at the current scenario, the future of bioformulations seems very bright. Due to awareness about hazardous effects of chemical formulations on human health and environment, acceptance of these formulations by general public has been increased. Now, it is time that governments should interfere, and with the help of regulatory authorities, the use of these formulations should be enforced in countries where farmers are not using them at large scale. Researchers should also play their role by searching for more diverse bacterial and fungal organisms rather than sticking to only two to three traditional genera.

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Regulation of Biopesticides: Global Concerns and Policies

16

Naveen Kumar Arora, Maya Verma, Jai Prakash, and Jitendra Mishra

Contents

Abstract

In the last few years, biopesticides have emerged as suitable alternatives to synthetic chemical pesticides. They are cheaper and pose no threat to agroecosystems. This is why their demand and production are also increasing at global level. The law and policies regulating their use and development vary from country to country, and in-depth analysis shows that there is no uniform regulatory model that can simplify their regulation and registration process. Although by the effort of some global agencies such as the International Organization for Biological Control (IOBC), European and Mediterranean Plant Protection Organization (EPPO) and Organization for Economic and Co-operative Development (OECD), some flexibility to biopesticide regulation have been provided, but in comparison to chemical pesticides, which have firm market and established nonoverlapping laws, biopesticides lag behind. This chapter provides comprehensive details of regulation systems adopted around the globe and to address shortcomings of existing system; besides this emphasis is also given to adopt innovative practices that could pave way for regulations which are simpler and more universal.

N.K. Arora (✉) • M. Verma • J. Prakash • J. Mishra
Department of Environmental Microbiology, School for Environmental Sciences, Babasaheb Bhimrao Ambedkar University, Lucknow 226025, Uttar Pradesh, India
e-mail: nkarora_net@rediffmail.com

N.K. Arora et al. (eds.), *Bioformulations: for Sustainable Agriculture*,
DOI 10.1007/978-81-322-2779-3_16

16.1 Introduction

Governments around the globe want to reduce the use of chemicals in agriculture due to their harmful effects on environment, and in this regard, they proposed some rules to minimize the use of chemical pesticides. Currently, various countries have diverse policies and laws to promote the use of biological pesticides or biopesticides. Concerns for green and safer food systems have risen globally, and countries have starkly imposed laws regarding the use of synthetic chemicals in food commodities and other allied products (Singh and Arora 2016). In this situation biopesticides as better alternatives gained much attention (Mishra et al. 2015). Biopesticides are mostly based on pathogenic microorganisms and target-specific, ecofriendly and effective solutions for pest problems in crop production (Gupta and Dikshit 2010). Although the use of biopesticides is practically simple and have advantages over chemicals, it has been observed that regulatory systems governing their use and production are somehow more complicated than chemical pesticides (Plimmer 1993). In spite of this discrepancy, the overall market of biopesticides has increased and numbers of registered products are also increasing (Sinha and Biswas 2008). At global level there are many policies and laws which not only promote biopesticide use but also regulate them. Several countries are also trying to overcome difficulties and creating a favourable business environment for commercialization of biopesticides. Clear and transparent administrative processes will provide flexibility to existing investors and at the same time also attract new investors in the biopesticide industry. However, in some countries, appropriate administrative guidelines have not been made available through regulations, and this creates problems in introduction of new biological products in the market (Ochieng 2015). Amongst the major challenges faced by biopesticide companies, registration part is the biggest one. It has been found that inappropriate evaluation methods and disproportionate data requirements often result in a long and lengthy registration process that is unnecessarily very costly and time consuming (Ehlers 2006; AGBR 2015). This discourages especially many small and medium enterprises from applying for such lengthy and costly registration processes (AGBR 2015). In this chapter we have tried to summarize the various regulation processes involved in biopesticide registration around the globe and to further address the problem related to intricate process of registration.

16.2 Regulation of Biopesticides: A Legal Framework

From global regulatory and registration perspective, the most accepted definition of the term biopesticide is by the US Environmental Protection Agency (USEPA) (Libman and MacIntosh 2000). The Division of Biological and Pollution Prevention (BPPD) of USEPA separates the term 'biopesticides' into three broad categories: microbial pest control agents, biochemical pesticides and genetically engineered organisms (GEOs). But due to its transgenic nature, GEOs are not recognized by the European Union (EU) as biopesticides. The current term used in the EU in place of biopesticides is biocontrol agents (BCAs). In most of the countries, these are regulated by governmental agencies for their authenticity and use in the agricultural field. As these products mostly contain live organisms or their derived products, hence, there are problems in regulatory processes and also hurdles in import and export (Mishra et al. 2015). It has also been observed that often, registration cost is very high. Microbial biopesticides have been commercially available for over 30 years, but they have less than 1 % share in the global market for agricultural crop production (Hajek 2004). There is a need to develop novel techniques to improve or modify existing biopesticides that will accelerate their higher production (Leng et al. 2014). Data of current sale levels of biopesticides suggest that generally, registration costs amount to 40 % of annual sales (ACP 2004). The testing cost for product efficacy to fulfil registration requirements is also very high.

Biopesticide regulation is a very important process but it is also very complex and dynamic field. According to Guest (2015), the development of predictive and efficient regulation processes for biopesticide is an important issue, and it must be ensured that the biopesticide product is safe and consistent without limiting commercialization, whereas market growth is also influenced by political and societal pressures (AGBR 2015). The data requirements in a dossier form are again a cumbersome process in the development of biopesticides. Ravensberg (2011) discussed some of the reasons related to unsuccessful submission of data for biopesticide development in European context but some of these are globally reported:

1. Requirements of data are not clearly defined and often adapted same as for in case of chemicals.
2. Guidance documents and test methods are also set up as for chemical substances.
3. End points of risk assessments are not clearly established.
4. Regulators and risk assessors lack expertise.

Waage (1997) also opposed to follow the chemical pesticide model for regulating biopesticide and argued that the entire pesticide regulatory process does not want to adapt the new opportunities which biopesticides provide.

Although biopesticides are used globally, the regulation processes and authorities involved vary at regional or national levels (Table 16.1). Various regulatory bodies and agencies such as International Organization for Biological Control (IOBC), European and Mediterranean Plant Protection Organization (EPPO) and Organization for Economic and Co-operative Development (OECD) have from time to time tried to resolve the registration hurdles faced by countries but the success is limited. The application of newer biopesticides may require new regulatory and economic challenges due to their highly diverse nature, which must be addressed jointly by the social and natural scientists, policymakers and the industries (Kumar 2015). The main aims of the regulations are, firstly, protection of human and environment (for safety reasons) and, secondly, the characterization of products and thereby ensure that manufacturers supply consistent and reliable quality products (Chandler et al. 2011).

16.3 Regulations Worldwide

The registration of biopesticides is perhaps the most intricate part around the world. There has been a rise noticed in the recent past for registered biopesticide products but this could be more if harmonization of registration process happens globally. Various types of authorities and regulations are emerging to regulate the biopesticides but very little flexibility is provided. This section summarizes the regulations being followed around the globe in different countries and continents.

16.3.1 Asia

Asian market presents a large opportunity for biopesticides with growing and emerging economies such as China and India and established one such as Japan (http://www.reportsnreports.com/reports/319510-global-biologicals-market-researchreport.html). Besides these other Asian countries, Pakistan, Bangladesh and South Korea are also emerging in the field of biopesticides.

16.3.1.1 China

In 1997, the Regulation on Pesticide Administration law was introduced to govern pesticide production and use in China, and the law mandates the registration of biopesticides before entering the market (Kabaluk et al. 2010). The Chinese Ministry of Agriculture (MOA) along with other ministries is responsible for registration, production and commercial management of pesticides (Fang 2014). The Institute for the Control of Agrochemicals, Ministry of Agriculture (ICAMA 2008) is the national regulating authority which supervises the pesticide registration including biopesticides. Only registered and authorized companies (no research institutes, universities or other research groups) under the General Administration of Quality Supervision,

Table 16.1 Biopesticide regulations at glance

S. no.	Name of the country	Major regulatory actions/bodies	Contacts for further information
1.	India	The Insecticide Act (1968) regulates biopesticide for its induced production and application. CIB and RC work under this act as highly powerful bodies to regulate biopesticides. ICAR also has role in biopesticides regulation.	www.cibrc.nic.in, http://agricoop.nic.in/imagedefault/policy/NCF3.pdf
2.	China	The Regulation on Pesticide Administration (1997) was introduced to regulate pesticide/biopesticide and agrochemical market. Biopesticides are regulated by MOA in addition with other Ministries. ICAMA is the national regulating authority, which supervises the pesticide registration.	ICAMA (2008) and Fang (2014)
3.	South Korea	Registration of pesticides at first level is governed by AMD and RDA, and then it is evaluated by PSED and NAAS. The AMD has a council which is concerned about safety of agrochemical. The microbial pesticide regulations, 2005, have guidelines for biopesticides, and in 2009 it worked as acts for management of agricultural chemicals (RDA 2009).	http://www.mordorintelligence.com/industry-reports/south-korea-biopesticides-market-industry, www.rda.go.kr
4.	Pakistan	The PARC is responsible for monitoring the pesticides (Jabbar and Mallick 1994) and PBR (2005) regulates the marketing or trading of living microorganisms or any related products for any purpose. PBR, Rule 21, regulates the GEM, cells or products and states that these products should be approved by NBC.	Jabbar and Mallick (1994) and PBR (2005)
5.	Kenya	Kenya's pesticide regulatory authority is PCPB which is supported by a range of stakeholders such as the UK DFID. The Agriculture Act (Cap 318) is responsible for the imports and exports of live organisms. The KEPHIS Act, Cap 512 2013, and Plant Protection Act regulate the imports and exports of live organisms. Pest Control Products Act also participates in registration and regulations for microbial pesticides.	Wabule et al. (2004), Kimani (2014), KEPHIS Act, cap 512/2013
6.	Tanzania	In Tanzania, the regulatory body for the pesticide regulation is named as TPRI, under the Ministry of Agriculture.	Stadlinger et al. (2013)
7.	Nigeria	In Nigeria NAFDAC is an agency under the Federal Ministry of Health which is responsible for the control of the manufacture, sale and distribution of fertilizers, biofertilizer and biopesticides.	https://vmapnafdac.wordpress.com/
8.	UK	In the UK, the main regulatory body for biopesticide is CRD/PSD. The CRD is a new Directorate of the HSE which regulates pesticides, biocides, detergents, and chemicals under REACH. 'Biopesticide scheme' has also important role in regulation of biopesticides.	http://www.pesticides.gov.uk/, http://www.hse.gov.uk/pesticides/

(continued)

Table 16.1 (continued)

S. no.	Name of the country	Major regulatory actions/bodies	Contacts for further information
9.	Ukraine	In Ukraine, the MSIPP is responsible for the registration of biopesticides and all other plant protection products. Most of these organizations fall under the research institutes of UAAN and NANU and the latter of which are governed by Ministry of Education and Science.	www.msp.ua/state-registration-of-pesticides-ukraine.htm
10.	Netherlands	Like biopesticide the scheme in the UK, the Genoeg scheme in the Netherlands worked for biopesticide regulation. Genoeg is a 'bottom-up' process whereby a coalition of agencies and other interested parties are involved in creating new regulations. This scheme was developed to enhance the biopesticide production by efficient regulatory actions.	www.genoeg.net
11.	Germany	At national level the regulation in Germany (EC) No. 1107/2009 is implemented through the GPPA 2012. The PPA covers plant protection products, adjuvants and plant resistance improvers. In 2003 PAN launched the OISAT programme in Germany and the aim of this service is to limit the use of hazardous pesticides by providing safer alternatives.	EC (2009), German Plant Protection Act 2012
12.	USA	USEPA is the main authority to regulate the use, sale and distribution of biological pesticides. USEPAs have mainly three parts: (1) FIFRA 1947, (2) FFDCA 1938 and (3) FQPA 1996. US-BPIA promotes industry standards for biopesticides. BPIA is working with the EPA to register biopesticides and also worked as a leading source of reliable information for biopesticides.	www.epa.gov/pesticides/biopesticides, http://www.pesticides.gov.uk/environment.asp, http://www.biopesticideindustryalliance.org/
13.	Canada	PMRA regulates biopesticides, which involves in pesticide evaluation, registration and developed joint guidelines for biopesticides with EPA, OECD. The regulation of non-indigenous macrobials is administered by CFIA under the authority of the plant protection act 1990.The Agriculture and Agri-Food Canada Pest Management Centre is committed to improving access to biopesticides as part of its Pesticide Risk Reduction Program.	www.hc-sc.gc.ca, AAFC (2009), http://www.inspection.gc.ca, CFIA (2013)
14.	Cuba	In 2007, a formal process for registration was published and four institutions CNSV, CICA, CNSB EQDNCP are regulating biopesticides, and one or more involved with registration process.	Gaceta Oficial No. 016 Cuba (2007)

(continued)

Table 16.1 (continued)

S. no.	Name of the country	Major regulatory actions/bodies	Contacts for further information
15.	Colombia	In Colombia PALO are responsible for regular inspection on biopesticide trade and conduct regular direct inspection on quality control. Colombia has a specific regulation for biopesticides, which was established in 1994 and updated in 2004. Presently only acute toxicological studies are required and reviewed by The Ministry of Health.	Cotes (2010)
16.	Australia	Biopesticides regulation is governed by NRS which is a partnership programme between the Australian state and territory governments. Currently COAG is most considerable regulatory body. The regulatory system is controlled by an authority named as APVMA 1992, which was previously known as NRA	http://apvma.gov.au/ King et al. (2013)
17.	New Zealand	Biopesticides mainly microbial pesticides are regulated by HSNO Act, for their registration. Once approved by HSNO, biopesticides also need to be registered under ACVM Act 1997.	www.nzfsa.govt.nz/acvm, Pottinger and Morgan (2008), http://www.ermanz.govt.nz/, Agricultural Compounds and Veterinary Medicines Act, 1997

Inspection and Quarantine of the People's Republic of China have the right to apply for the registration of pesticides (Kabaluk et al. 2010). The Chinese Ministry of Forestry encourages the use of biopesticide by providing funds for pest control in forest. Many local government bodies encourage farmers to use biopesticides for safe agricultural practices and crop quality.

16.3.1.2 India

The Indian government has various rules and regulations for promoting registration and production of biopesticides on large scale. The National Agricultural Technology Project (NATP) led the Integrated Pest Management (IPM) project (1998–2005), and the National Farmer Policy (2007) also promoted the use of biopesticides in agriculture. The Insecticide Act (1968) induced the higher production and application of biopesticides by simplifying the registration and regulation process of biopesticides. The Central Insecticides Board (CIB) and the Registration Committee (RC) worked under this act (www.cibrc.nic.in) and both are highly powerful bodies for biopesticide regulation (Kabaluk et al. 2010). CIB is the Apex Advisory Committee having experts of all disciplines/fields concerned. Based on the OECD guidelines, the CIB has streamlined the guidelines and data requirements not only for registration but also minimum infrastructural facilities for the production of biopesticides (NAAS 2013). The RC grants registrations, after scrutinizing and verifying claims with respect to their bio-efficacy and safety to human beings and animals. The National Agricultural Research System plays a leading role in promoting biopesticides. This system includes many institutes of Indian Council of Agricultural Research (ICAR) (www.icar.org.in/) and various agricultural universities in the country (Rabindra 2005).

16.3.1.3 Japan

Japan was originally amongst the pioneers in terms of biopesticide use. In the last few years, Japanese research in biocontrol has resulted in the identification and characterization of several new insect pathogen delivery systems and formulation development. Each year sales of biopesticides in Japan are over $9 million (Kunimi 2007). In Japan, the Japanese Agricultural Standard (JAS) Law (2000) governs agricultural chemicals, fertilizers and soil improvement substances, and biopesticide formulation is also included in this list.

16.3.1.4 Pakistan

The Pakistan Agricultural Research Council (PARC) is assigned the task of monitoring the pesticides (Jabbar and Mallick 1994). The Pakistan Biosafety Rules, 2005 (PBR 2005, rule 11), proposed that a licence is required from the Pakistan EPA for marketing or trading of living microorganisms, substances or cells or any related products for any purpose in the country. PBR, Rule 21, regulates the genetically engineered microorganism (GEM), cells or products and states that these products should be approved before being introduced in the market by the National Biosafety Committee (NBC).

16.3.1.5 South Korea

In South Korea the registration of pesticides at the first level is governed by the Agromaterials Management Division (AMD) and the Rural Development Administration (RDA), and then it is evaluated by Pesticide Safety Evaluation Division (PSED) and National Academy of Agricultural Science (NAAS). The AMD has a dedicated council for agrochemical safety for the final decision and for reporting to the applicant's dossier (http://www.mordorintelligence.com/industry-reports/south-korea-biopesticides-market-industry). The microbial pesticide regulations, 2000, were replaced in 2005 having new guidelines for biopesticides, and in 2009 it was transferred to the acts for management of agricultural chemicals (RDA 2009).

16.3.1.6 Other Countries

Bangladesh has some rules and regulations for biopesticide commercialization in the market. Any person in Bangladesh can import biopesticides if they have documents such as import licence, repack licence, wholesale licence, repacking factory, environment licence, etc. Besides these documents, product label must be approved by the registration authority with the trade name of the pesticide, genetic name, doses, pest, crop, first aid, toxic label, name of principle, waiting period, manufacture, expiry date and price for biopesticide commercialization (Ahsan 2012).

Thailand has made significant progress in promoting the production, application and registration of biopesticides (Grzywacz 2003). The Department of Agriculture (DOA) under the Ministry of Agriculture and Cooperatives (MOAC) has been promoting the use of biopesticides by launching various programmes such as organic farming, IPM and good agricultural practice (GAP) (Panuwet et al. 2012). In Vietnam various state research institutions such as the National Institute of Plant Protection (NIPP) and Vietnam Agricultural Science Institute (VASI), in Hanoi, have a role in biopesticide business (Jakel 2003).

16.3.2 Africa

Several countries in Africa use various guidelines to develop systems for registration and regulation of biopesticides in pest and disease management. Some African countries are taking a proactive role in developing capacity for regulating microbial pesticides. In 2012, six country representatives from Kenya, Uganda, Ethiopia, Tanzania, Nigeria and Ghana in the West African region undertook a regional stocktaking of the regulatory environments as part of the Commercial Products (COMPRO II) project, which is managed by the International Institute of Tropical Agriculture (IITA). The project's aim is to improve the regulation of biofertilizers and biopesticides (Simiyu et al. 2013).

16.3.2.1 Kenya

Kenya has developed biopesticide-specific registration regulations, representing a proportional and reasonable system that correctly assesses the safety and risks associated with microbial pesticides (Kabaluk et al. 2010). Kenya's pesticide regulatory authority is the Pest Control Products Board (PCPB) which is supported by various stakeholders such as the UK Department for International Development (UK DFID). The Agriculture Act Chapter (Cap 318) is responsible for the imports and exports of live organisms. The Kenya Plant Health Inspectorate Service (KEPHIS) Act, Cap 512/2013, and Plant

Protection Act are working for the imports and exports of live organisms. The Pest Control Products Act is also involved in registration and regulations for microbial pesticides (Kimani 2014).

16.3.2.2 Nigeria

In Nigeria the National Agency for Food and Drug Administration and Control (NAFDAC) is an agency under the Federal Ministry of Health, which is responsible for the control of the manufacture, sale and distribution of fertilizers, biofertilizer and biopesticides (https://vmapnafdac.wordpress.com/).

16.3.2.3 South Africa

South Africa has laws and guidelines for regulations of registered, sold and used biological control agents. The registrations of biological remedies are regulated by the Department of Agriculture, Forestry and Fisheries (DAFF) under the Act 36 of 1947 (www.daff.gov.za) (DAFF 2010).

16.3.2.4 Tanzania

In Tanzania, the regulatory body for the pesticide regulation is named as Tropical Pesticides Research Institute (TPRI), under the Ministry of Agriculture (Stadlinger et al. 2013). It has a mandate to register or deregister pesticide products under the country's Plant Protection Act. The field trial findings are submitted for the Pesticide Approval and Registration Technical Subcommittee (PARTS), and if it is approved, it is submitted to the National Plant Protection Advisory Committee for review (http://www.farmchemicalsinternational.com/crop-protection/africa-registration-guide-kenya-drives-to-harmonize-pesticide-registration).

16.3.2.5 Uganda

The Uganda National Agricultural Chemicals Board (UNACB) and the Agricultural Chemical Control Technical Committee (ACCTC) have the mandate to build capacity to inspect, certify agrochemical trade in Uganda (http://www.farmchemicalsinternational.com/crop-protection/africa-registration-guide-kenya-drives-to-harmonize-pesticide-registration).

16.3.3 European Union

It is the second largest continent on the basis of biopesticide production and use. In EU, microorganisms, botanicals and pheromones were regulated under the Directive 91/414/EEC (EU 1991), which was originally developed for chemical pesticides (Regnault-Roger et al. 2012). The Directive 91/414 was amended by 2001/36/EC (EC 2001) and 2005/25/EC (EC 2005) to add the specific requirements for microorganisms, whereas new legislation regarding plant protection was further added in the EU in 2009, and due to this, the following four legislations: (1) Regulation (EC) No 1107/2009, (2) Directive 2009/128/EC, (3) Directive 2009/127/EC and (4) Regulation (EC) No 1185/2009, are also included. The new Regulation (EC) No 1107/2009 applies in all member states from 2011 and replaces Directive 91/414/EEC (Meeussen 2012). The registration of biopesticides in EU countries seems to be more difficult than the rest of world as the dossier is submitted along with toxicological and environmental testing; it also requires efficacy evaluation. Under Regulation (EC) No 1107/2009, registrations of products are made by three zones following geographic and climatic criteria (Hauschild 2012). These zones are Zone A (North): Denmark, Estonia, Latvia, Lithuania, Finland and Sweden; Zone B (Central): Belgium, Czech Republic, Germany, Ireland, Luxembourg, Hungary, the Netherlands, Austria, Poland, Romania, Slovenia, Slovakia and the UK; and Zone C (South): Bulgaria, Spain, Greece, France, Italy, Cyprus, Malta and Portugal.

Applicants have to submit the dossier for the registration of plant protection products (PPPs) to a 'zonal rapporteur member state' (zRMS), which evaluates the dossier. Recently Regulation (EC) No 283/2013 implements Regulation (EC) No 1107/2009 for establishing data-related issues (EC 2013).

16.3.3.1 Germany

Germany is included in the largest central zone of 13 member states. At national level Regulation (EC) No 1107/2009 is implemented through

the German Plant Protection Act (GPPA) 2012. This underwent a major revision in 2012. The Plant Protection Act (PPA) covers plant protection products, adjuvants and plant resistance improvers. In 2003 the Pesticide Action Network (PAN) launched the Online Information Service for Non-chemical Pest Management in the Tropics (OISAT) in Germany for limiting the use of hazardous pesticides by providing safer alternatives to poor farmers (Bissdorf 2008).

16.3.3.2 Netherlands

The Netherlands is also inclined to increase the use of biopesticides, and like the biopesticide scheme in the UK, the Genoeg scheme in the Netherlands worked for biopesticide regulation. Genoeg scheme is a 'bottom-up' process whereby a coalition of agencies and other interested parties is involved in creating new regulations (www.genoeg.net). The Genoeg scheme in the Netherlands was developed to enhance the biopesticide production by efficient regulatory action (http://www.pesticides.gov.uk/environment.asp).

16.3.3.3 Russia

In Russia the state registration of microbial pesticides is regulated by the Russian Agricultural Control (RAC). RAC not only administers the registration of pesticides but also regulates their use, production, sale, transportation, storage, disposal, advertising, import and export (Kabaluk et al. 2010). The All-Russian Institute for Plant Protection (VIZR) in St. Petersburg, falling under the Russian Agricultural Academy (RAN), is involved in biopesticide research and development, but it is also involved in various aspects of the registration process (Kabaluk et al. 2010).

16.3.3.4 Ukraine

In Ukraine, the Main State Inspection of Plant Protection (MSIPP) is responsible for the registration of biopesticides and all other plant protection products. Most of organizations fall under the research institutes of the Ukrainian Agricultural Academy of Sciences (UAAN), the National Academy of Sciences (NANU) or the various agricultural universities, the latter of which are governed by the Ministry of Education and Science (Kabaluk et al. 2010). The two best known institutions conducting biopesticide research and development are the Institute of Plant Protection (UAAN) and National University of Life and Environmental Sciences of Ukraine (National Agrarian University of Ukraine) (www.msp.ua/state-registration-of-pesticides-ukraine.htm).

16.3.3.5 United Kingdom (UK)

In the UK, the main regulatory body responsible for plant protection products, including biopesticides, is the Chemicals Regulatory Directorate (CRD)/Pesticide Safety Directorate (PSD) (http://www.hse.gov.uk/pesticides/). The CRD is a new Directorate of the Health and Safety Executive (HSE) which is responsible for the regulation of pesticides, biocides, detergents, and chemicals under the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) Regulation (http://www.pesticides.gov.uk/guidance/industries/pesticides/advisorygroups/pesticidesforum). PSD is an agency of the Department for Environment, Food and Rural Affairs (DEFRA) and deals with the registration of agricultural pesticides (DEFRA 2006). The UK regulatory system was developed according to a chemical pesticide model, and this may have acted as a barrier to biopesticide commercialization (ACP 2004). The biopesticide scheme was developed as an important project in 2003, and the main aim of this project was to enhance the biopesticide production (http://www.pesticides.gov.uk/environment.asp). This scheme has introduced the role of biopesticide champion in 2006 (Chandler et al. 2011) for biopesticide registration and regulation.

16.3.4 North America

This continent has the highest share in biopesticide market. The North American Free Trade Association (NAFTA) is a regulatory authority in both the USA and Canada committed to joint

reviews and work sharing of biopesticide evaluation (NAFTA 2009).

16.3.4.1 Canada

In Canada definitions and registration requirements are closely aligned with those used by the USEPA. Microbials, semiochemicals and nonconventional pest control products (NCPCPs) are principally regulated by the Health Canada's Pest Management Regulatory Agency (PMRA 2007) under the federal authority of the Pest Control Products Act 2002. The regulation of nonindigenous macrobials is administered by the Canadian Food Inspection Agency (CFIA) under the authority of the Plant Protection Act 1990. Invertebrate biological controls are not registered by the PMRA. PMRA regulates all pesticides including biopesticides in Canada. It is involved in pesticide evaluation and registration and developed joint guidelines for biopesticides with EPA and OECD (AGBR 2015). The PMRA website also provides comprehensive information on pest control products for registrants, regulators, researchers and the general public (www.hc-sc.gc.ca). Agriculture and Agri-Food Canada Pest Management Centre is committed to improve access to biopesticides as part of its Pesticide Risk Reduction Programme (AAFC 2009).

16.3.4.2 United States (USA)

The USA has significant proportion of the global biopesticide market. In the USA the EPA shows a comprehensive and complex regulatory system for registration and regulation process of biopesticides (USEPA 2010), and this system requires unique properties for registration than other regulatory systems (Harman et al. 2010; Chandler et al. 2011). The regulation of biopesticides is a responsibility of the Office of Chemical Safety and Pollution Prevention (OCSPP) and the Office of Pesticide Programs (OPP), and there are three divisions within OPP that are involved in the registration of pesticides: (1) Antimicrobial Division, (2) Registration Division and (3) Biopesticides and Pollution Prevention Division (BPPD) (Matthews 2014). Since biopesticides cause fewer risks than chemical pesticides, EPA generally requires much less data to register a biopesticide than to register a conventional pesticide (Kumar 2012). USEPAs have mainly three parts: (1) the Federal Insecticide, Fungicide and Rodenticide Act (FIFRA) 1947; (2) the Federal Food, Drug and Cosmetic Act (FFDCA) 1938; and (3) the Food Quality Protection Act (FQPA) 1996. In the USA BPIA (Biopesticide Industry Alliance) promotes industry standards for biopesticides by communicating the value of biopesticides in agriculture and other markets (http://www.biopesticideindustryalliance.org/). It also collaborates with the biopesticide regulating authorities in order to ensure transparent and appropriate registration and regulatory requirements.

16.3.5 South America

16.3.5.1 Argentina

The regulatory institutions in Argentina are as follows: the Servicio Nacional de Sanidad y Calidad Agroalimentaria (SENASA); Vegetal National Committee of South Cone (COSAVE); Secretary of Agriculture, Livestock, Fish and Food (SACPyA); and National Administration of Drugs, Food and Medical Technology (ANMAT). The 'Coordination of Agrochemical and Biological Products' department of SENASA issues the information relating to pesticide registration, restrictions, commercialization and use of agrochemicals and biological products (www.senasa.gov.ar). The National Agriculture Department and Environmental Policy Secretary (NADEPS) is involved in the regulatory system, and the National Agriculture Department (NAD) and Environmental Policy Secretary (EPS) are also involved in the regulation process in Argentina.

16.3.5.2 Brazil

In Brazil the registration of all the pesticides is done by the Brazilian Ministry of Agriculture, Livestock and Food Supply (MAPA). National Health Surveillance Agency (ANVISA) and the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) scrutinize the process, while in urban areas and public health

campaigns, products are registered with ANVISA, after scrutiny by MAPA and IBAMA. In Brazil, a decree establishing the criteria for registration of BCAs for organic agriculture was approved in 2009, and biocontrol products based on macro- and microorganisms are considered to be plant protection products with the regulations for registration and use as for chemical pesticides. In 2007, the Brazilian Biocontrol Manufacturers' Association (ABCBIO) was created to organize this sector (abcbio.org.br).

16.3.5.3 Colombia

In Colombia, the Provincial Agricultural Legislation Office (PALO) is responsible for regular inspection of biopesticide trade and conducts regular direct inspection on quality control. Colombia has a specific regulation for BCAs (biopesticides) since 1994, which was updated in 2004. Presently only acute toxicological studies are required and reviewed by the Ministry of Health (Cotes 2010).

16.3.5.4 Cuba

In Cuba a specific IPM Act (1982) has induced the use of biopesticides (Pérez and Vázquez 2002). The biopesticide registration process was developed according to standards and guidelines of the three authorities named as the (i) Food and Agriculture Organization (FAO 1988), (ii) OECD (1996) and (iii) USEPA (1996) (Díaz 2003). In 2007, a formal process for registration was published (Gaceta Official No. 016 Cuba 2007) and four institutions are regulating biopesticides and one or more involved with registration process:

(i) The Central Registrar of Pesticides (CNSV) – approves and registers new active ingredients and new products.
(ii) The Centre for Environmental Inspection and Control (CICA) – regulates environmental aspects for production of biopesticides.
(iii) The National Centre for Biological Safety (CNSB) – regulates research, production, trials, releases, import and export of biopesticides and production facilities.
(iv) External Quarantine Department of the National Centre of Plant Health (EQ-DNCPH) – regulates the import and export of materials under quarantine (including biopesticides).

16.3.6 Oceania

16.3.6.1 Australia

In Australia, the regulation of biopesticides is governed by the National Registration Scheme (NRS) for the application and commercialization. Currently, the Council of Australian Governments (COAG) is the most important regulatory body amongst various regulatory bodies running in Australia (King et al. 2013). The regulatory system of biopesticides is done by the Australian Pesticides and Veterinary Medicines Authority 1992 (APVMA 1992) which has a role in registration of biopesticides having unique data (http://apvma.gov.au/), and this authority was previously named as National Registration Authority (NRA). In 2005 some guidelines were published with additional requirements for microbial pesticides, and these guidelines consider evaluation of potential hazards of microbes such as toxicity, pathogenicity, host range and impact on native flora and fauna (APVMA 2005).

16.3.6.2 New Zealand

In New Zealand biopesticides mainly microbial pesticides are regulated by the Hazardous Substances and New Organisms (HSNO) Act 1996. Release and importation of all new organisms, including microbes for biocontrol, have been regulated under this act since 1998, and prior to 1998, unknown microbial species must have been approved by the Environmental Risk Management Authority (ERMA) before commercialization, even if the species were isolated from material within the country (Pottinger and Morgan 2008; ERMA 2010). Once approved by HSNO,

biopesticides also need to be registered under the Agricultural Compounds and Veterinary Medicines (ACVM) Act 1997 (www.nzfsa.govt.nz/acvm), which has replaced the Pesticides Act 1979. The approval of the existing microbial products for their identity and purity for safe importation is done by the Ministry of Agriculture in New Zealand.

Table 16.1 summarizes the regulations of biopesticides in various countries around the globe.

16.4 Limitations of Current Regulation Systems

There are many systemic problems arising in regulation of biopesticides, and one main problem is that it is based on the conventional chemical pesticide models (Greaves 2009). According to Chandler et al. (2008), in the EU system, the regulatory failure arises from the application of an inappropriate synthetic pesticide model and lack of regulatory innovation. Besides this, registration and commercialization of biopesticides and their evaluation is also a lengthy process. EU system takes much time to complete registrations, and the industry criticizes that the present registration period, especially for microbial biological control, is costly and time consuming (Bailey et al. 2010). For example, the mean time taken by the EU was 75 months compared with 28 months in the USA (Hokkanen and Menzler-Hokkanen 2008). The US system provides flexibility, and in some cases, it invites applicant for pre-submission meeting where the applicant is advised on which studies are necessary, based on preliminary data and literature available (Mubyana-Jhon and Taylor 2015).

Biopesticide production system is underdeveloped and underutilized in Asia because there are several constraints such as technical, social and institutional that limit commercial production of new biopesticides (NAAS 2013). In developing countries like in Asia and Africa, there is a problem of quality control because of which the trust of farmers is lacking. This situation can be corrected only by proper regulatory system. Although biopesticide production and application in India is in progress but in comparison to chemical pesticides, the growth is not up to mark. In a study Rabindra (2005) estimated that the current production of microbial pesticides meets less than 10 % of the identified need. Nearly 500 biopesticides are registered by CIB and available in the Indian market, but quality control is a major issue related to most of the products (NAAS 2013).

Mensink and Scheepmaker (2007) suggest that even though data requirements are increasingly transparent and harmonized for more efficient regulatory procedures, there is lack of sufficient guidance on evaluation and use of biological products to conduct a premarket assessment of the environmental safety. Regulatory agencies realize that biopesticides are fundamentally different than chemical pesticides and should not be compared with the same standards of safety and efficacy; it is challenging to design a system for evaluation that is equally fair to both biopesticides and chemical pesticides (Bailey et al. 2010). One difficulty is that the regulatory processes assess single products, while microbial pesticides are very complex and diverse in nature (Hubbard et al. 2014). Ravensberg (2011) gave recommendations on how to build a dossier and which sources, in addition to the guidelines, can be used to better understand what exactly the authorities require. A data requirement in the USA and Canada is somewhat common except that PMRA requires a complete dossier on efficacy and phytotoxicity data, and the EPA does not.

Another complex issue is surrounding the regulation of biopesticides having multiple modes of action. For example, species of the fungus *Trichoderma*, which are used as biopesticides against soilborne plant pathogenic fungi, are able to parasitize plant pathogenic fungi in the soil (Gupta and Dikshit 2010); they also produce antibiotics (Ghisalberti and Sivasithamparam 1991; Vey et al. 2001) and fungal cell wall-degrading enzymes (Bech et al. 2015). *Trichoderma* compete with soilborne pathogens for carbon, nitrogen and other factors (Limón and Codón 2004), and they can

also promote plant growth by the production of auxin-like compounds (Vinale et al. 2008; Nega 2014). Some *Trichoderma* products have been sold on the basis of their plant growth-promoting properties, rather than as plant protection products (Nega 2014), and so have escaped scrutiny from regulators in terms of their safety and efficacy (Bailey et al. 2010). Similar is the case with *Pseudomonas*. Fluorescent *Pseudomonas* can be used as plant growth-promoting agent as well as in biocontrol (Negi et al. 2005; Mehnaz 2013; Tewari and Arora 2014, 2016). However, there are no particular regulatory mechanisms to check this. There are several technological and policy gaps in effective utilization of biopesticides which need to be addressed properly in order to minimize the use of chemical pesticides and to promote the use of biopesticides (Kumar 2015).

16.5 Innovation for Better Regulation Schemes

Innovation in current regulation system of biopesticides is required as an important need for higher production of agrobiologicals at global level (Arora et al. 2012). The regulation situation at present varies across countries; some countries have developed system, some having progress in regulatory framework, and few have no proper regulations for biopesticides (Simiyu et al. 2013). The appropriate regulatory system is that which has concern about public safety and environmental impact, i.e. it must be ecofriendly, scientific and technologically innovative for pest control (Greaves 2009). Biopesticides have not been used more widely; the solution of this matter may be lowering registration costs and eliminating efficacy (Greaves 2009). Another appropriate plan for better regulation of biopesticides is that the governments can make regulations globally by organizing meetings, workshops, and conferences regarding uplifting the status of biopesticides (Mishra et al. 2015).

Some factors that may promote the necessary regulatory innovations ranging from government intervention are exogenous pressure and some within regulatory bodies are endogenous pressure (Greaves 2009). Various institutions have done some preliminary research about the industrialization of biopesticides and institutional changes may be significant; however, no systematic reports have appeared so far; therefore, there is requirement of guidelines to promote the collaboration of enterprises and research institutes (Leng et al. 2014).

Harmonization of biopesticide regulatory guidelines in the world is very important for the innovative approach for the production and commercialization of biopesticides, and OECD is important for harmonization at international level (Holm et al. 2005). The OECD and the World Health Organization (WHO) have an influence on pesticide regulation and their role is very important (NAAS 2013). The OECD project on biopesticides was initiated in 1999 to help its member countries to harmonize the methods and approaches used to assess biological pesticides (Sigman 2005). Currently the OECD has 34 member countries, and more than 70 developing and transition economies are engaged in working relationships with the OECD. The working group of OECD on pesticides includes the (i) Registration Steering Group (RSG), (ii) Risk Reduction Steering Group (RRSG) and (iii) Biopesticides Steering Group (BPSG) (http://www.oecd.org/chemicalsafety/pesticides-biocides/). The BPSG has achieved a lot of progress towards harmonization and work sharing through the development of guidance and working documents (Richards and Kearns 1997). The headquarters of OECD group is situated in Paris, France, and assists EU governments for assessing biopesticide risks to humans and the environment very carefully (http://www.biopesticideindustryalliance.org/).

Globally, the regulation of pesticides has engaged the attention of OECD, Food and Agriculture Organization (FAO) and EU both generally and particularly (Greaves and Grant 2011; FAO 2012). EPPO is an intergovernmental organization in Paris, funded by contributions from the member governments (www.eppo.int). In 2010, the IOBC of noxious animals and plants reviewed the rapid expansion of the application of microbial pesticides and advancement in their

regulatory systems worldwide (IOBC 2010). The major steps towards the promotion of harmonization for biopesticide regulation and facility developments for work sharing between governments have been taken by various bodies including OECD, North American and European (AGBR 2015). The BPSG of OECD, the FAO, the European Commission (EC), the IOBC, the EPPO, the North American Plant Protection Organization (NAPPO) and the NAFTA have worked as most important intergovernmental organizations for biopesticide regulation and innovation (AGBR 2015).

16.6 Conclusion and Future Prospects

Commercialization of biopesticides is rapidly growing all over the world (Kumar 2012), but in absence of effective regulations and some other constraints (Arora 2015), the growth is not as expected. Effective regulation can also prevent spurious biopesticides in the market (NAAS 2013). Regulation of biopesticides is an obstacle in production and commercialization of these products. As is evident from aforesaid discussion, regulation criterion varies according to countries. To resolve this barrier, it is recommended to develop a common regulatory system. Moreover, it is also required to improve communication and information exchange between regulatory bodies, scientists and industries to review the possible risks associated with microbial biopesticides. Guidelines used to evaluate biopesticide efficacy, field testing and quality also need to be revised, as most of the time, it is conducted by non-expert persons especially in developing countries. Regulatory bodies are required to be established to not only ensure fast track registration of biopesticide products with justified regulations and transparent procedures but also promote the adoption of new safer technologies in the development of commercial products. The criterion adopted in policymaking for regulation should be identical for all countries and proposed according to nature of agrobiologicals and not as for chemical pesticides.

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