R. Z. Sayyed · M. S. Reddy
Sarjiya Antonius *Editors*

Plant Growth Promoting Rhizobacteria (PGPR): Prospects for Sustainable Agriculture

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Foreword

Dear editors, authors, and readers,

It gives me immense pleasure in writing this foreword for the book titled *Plant Growth Promoting Rhizobacteria (PGPR): Prospects for Sustainable Agriculture*. The book is edited by two well-known scientists in the field of agriculture, plant pathology, and research on plant growth-promoting rhizobacteria (PGPR). The use of alternative methods to fungicide use is not only important in reducing harmful environmental impacts due to chemicals, but it also reduces the costs a grower may incur during a field season. As we know, there are millions if not billions of microorganisms in our soils that can improve plant health. We had only "scratched the surface" until newer methods, especially omics, came around and helped us to identify un-culturable microorganisms and further our understanding of how these PGPRs are helping agricultural and natural systems. As we are dealing with biological organisms, it is important to note that the success of such PGPR use and application relies on the knowledge we have on such organisms, their habitat and needs, and how each biological process can be changed with the environmental factors and climate change.

This book illustrates some excellent papers from researchers who are dedicating their research career to finding such soils, microorganisms, and delivery methods to improve plant health around the world. The aim of the book and its content is to educate the reader with knowledge on how agricultural sustainability and profitable production can be achieved with PGPR either as single organisms or as consortia.

The book is divided into two parts. The first part relates to how PGPRs can be utilized for plant growth promotion and nutrient uptake. It takes a holistic look at PGPRs recovered as endophytes or from soils and their biochemical parameters to formulations to improve application. The second part discusses how PGPRs are known to and can be used as biological control agents in mitigating diseases caused by plant pathogens and abiotic and biotic stress. The book is a great compilation of these areas, and I would encourage young and experienced researchers to read chapter by chapter to see the present status in accomplishments and challenges within agricultural systems and PGPRs.

I wish its editors every success with the launch of this book and thank them for their dedication to agricultural sustainability around the world.

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Preface

This book was concesptualized during finalizing the proceedings of our Fifth Asian PGPR International Conference held in Bogor, Indonesia, on 16–19 July 2017. The prime aim and objective of this book is to highlight salient features on the application of PGPRs in agricultural crop plants to lend a hand to scientists throughout the world working in this field. Plant growth-promoting rhizobacteria (PGPR) are root-colonizing bacteria that exert direct or indirect beneficial effects on plant growth and development. PGPR shows an important role in the sustainable agriculture industry. The increasing demand for crop production with a significant reduction of synthetic chemical fertilizers and pesticides use is a big challenge nowadays. Excessive application of chemical fertilizers leads to several environmental hazards, causing damages to human, animal, and ecosystem health. Such environmental, agricultural, and health issues alert us for serious attention toward environmentally friendly PGPR. PGPRs are the group of microorganisms which colonize and have a symbiotic relationship with the plant roots and promote plant growth via various plant growth-promoting substances and also act as biofertilizers, biostimulants, and biofungicides. A large number of microorganisms such as bacteria, fungi, and other microbes coexist in the plant root rhizosphere; however, the most abundant beneficial microorganisms are bacteria. It is well-established that only 1–3 % of bacteria promote plant growth in the soil as PGPR. Beneficial effects of PGPRs have also been attributed to alterations in the microbial ecology of the rhizosphere. PGPR are considered as an effective competitor in the soil root zone to help in plant growth promotion or disease suppression. Increasing disease resistance through induced systemic resistance (ISR) or acquired systemic resistance (ASR), PGPR also helps the plant to overcome various biotic and abiotic stresses. The principle of biotic stress involves the competing microbes, pathogens, and parasites, while abiotic stresses include salinity, extreme (high/low) temperature, heavy metal ions, drought, and presence of toxic metabolites or pesticides. PGPR serve as biocontrol agents for controlling and suppressing the growth of several plant pathogens. More importantly, they play a key role in bioremediation too.

Global concern over the use of chemicals in agriculture has diverted the attention of researchers toward sustainable agriculture by utilizing the multiple potentials of PGPR. Therefore, management of pests and diseases for healthy agroecosystem and safety is the need of the hour. The use of PGPR as biofertilizers, biopesticides, and

biostimulants has gained considerable attention among researchers, agriculturists, farmers and policymakers, and consumers.

The multiple potentials exhibited by PGPR for increasing agro-productivity are the need of the hour for feeding the growing world population under the present climate scenario. Thus, sustainable agricultural vision could be achieved through the use of PGPR, for disease suppression and drought, salt, and heavy metal stress tolerance of the plant varieties with better nutritional value. PGPR have been researched as reliable, eco-friendly, cost-effective alternatives for plant growth promotion and phytopathogen control. For an effective bioinoculant candidate, PGPR strains should possess high rhizosphere competence, be safer to the environment, be compatible with cultural practices, have broad-spectrum activity, and be tolerant to various biotic and abiotic stresses. In view of this, the need for a better PGPR to complement to increase agro-productivity is one of the crucial factors for the world economy which has been highlighted.

Plant Growth Promoting Rhizobacteria (PGPR): Prospects for Sustainable Agriculture is a timely effort for sustainable agriculture. This book is composed of 25 chapters encompassing various aspects of the influence of PGPR in agricultural crop plants. The authors of the chapters are from various countries which include India, Indonesia, Malaysia, South Korea, Pakistan, Kazakhstan, China, the Philippines, Germany, etc. and discuss about the PGPR role in growth promotion of various crop plants, suppression of wide range of phytopathogens, their formulation, effect of various factors on growth and performance of PGPR, assessment of diversity of PGPR through microsatellites, and their role in mitigating biotic and abiotic stress. We hope that this book will be beneficial for students, teachers, researchers, and entrepreneurs involved in PGPR and allied fields.

It has been a pleasure in editing this book, and we are thankful to all the contributors for attending the Fifth Asian PGPR International Conference held in Bogor, Indonesia, and contributing their research in a timely manner to enable the compilation in this book form. Our sincere appreciation and thanks to Prof. Dilantha Fernando, dean at the University of Manitoba, Canada, and president of Canadian Phytopathological Society, Canada, for endorsing our efforts in the foreword. Special thanks to the team of Springer in India, especially Dr. Mamta Kapila and Ms. Shukla Raman, for their cooperation and assistance in finalizing this book.

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Riyaz Sayyed is an Associate Professor and the Head of the Department of Microbiology, PSGVP Mandal's Arts, Science College, Shahada, Maharashtra, India. Currently, he serves as President of the Indian Chapter of the Asian PGPR Society for Sustainable Agriculture. He has 15 years of research expertise in siderophore-based PGPR and has authored 91 peer-reviewed research papers and 61 books. He is a recipient of many prestigious awards and honors from various research societies and the Government of India. In addition, he is an Associate Editor of the journal *Environmental Sustainability* (Springer).

M. S. Reddy is the Founder and Chairman of the Asian PGPR Society for Sustainable Agriculture, a consultant for several national and international agencies, a successful businessman, an investor, and entrepreneur. Over the past 40 years, he has served as a Professor at the Department of Entomology & Plant Pathology, Auburn University, USA, and has established a body of multidisciplinary, internationally recognized research in the area of PGPR and poverty alleviation. Dr. Reddy has received many prestigious awards from many countries around the world. In addition to having authored or co-authored over 250 publications, he serves as an Editor, Associate Editor, and Reviewer for various journals.

Sarjiya Antonious is the Head of the Microbiology Laboratory, Indonesian Institute of Sciences, Indonesia, and currently serves as President of the Indonesian Chapter of the Asian PGPR Society. He has more than 15 years of research expertise in industrial biofertilizer development and has authored or co-authored many peer-reviewed papers in the fields of microbiology and soil biochemistry. As a professional biofertilizer industrial consultant for biofertilizers, several of his inventions have been patented and adopted by national stakeholders and licensed for agro-industries. He has received many prestigious awards, including a technology transfer (ALTEK) award and honors from international research organizations.

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Part I

PGPR for Sustainable Plant Growth and Nutrition

Diversity and Plant Growth-Promoting Potential of Bacterial Endophytes in Rice

1

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Contents

Abstract

Rice seed is colonized by endophytic bacterial communities which becomes a source of endophytes and allows for a diverse bacterial community to occur inside the plant host. These endophytes can provide beneficial effects to growth and development of the host plant. Furthermore, the diversity of endophytic bacteria is dynamic within the host plant. Variations as well as density of populations of endophytic bacteria usually decline from the root to the leaf region of the plants. This review gives an overview of the plant growth-promoting mechanisms of endophytes with respect to their intensely studied plant growth-promoting rhizobacteria (PGPR) counterparts. The community dynamics and diversity of

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bacterial endophytes associated with rice and its microniches including roots, shoots, and the seeds were also discussed. This review also delves into the proposed concept of a potential core microbiota of rice, bacterial endophytes that are highly associated with rice, as well as the interesting abilities of seed endophytes to be transmitted and conserved through generations of rice hosts via the seeds.

Keywords
PGPR · Endophyte · Rice · Diversity · Transmission

1.1 Introduction

In terms of plant-endophyte interactions, an endophytic bacterium is defined as a bacterium isolated from surface-sterilized plant tissue or isolated from the internal tissues of plants, and it does not cause visible injury to the host plant (Hallmann et al. 1997). Endophytic bacteria colonize internal tissues of the host plant without any damaging effect or activation of intense and prolonged defense responses, sometimes in high numbers (Reinhold-Hurek and Hurek 2011). Hallmann et al. (1997) added that bacterial endophytes include symbionts as well as internal colonists with apparently neutral effect. Endophytes also include bacteria that fluctuate between epiphytic and endophytic lifestyle. Members of the genus *Methylobacterium* are dominant phyllosphere colonizers, but they were also documented to be present in the interior of the leaves and stem of the rice plant (Mano and Morisaki 2008).

As an extension, bacterial endophytes can also be categorized as obligate and facultative according to their life strategies. Endophytes that are completely dependent on their host are obligate, while those that could partly exist outside their host plants are facultative (Hardoim et al. 2008). Hardoim et al. (2008) also added the concept of competent endophytes. These endophytes are equipped with genes and physical and physiological adaptations necessary for the continued plant-endophyte relationship and interactions.

Rice (*Oryza sativa*) as a plant host for endophytes is also one of the more studied plants among others since it is one of the major cereal crops where increasing consumption occurs worldwide. As reviewed by Mano and Morisaki (2008), researchers have commonly isolated *Pantoea* (seeds), *Methylobacterium* (shoots), *Azospirillum* and *Herbaspirillum* (stems and roots), and *Burkholderia* and *Rhizobium* (roots). Studies of the culturable bacterial floras in roots, leaves, and seeds of *Oryza sativa* by Mano et al. (2006, 2007) showed certain patterns of endophytic diversity. The floras differed greatly according to the outside environment, the atmosphere in the case of the rice shoot and the soil in the case of the root. They surmised that endophytic bacteria are a subset of the epiphytic communities since many endophytic bacteria in the rice plant seem to penetrate the endosphere coming from the surface of roots and shoots. On the other hand, they also showed that there are endophytic bacteria which have been isolated so far only from inside the rice

plant. This opens up probabilities on the origins of these endophytes aside from the epiphytic bacterial populations.

The study on bacterial endophytes has been dramatically increasing recently due to the perceived importance of these microorganisms to their plant host. This review is an overview and update of bacterial endophytes of rice and its microniches including roots, stems, leaves, and reproductive organs. The plant growth promotion of rice bacterial endophytes is also discussed in reference to their well-documented PGP rhizobacteria. Lastly, the unique ability of seed bacterial endophytes of rice to be transmitted and conserved throughout generations of rice plants is also presented with the proposal that rice contains "core microbiota" highly associated with the rice host.

1.2 The Diversity of Endophytes in the Plant Endosphere

A compilation of bacterial endophytic sequences of plants was done by Hardoim et al. (2015) comprising a database of 56% and 44% from cultured and uncultured bacteria, respectively, with a total of 7348 16S rRNA sequences. Their data showed a diverse bacterial community with 23 phyla, 2 of which are from *Archaea* (29 sequences). Despite the astounding diversity and overall distribution of endophytes, majority (96%) of the overall endophytic bacterial sequences belong to *Proteobacteria*, *Actinobacteria*, *Firmicutes*, and *Bacteroidetes*, with a distribution of 54%, 20%, 16%, and 6%, respectively.

Among the *Proteobacteria*, *Gammaproteobacteria* dominates most of the prokaryotic endophytes with 26% of the total sequences, followed by 18% from *Alphaproteobacteria* and 10% from *Betaproteobacteria*. Representative groups of *Gammaproteobacteria* include *Pseudomonas*, *Enterobacter*, *Pantoea*, *Stenotrophomonas*, *Acinetobacter*, and *Serratia*. *Rhizobium* and *Bradyrhizobium* as well as *Methylobacterium* and *Sphingomonas* dominate among *Alphaproteobacteria*, while *Burkholderia*, *Massilia*, *Variovorax*, and *Collimonas* are well-known representatives of *Betaproteobacteria*. Among the Gram-positive endophytes, class *Actinobacteria* is well represented by *Streptomyces*, *Microbacterium*, *Mycobacterium*, *Arthrobacter*, and *Curtobacterium*. Class *Bacilli* includes the genera *Bacillus*, *Paenibacillus*, and *Staphylococcus*.

1.3 The Microniches of the Rice Endosphere and Their Inhabitants

The diversity of bacterial endophytes inhabiting the plant microbiomes is said to be a soil bacterial community subcategory (McInroy and Kloepper 1995; Idris et al. 2004; Sheng et al. 2008). Bacterial colonization usually originates from the soil rhizosphere and then to the rhizoplane of roots. Endophytic colonization from the roots happens at colonization sites particularly the lateral root junctions and emergence, root tips, wounds, and root cracks (Hardoim et al. 2008). Competent bacteria

penetrate the endoderm into the Casparian strips systematically dispersing to the shoots utilizing the vascular elements especially xylem, but may also pass in between cells (Hallmann et al. 1997).

Culturable rhizosphere bacteria commonly range from 10^7 to 10^9 CFU g^{-1}. The population density decreases to 10^5–10^7 CFU g^{-1} FW in the rhizoplane region (Compant et al. 2010). Following colonization of the soil surrounding the root and the root surface, some endophytes could penetrate the root endosphere up to10^5–10^7 CFU g^{-1} (Compant et al. 2010). These bacteria may systematically inhabit the plant endosphere, and reach a cultivable population density of up to 10^4 CFU g^{-1}FW under normal settings, and go to the upper shoot parts (Compant et al. 2010). Specialized endophytes could also spread to the reproductive plant structures reaching around10^3 CFU g^{-1}FW (Compant et al. 2010). The seeds of rice can also be populated by culturable bacterial endophytes ranging from 10^3 to 10^6 CFU g^{-1}FW (Walitang et al. 2017, 2018a, b).

Rice (*Oryza sativa*) with an increasing consumption worldwide is an important plant host for bacterial endophytes. As reviewed by Mano and Morisaki (2008), researchers have commonly isolated specific bacterial groups inhabiting different structures of the host plant. Studies of the culturable bacterial floras in roots, leaves, and seeds of rice (*Oryza sativa*) by Mano et al. (2006, 2007) showed certain patterns of endophytic diversity. There are endophytic groups that are more generally isolated in specific plant structures, while there are also groups consistently found throughout the plant host. The table below is a summary of bacterial endophytes in association with rice (Table 1.1).

1.4 The Presence of Core Bacterial Endophytes of Rice

Core microbiota are organisms that are ubiquitous in all studied samples. Increasing studies on plant-microbe interaction indicates high association of core endophytes and their integral importance to their plant host. There are evidences on the occurrence of core bacterial endophytes found intergenerationally in specific rice genotypes or across genotypes in subspecies of *Oryza sativa*. There are potentially similar evidence also found in other host plants such as in maize (Johnston-Monje and Raizada 2011). Studies done by Walitang et al. (2017, 2018a) showed that there are culturable bacterial endophytes that are found in different genotypes of *Oryza sativa* subspecies indica including members belonging to *Microbacterium testaceum*, *Xanthomonas sacchari*, and *Flavobacterium acidificum*. In addition, core microbial groups are prominent characteristics of seed bacterial communities supporting the previous study stated above. The core endophytic bacterial groups in the seeds of *Oryza sativa* subsp. *indica* include members as mentioned in Table 1.1 (Walitang et al. 2018a). There are also previous studies indicating core microbial groups in other subspecies and genotypes of rice. For example, Hardoim et al. (2011) showed that *Oryza sativa* under the subspecies indica tend to maintain similar bacterial groups across genotypes in their samples. In addition, Hardoim et al. (2012) found out that there are particular groups of bacteria such as *Stenotrophomonas maltophilia* that are cultivated

Table 1.1 Known endophytic genera detected in different endosphere regions of the rice plant from different studies

Genera of endophytes	Endosphere region of detection	Citation
Curtobacterium	Seeds	Ruiza et al. (2011)
Serratia	Whole plant	Tan et al. (2001)
Burkholderia, *Cronobacter*, *Klebsiella*, *Pantoea*, *Pseudomonas*, *Sphingomonas*	Leaves, seeds, roots	Loaces et al. (2011)
Actinophytocola, *Actinoallomurus*	Roots	Indananda et al. (2011)
Acidimicrobiales, *Actinaurispora*, *Actinoplanes*, *Actinospica*, *Amycolatopsis*, *Dietzia*, *Fodinicola*, *Frankia*, *Lapillicoccus*, *Marmoricola*, *Mycobacterium*, *Nocardioides*, *Streptomyces*	Stems and roots	Tian et al. (2007)
Aureimonas, *Bacillus*, *Brachybacterium*, *Brevundimonas*, *Erythrobacteraceae*, *Aerococcus*, *Burkholderia*, *Chryseobacterium*, *Rhodoglobus*, *Enterobacter*, *Erythrobacter*, *Methylobacterium*, *Patulibacter*, *Altererythrobacter*, *Curtobacterium*, *Altererythrobacter*, *Xanthomonas*, *Brachybacterium*, *Flavobacterium*, *Ochrobactrum*, *Pantoea*, *Pseudomonas*, *Sphingomonas*	Leaves	Ferrando et al. (2012)
Acinetobacter, *Aeromonas*, *Agromyces*, *Bacillus*, *Citrobacter*, *Curtobacterium*, *Deinococcus*, *Dyella*, *Enterobacter*, *Escherichia-Shigella*, *Flavobacterium*, *Frigoribacterium*, *Microbacterium*, *Herbaspirillum*, *Mycobacterium*, *Neisseria*, *Ochrobactrum*, *Paenibacillus*, *Pantoea*, *Plantibacter*, *Pseudomonas*, *Pusillimonas*, *Rhizobium*, *Sphingobium*, *Stenotrophomonas*	Root, shoot, leaves	Hardoim et al. (2012)
Acidovorax, *Anaeromyxobacter*, *Aquamicrobium*, *Methylocaldum*, *Anaeromyxobacter*, *Caulobacter*, *Aquitalea*, *Armatimonadetes*, *Azospira*, *Azospirillum*, *Bacillus*, *Bradyrhizobium*, *Bryobacter*, *Burkholderia*, *Chitinophagaceae*, *Nitrospiraceae*, *Polymorphospora*, *Christensenellaceae*, *Paenibacillus*, *Chromobacterium*, *Clostridium*, *Dickeya*, *Dyella*, *Enterobacter*, *Geobacter*, *Geothrix*, *Haematobacter*, *Herbaspirillum*, *Isoptericola*, *Methylovulum*, *Microbacterium*, *Holophagaceae*, *Nevskia*, *Novosphingobium*, *Orientia*, *Paludibacter*, *Pantoea*, *Pelobacter*, *Rhizobium*, *Rhizomicrobium*, *Ruminococcaceae*, *Bradyrhizobium*, *Sphingomonas*, *Staphylococcus*, *Tepidicella*, *Treponema*, *Aquitalea*, *Cedecea*, *Ideonella*, *Pandoraea*, *Pelomonas*	Roots	Zhang et al. (2013)
Azospirillum, *Enterobacter*, *Herbaspirillum*, *Ideonella*	Stem	Elbeltagy et al. (2001)
Enterobacter, *Rhizobium*	Roots	Peng et al. (2008)

(continued)

Table 1.1 (continued)

Genera of endophytes	Endosphere region of detection	Citation
Acinetobacter, *Burkholderia*, *Enterobacter*, *Pseudomonas*	Stems	Chaudhary et al. (2012)
Bacillus, *Clostridium*, *Enterobacter*, *Incertae sedis*	Stem	Minamisawa et al. (2004)
Acidovorax, *Bacillus*, *Burkholderia*, *Chryseobacterium*, *Deinococcus*, *Paenibacillus*, *Pantoea*	Roots and leaves	Yang et al. (2008)
Bacillus, *Caulobacter*, *Kocuria*, *Lysinibacillus*, *Methylobacterium*, *Micrococcus*, *Pantoea*	Seeds	Kaga et al. (2009)
Burkholderia, *Rhizobium*	Roots	Singh et al. (2006)
Acinetobacter, *Alkanindiges*, *Bdellovibrio*, *Brevundimonas*, *Burkholderia*, *Comamonadaceae*, *Methylobacterium*, *Comamonas*, *Deinococcus*, *Delftia*, *Ensifer*, *Enterobacter*, *Flavobacterium*, *Geobacter*, *Herbaspirillum*, *Holophagaceae*, *Acidaminobacter*, *Massilia*, *Methylophaga*, *Stenotrophomonas*, *Lachnospiraceae*, *Methanospirillum*, *Methyloversatilis*, *Novosphingobium*, *Sideroxydans*, *Papillibacter*, *Phenylobacterium*, *Pseudomonas*, *Planomicrobium*, *Achromobacter*, *Methanoregula*, *Pleomorphomonas*, *Rhizobacteria*, *Sideroxydans*, *Sphingobacterium*, *Hydrogenophaga*, *Sulfurospirillum*	Roots	Sun et al. (2008)
Bradyrhizobium	Whole plant	Chaintreuil et al. (2000)
Microbacterium, *Curtobacterium*, *Bacillus*, *Paenibacillus*, *Rhizobium*, *Sphingomonas*, *Xanthomonas*, *Herbaspirillum*, *Pseudomonas*, *Kosakonia*, *Enterobacter*, *Pantoea*, *Flavobacterium*, *Stenotrophomonas*, *Ralstonia*, *Delftia*	Seeds	Walitang et al. (2017, 2018a, b)

intergenerationally from the same rice host species. Ferrando et al. (2012) also found that there is an occurrence of strongly associated similar endophytes in rice leaves in different cultivars of *Oryza sativa*. Table 1.1 also shows that there are certain groups even represented by specific species of bacteria that are widespread in different rice cultivars. For example, *Curtobacterium* sp. (Ruiza et al. 2011; Hardoim et al. 2012; Ferrando et al. 2012; Walitang et al. 2017, 2018a, b) and *Herbaspirillum* spp. (Hardoim et al. 2012; Zhang et al. 2013; Elbeltagy et al. 2001; Sun et al. 2008; Walitang et al. 2017, 2018a, b) are commonly found from ancient to modern cultivars of rice. The widespread occurrence of these bacterial groups potentially indicates their status as core microbial groups highly associated with their rice host.

The presence of core endophytes of rice, at least in *Oryza sativa* subsp. *indica*, brings us many unanswered questions as to the functional, ecological, and evolutionary importance of these core microbial groups to their rice host. However, increasing evidence show that core microbial groups are crucial to their rice host. For example, Walitang et al. (2018a) found that some core microbial groups become dominant under saline conditions in salt-sensitive and salt-tolerant rice cultivars. These dominant core microbial groups such as *Pantoea*, *Flavobacterium*, and *Kosakonia* were also highly tolerant to both salt stress and osmotic stress (Walitang et al. 2017) indicating their competence and potential contribution to the host health and development under normal and stress conditions. Interestingly, widespread occurrence of some core microbial groups particularly residing in the rice seeds could be explained through continued transmission, conservation, and dispersal from the ancient parental ancestors to the modern rice varieties of rice via seeds. Walitang et al. (2018b) found that endophytes are passed on and conserved even after crossbreeding, human selection, and repeated inbreeding of their rice host. Rice parental lines greatly contributed to the seed endophyte communities of the subsequent generations of rice. In addition, seed endophytic communities show resilience to change in terms of the presence of the same bacterial groups even if the rice host is recultivated in different ecogeographic locations.

1.5 Transmission and Conservation of Rice Endophytes Through the Seeds

As summarized by Truyens et al. (2015), bacterial endophytes can get in seeds through different mechanisms. Vascular connections from maternal parent allow connection and transmission of bacterial endophytes from the vegetative parts into the seed endosperm. Gametes also allow transmission through direct colonization of the resulting embryo and endosperm. Specialized shoot meristems could also transmit endophytes to the seeds when these structures are colonized by endophytes and become reproductive meristems giving rise to seeds and ovules. External sources of potential endophytes would also enter through the reproductive organs or the vegetative parts of the plants.

Seeds can act as carriers and vectors for endophytic bacteria (Mastretta et al. 2010). Bacterial endophytes colonizing reproductive plant parts then into the ovules and seeds are very intriguing because they are passed on from parent to offspring (Compant et al. 2010). Some authors even suggest that endophytes come from the seeds (Mundt and Hinkle 1976; McInroy and Kloepper 1995). Studies have already proven that seed bacteria can be vertically passed on to the succeeding lineages of plant hosts. In some cases (Hardoim et al. 2012; Walitang et al. 2018b), similarity between the parents and the successive offspring could be from 45% to 70%. They also noted that bacteria residing in the seeds are generally adapted to plant tissues initially colonizing the root interior and then rapidly migrating to the stem and leaves and could even differentially respond according to the prevailing host conditions. Liu et al. (2012) also showed that even after the process of natural crossbreeding in

maize, the most abundant bacterial endophytes of the parents are transmitted to the next-generation seeds and could also become the dominant endophytes of that community. In addition, vertical transmission of maize seed endophytes between immediate generations range from 10% to 40% (Johnston-Monje and Raizada 2011). These results corroborate with the observations in rice (Walitang et al. 2018b). The passing on and conservation of bacteria in the seeds allowed an astounding reflection of the phylogenetic relationship of the host maize plants observed in the composition of its endophytic community. Other studies have also isolated same bacterial strains from one seed generation to another rather than looking at transmission of communities of bacteria (Johnston-Monje and Raizada 2011; Hardoim et al. 2012).

1.6 Plant Growth Promotion (PGP) of Rice Endophytes

The plant growth promotion of bacterial endophytes is vividly classified into direct and indirect mechanisms. Direct mechanisms entail capacitating certain nutrient acquisition or providing the host organism with compounds produced by the endophyte (Lodewyckx et al. 2002). Examples of these are the synthesis of plant growth hormones and regulators, suppression of ethylene production related to stress, conversion of atmospheric nitrogen to nitrogenous compounds that plant could absorb, and the solubilization of nutrients such as iron and phosphorus (Weyens et al. 2009). Indirect mechanisms include enhancing host health and vigor against plant pathogens (Lodewyckx et al. 2002), and these include pathogen antagonism, active antibiosis, activation of host defense responses, and prevention of pathogen secretions (Weyens et al. 2009).

Endophytic bacteria may act as biofertilizers altering plant productivity (Vessey 2003; Weyens 2009). Perhaps the most studied aspect of PGP is nitrogen fixation. Bacterial endophytes capable of fixing nitrogen include *Azoarcus* sp., *Herbaspirillum* ssp., and *Acetobacter diazotrophicus*. They can colonize the plant endosphere, but not activating plant responses leading to disease symptoms (Reinhold-Hurek and Hurek 1998). Significant N_2 fixation has been demonstrated in which plants could sustain part of their nitrogen requirements from endophytes (Boddey et al. 1995; James 2000). The oxygen-sensitive nitrogenase enzyme in nitrogen-fixing endophytes catalyzes the reduction of atmospheric N_2 to ammonia (NH_3). Symbiotic nitrogen-fixing bacteria can induce nodule formation (James 2000), but some diazotrophic endophytes may not form any nodules (Reinhold-Hurek and Hurek 1998).

Phosphorus and iron, aside from nitrogen, are the nutrients which are present in limiting available concentration in the soil. Phosphate solubilization and siderophore production by bacterial strains can help in acquisition of phosphorus and iron, respectively (Puente et al. 2009; Costa and Loper 1994). Total soil phosphorus content may be in large amount, but only a small proportion of this is available to plants (Stevenson and Cole 1999). Majority of the total soil phosphorus is found in insoluble forms which plants cannot absorb (Ae and Shen 2002). Soluble forms of

phosphorus are the monobasic ($H_2PO_4^-$) and the dibasic ($H_2PO_4^{2-}$) anions (Schachtman et al. 1998). Phosphorus could be mobilized in the soil by either organic or inorganic phosphate solubilization, and bacteria solubilizing phosphorus employ different mechanisms in order to turn insoluble phosphorus to soluble phosphates for absorption of the plant (Sharma et al. 2013). Typically, phosphate solubilizing bacteria produce organic acids with low molecular weight in order to facilitate conversion of inorganic phosphorus to soluble forms (Rodríguez and Fraga 1999; Rodriguez et al. 2004). Evidence is increasing on the role of phosphate solubilization by endophytes compared to their well-established rhizosphere counterparts. Puente et al. (2009) showed that seed endophytes of cactus enhanced phosphate solubilization of pulverized rock with the production of organic acids allowing the growth of cactus seedlings in extreme conditions. Johnston-Monje and Raizada (2011) added that some seed endophytes of corn were able to get out from the endosphere to the root and inhabit the root rhizosphere indicating potentials of allowing mineral acquisition through phosphate solubilization and siderophore production. On the other hand, iron (III) in the environment is sparingly soluble leading to low availability (Ma et al. 2011). Iron as opposed to phosphorus could be mobilized in the soil through bacterial synthesis of chelating agent specific to ferric ion under low iron conditions. The role of these chelating agents or siderophores is to increase the availability of iron by scavenging (Neilands 1995). Evidence suggest that bacteria-synthesized Fe^{3+}-siderophore complexes can undergo multi-species recognition particularly among bacteria and plant species (Sharma and Johri 2003; Ma et al. 2011), although bacteria, fungi, and other plants could also produce their own siderophores (Hider and Kong 2010).

Bacterial endophytes modulate plant growth and development through production of hormones such as auxins, cytokinins, and gibberellins (Taghavi et al. 2009; Tanimoto 2005). During abiotic stress conditions, plants balance the levels of endogenous phytohormones to cope up with the stress exerted by the environmental conditions. On the other hand, microorganisms in association with plants can also modulate levels of phytohormones (García de Salamone et al. 2005) and thereby alter the plant's physiological response to stress (Glick et al. 2007a).

Auxin or indole-3-acetic acid (IAA) is an extensively studied and characterized phytohormone produced by plant-associated bacteria which has been authenticated to enhance plant growth and development (Vessey 2003) by enhancing root growth as well as proliferating and elongating root hairs (Taghavi et al. 2009). Auxins have multiple functions in the plants related but not limited to cell division modulation, seed germination, root and vegetative growth, and stress resistance (Spaepen and Vanderleyden 2011).

Cytokinins are plant hormones associated with enhancing cell multiplication, increase in the cell and tissue size. They are also capable of controlling stomatal opening, stimulating shoot growth, as well as decreasing root growth (Salisbury 1994). Under certain stress conditions, stomatal closure related to cytokinin concentration level has a consequent effect on the growth of the shoot and root (Hare et al. 1997). Arkhipova et al. (2007) showed the beneficial effect on plant growth after inoculation of cytokinin-producing bacteria under moderate drought stress conditions. Gibberellins

(gibberellic acid), another group of phytohormones, could alter stem morphology (Salisbury 1994). Although with lesser importance than auxins, gibberellins are also crucial for normal development of the root (Tanimoto 2005).

Ethylene plays a vital role in growth and development of plants in plethora of ways. Abeles et al. (1992) summarized major functions of ethylene related to root growth (initiation and elongation inhibition), fruit ripening, seed germination, leaf abscission, inhibition of plant and mycorrhiza interaction, and ethylene control of stress. Hardoim et al. (2008) show that plant ethylene biosynthesis could be modulated by ACC deaminase-producing endophytes affecting diverse functions of ethylene, especially those related to "stress ethylene." The impact of various stresses such as high temperature, flooding, drought, salinity, and pathogen attack on plants leads to the rise in ethylene levels, often termed as "stress ethylene" (Abeles et al. 1992; Morgan and Drew 1997). There is also an indication that ethylene is connected to endophytic colonization of plant endosphere and is most probably connected to the plant signaling pathways (Iniguez et al. 2005).

The increased synthesis of ethylene can aggravate conditions of stress or can help the plants to adapt under limiting and stressful environmental settings. These contradictory plant responses could be described by a model where a rapid response is stimulated on plants that are exposed to stress by producing a small peak of ethylene that initiates a defensive response (Glick et al. 2007b; Ciardi et al. 2000). During the course of persistent stress conditions, a larger peak of ethylene occurs that may result in detrimental effect on plant growth and survivability.

The discovery of 1-aminocyclopropane-1-carboxylate (ACC) deaminase-producing soil bacteria (Honma and Shimomura 1978) leads to studies of this enzyme as a common indicator of PGPB (Glick et al. 2007b). The mechanisms of ACC deaminase-producing bacteria to modulate plant ethylene levels could be through cleaving ACC into α-ketobutyrate and ammonia (Glick et al. 2007a) or via inhibition of the actions of ACC synthase and/or b-cystathionase. These latter enzymes play a key role in ethylene biosynthesis in plants (Sugawara et al. 2006). Since endophytes reside inside the plants, close proximity of ACC deaminase bacteria to the plant cells leads to an enhanced modulation of ethylene levels as the enzyme is not known to be transported outside of the bacterial cell (Glick et al. 2007a). The widely accepted mechanism of regulation of plant ethylene level by ACC deaminase-producing bacteria is to cleave the precursor of ethylene, ACC into ammonia, and a-ketobutyrate before its oxidation by the plant ACC oxidase (Glick et al. 1998).

Many PGPB have the ability of synthesizing an array of antibiotics commonly associated in preventing the proliferation of plant pathogens. These PGPB have been shown to mediate synthesis of allelochemicals for biocontrol activity such as siderophores, antibiotics, volatiles, and lytic and detoxification enzymes (Compant et al. 2005). Antifungal activities were also noted in seed endophytes of rice (Mukhopadhyay et al. 1996; Ruiza et al. 2011). Endophytes that can synthesize chitinases, cellulases, β-1,3-glucanases, proteases, and lipases act as biocontrols since they can kill pathogenic fungi by enzyme-mediated cell lysis (Kamensky et al. 2003; Ordentlich et al. 1988). PGPB can limit the phytopathogen's ability to

proliferate by preventing them from acquiring sufficient amount of iron through siderophores (Kloepper et al. 1980). Physical competition for the limited space in the plant endosphere between endophytes and invading pathogens could also limit disease incidence and severity. Bacterial endophytes could rapidly colonize the plant internal tissues and utilize most of the available nutrients, creating competition and inhibiting growth of pathogens (Reinhold-Hurek and Hurek 2011). Quorum sensing could also be an important mechanism on the antifungal activities of endophytes (Ruiza et al. 2011) since endophyte quorum sensing could lead to induction of gene expression in plants (Mathesius et al. 2003; Rosenblueth and Martínez-Romero 2006).

It is known that some endophytic bacteria are capable of triggering induced systemic resistance (ISR) similar to systemic acquired resistance (SAR). SAR has been shown to develop in plants after activating defense mechanism in response to pathogenic infection leading to hypersensitive reaction isolating the pathogens by forming localized necrotic lesion (Kloepper and Ryu 2006). The basic difference between ISR and SAR is that the inducing bacterium does not cause hypersensitive response and does not show symptoms on the plant (Compant et al. 2005). Also, ISR has been shown to be effective against plethora of pathogens. Induction of defense responses by colonizing endophytes creates a "priming" state in the host plant. Plants can quickly and more strongly react to a second pathogen attack by inducing defense mechanisms. When ISR is activated, it does not target specific pathogens, but rather controls further infection in a range of multiple types of pathogens. The jasmonate and ethylene bio-signaling are responsible for modulating ISR in plants (Verhagen et al. 2004). ISR could be activated even without the any direct interaction between the inducing PGPB and the pathogen (Bakker et al. 2007). Endophytes may also initiate ISR through their *O*-antigenic side chain of the outer membrane protein lipopolysaccharide, flagellar proteins, pyoverdine, chitin, β-glucans, cyclic lipopeptide surfactants, and salicylic acid.

1.7 Future Prospective of Rice Endophytes

The roles and mechanisms of bacterial endophytes as PGP are still far from clearly elucidated. Plant bacterial endophytes seem to overcome many obstacles in the practical applications of biofertilizers. Plant endophytes show rhizosphere and endosphere competence. They have endophytic adaptation for colonization, survival, and maintenance inside the plant host. Further studies are necessary in order to comprehend plant-microbe interaction, endophyte-pathogen-plant interactions, the nature of bacterial endophytes, and the dynamic changes in the structure and diversity of plant endophyte communities in the whole plant and in the different microniches inside the host plant.

With the increasing studies on rice endophytes and rice-associated bacteria, it is becoming apparent that there are bacterial groups which are highly integrated and co-existing with rice as a host. It is also interesting to study how these rice-associated endophytes and bacteria are maintained, transmitted, and even conserved throughout the generations and lineages of the rice species.

1.8 Conclusion

Rice is home to diverse communities of bacterial endophytes and rice-associated bacteria. Among these bacterial communities are core microbial groups present in many rice cultivars potentially transmitted in every rice generation especially through the seeds and are conserved in the rice plant. These rice-associated core microbial groups are possibly critical to the growth, development, health, and survival of their rice host. Many bacterial endophytes of rice also show multiple plant growth-promoting potential indicating their central beneficial roles to the well-being of their rice host. Continued research on the mechanisms of plant-endophyte-pathogen interaction is necessary to understand the highly complex interaction occurring in rice as a holobiome of associated microbial groups.

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Plant Growth-Promoting Microbes for Sustainable Agriculture

2

Suhas P. Wani and S. Gopalakrishnan

Contents

Abstract

Microbes are an integral part of living soil not only in transforming nutrients in the soil but also with multiple functions in influencing soil health. There are specific microbes which help the plant to grow well in their presence by various mechanisms. The direct mechanism may include fixation of atmospheric nitrogen, synthesis of various phytohormones and enzymes, and solubilization of minerals in the soil, while the indirect mechanism includes inhibiting phytopathogens. Hence, such plant growth-promoting rhizobacteria (PGPR) need to be harnessed and exploited for sustainable agriculture. Some of the representative PGPR group includes *Azotobacter*, *Azospirillum*, *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Serratia*, *Streptomyces*, *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Frankia*, and *Thiobacillus*. Demonstrations of these PGPR and their beneficial traits under glasshouse and field conditions are documented for a range of crops including

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cereals, legumes, fruits, vegetables, herbs, and ornamentals. Several industries are commercializing the potential PGPR strains as biofertilizers and as biocontrol agents. However, successful commercialization of PGPR in many developing countries is a distant dream largely due to the lack of well-developed technology, quality carrier material, quality control legislation, training programs, and on-farm demonstrations. The development of quality PGPR inoculum and its application will definitely lead to an ideal sustainable agricultural system. Further, PGPR is known for not only reducing the emission of greenhouse gases (GHGs) and carbon footprint but also increasing nutrient-use efficiency.

Keywords
Plant growth-promoting microbes · Secondary metabolites · Biological control · Commercialization · Sustainable agriculture

2.1 Introduction

Food security, agricultural sustainability, energy renewability, and rural livelihoods depend largely on soil fertility. Land degradation and desertification due to anthropogenic activities causes an estimated loss of 24 billion tons of fertile soil around the world's croplands (FAO 2011). It is estimated that, by 2030, the ever-increasing human population and changing food habits would increase the demand for water by 30%, energy by 45%, and food by 50% (IFPRI 2012). These demands cannot be met unless the soil fertility is restored. In addition, environmental pollution, today's biggest public concern, is largely caused by the use of synthetic pesticides, fertilizers, and herbicides. This has led to seek an alternative strategy for synthetic pesticides and fertilizers (Glick et al. 2007). Therefore, a greener and cleaner approach toward crop protection and production is essential. The use of microbes for crop protection and production and soil health had been practiced for centuries. These beneficial microbes are called as "plant growth-promoting rhizobacteria (PGPR)" (Kloepper and Schroth 1978). These heterogeneous bacteria are usually found in the rhizosphere and root surfaces and provide benefits to the crops. In recent years this terminology PGPR has gained a simple expressive term as "plant probiotics" (Maheshwari 2012).

The rhizosphere soil is rich in nutrients due to the accumulation of sugars, vitamins, amino acids, organic acids, fatty acids, phenols, and plant growth promoters released from the roots by secretion, exudation, and deposition. This leads to the enrichment of microbes (10- to100-fold) which include bacteria, actinobacteria, fungi, and algae (Uren 2007). The role of rhizospheric microorganisms in plant growth promotion is widely reported (Vessey 2003; Tilak et al. 2005; Podile and Kishore 2006). Some of the representative rhizospheric microbes/PGPR include the genera *Acinetobacter*, *Agrobacterium*, *Allorhizobium*, *Arthrobacter*, *Azorhizobium*, *Bacillus*, *Bradyrhizobium*, *Brevibacterium*, *Chromobacterium*, *Burkholderia*, *Hyphomicrobium*, *Caulobacter*, *Flavobacterium*, *Erwinia*, *Gluconobacter*, *Mesorhizobium*, *Micrococcus*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Sinorhizobium*,

Sphingomonas, and *Streptomyces* (Vessey 2003; Vijayabharathi et al. 2016). In recent years, the plant growth-promoting traits of actinobacteria are getting much attention (Jog et al. 2012; Gopalakrishnan et al. 2016b).

2.2 Mechanisms of PGPR

PGPR employs a direct and indirect mechanism to enhance plant growth promotion and protection. The direct mechanisms include the production of growth hormones, solubilization of phosphorous, fixation of atmospheric nitrogen, and acquisition of iron. The indirect mechanisms include minimizing the deleterious effects of biotic stresses through production of low molecular weight compounds such as alcohols, ammonia, aldehydes, cyanogens, ketones, sulfides, cell wall-degrading enzymes, and secondary metabolites with antagonistic traits and competition for nutrients (Glick 2012; Dey et al. 2014).

2.3 Direct Plant Growth Mechanisms

2.3.1 Biological Nitrogen Fixation (BNF)

Nitrogen (N), an essential element for crop growth, is available easily in the air, as 78% of atmosphere is composed of N. However, plants cannot utilize gaseous form of this N. Currently, a variety of synthetic fertilizers are being used for enhancing agricultural productivity. Hence, biological alternatives are preferred as these are addressing economic, environmental, and renewable energy concerns. BNF is a process of converting atmospheric N into plant assailable N (such as ammonia) through a reaction between rhizobia and leguminous plants (such as chickpea, pigeon pea, and groundnut) (Wilson and Burris 1947). Legumes, the major symbionts of BNF, can meet their own N needs. A major portion of N fixed by legumes is harvested in grains. The succeeding crop(s) also get benefitted by N in the form of the root and shoot residues (Bhattacharyya and Jha 2012). Crops such as sugarcane, wheat, and rice have also the capacity to fix atmospheric N using free-living bacteria/diazotrophs such as *Azoarcus*, *Cyanobacteria*, and *Azospirillum*. However, the N fixed by legume-rhizobia symbiosis (13–360 kg N ha^{-1}) is far greater than the N fixed by free-living bacteria or diazotrophic nonsymbiotic systems (10–160 kg N ha^{-1}) (Bohlool et al. 1992). Rhizobia are treated on seeds of legumes for enhanced N fixation (Lindström et al. 1990). They can persist in soil for many years in the absence of their host (Sanginga et al. 1994). Actinobacteria such as *Streptomyces*, *Micromonospora*, *Corynebacterium*, *Agromyces*, *Arthrobacter*, *Propionibacterium*, and *Mycobacterium* also have been shown to exhibit BNF (Sellstedt and Richau 2013). Actinobacteria have also been demonstrated for their BNF capability by acetylene reduction assay (ARA), ^{15}N isotope dilution analysis, ability to grow on nitrogen-free medium, and identification of *Nif* genes via PCR amplification (Ghodhbane-Gtari et al. 2010).

2.3.2 Phosphate Solubilization

Phosphorus (P) plays an important role in cell metabolism and signaling in plants (Vance et al. 2003). P is present in bound form (with inorganic or organic molecules), but plants can take only H_2PO_{4-} and/or HPO_4^{2-}form of P (Smyth 2011). Hence, P is extensively used as a synthetic fertilizer, but their excessive and unmanaged application leads to negative impact on the environment (Correll 1998). The P-solubilizing bacteria mineralize the organic P by several enzymes of microbial origin, such as acid phosphatases, C-P lyase, D-α-glycerophosphate, phosphor hydrolases, phosphonoacetate hydrolase, and phytase which solubilizes the bound form, so that they are available to plants (Gügi et al. 1991; Abd-Alla 1994; Ohtake et al. 1996; McGrath et al. 1998; Skrary and Cameron 1998; Glick 2012). Plant growth-promoting (PGP) microbes such as *Bacillus*, *Pseudomonas*, and *Streptomyces* are widely known for P solubilization. Actinomycetes having high P-solubilizing traits have been reported, for instance, *Streptomyces* sp. mhcr0816 (TCP 1916 mg l^{-1}, RP 990 mg l^{-1}), *Streptomyces* sp. (RP 250 mg l^{-1}), and *Arthrobacter* sp. (RP 519 mg l^{-1}) are comparable to *Pseudomonas* sp. (TCP 1500 mg l^{-1}) or *Bacillus* (TCP 957 mg l^{-1}) strains (Hamdali et al. 2012; Jog et al. 2014). The role of P solubilization by *Mesorhizobium mediterraneum* in enhancing plant growth has been reported in barley and chickpea (Peix et al. 2001).

2.3.3 Phytohormone Production

Plant-associated bacteria are known to produce phytohormones (such as auxins) that regulate plant growth. Phytohormones affect morphological and physiological processes of plants even at lower concentrations (Arshad and Frankenberger 1998). They change growth pattern of the plants and result in branched and lengthier roots with greater surface area enabling the plants to access water and nutrients from deeper depths of soils. Several actinobacteria capable of producing auxins, gibberellins, cytokinins, and abscisic acid are shown in Table 2.1.

2.3.4 Iron Acquisition

In nature, iron exists as insoluble form of hydroxides and oxyhydroxides which are not accessible to plants. Siderophores (high-affinity iron-chelating compounds) can be of both the plant and the microbial origin and trap iron present in the soil (Rajkumar et al. 2010). The mechanism of microbial-origin siderophores in plant growth is not completely understood, but under low iron available conditions, PGP is assumed to involve one of the following mechanisms:

Table 2.1 List of PGP microbes producing growth hormones

Phytohormone/ ACC deaminase	PGP bacteria	References
Auxin /IAA	*Actinomyces* sp., *Bradyrhizobium*, *Bacillus megaterium*, *Frankia* sp., *Micrococcus*, *Methylobacterium oryzae*, *Nocardia* sp., *Rhizobium*, *Streptomyces* spp., *S. atrovirens*, *S. griseoviridis* K61, *S. lydicus* WYEC108, *S. olivaceoviridis*, *S. rimosus*, *S. rochei*, *S. viridis*	Kaunat (1969), Brown (1972), Wheeler et al. (1984), Abd-Alla (1994), Mahadevan and Crawford (1997), Tokala et al. (2002), Tsavkelova et al. (2006), El-Tarabily (2008), Khamna et al. (2010), Verma et al. (2011), Abd-alla (2013), Lin and Xu (2013) and Subramanian et al. (2014)
Gibberellin	*Actinomyces* sp., *Bacillus*, *Arthrobacter*, *Micrococcus*, *Nocardia* sp., *Streptomyces* sp.	Katznelson and Cole (1965), Kaunat (1969), Brown (1972), Merckx et al. (1987) and Tsavkelova et al. (2006)
Cytokinins	*Arthrobacter, Frankia* sp., *Leifsonia soli*, *Rhodococcus fascians*, *Pseudomonas*, *Streptomyces turgidiscabies*	Sang-Mo et al. (2014), Cacciari et al. (1980), Stevens and Berry (1988), Joshi and Loria (2007) and Pertry et al. (2009)
ACC deaminase	*Arthrobacter*, *Microbacterium azadirachtae* sp. nov., *Leifsonia soli* sp. nov., *Micrococcus* spp., *Rhodococcus* sp. R04, *Streptomyces* spp.	El-Tarabily (2008), Dastager et al. (2010), Madhaiyan et al. (2010a, b) and Nascimento et al. (2014)

Source: Swarnalakshmi et al. (2016)

1. Microbial-origin siderophores with high redox potential transfer their ferrous form of iron to a plant's transport system through apoplastic pathway in roots.
2. Iron chelation by microbial-origin siderophores from soil depends on its concentration, pH, and redox potential and does a ligand exchange with plant-origin siderophores. During this process, the iron-free plant-origin siderophores are initially bound to the receptor protein. This complex binds to a receptor, where the ion exchange between the two siderophores occurs (Crowley 2006).

Besides contributing to plant health, microbial-origin siderophores also involved in biocontrol of plant pathogens. It functions by acquiring iron, thereby competing with other pathogenic microbes in the vicinity and supplying it to the plant (Glick 2012). The production of high-affinity siderophores by PGP microbes is perceived as a means of biocontrol, as phytopathogens produce low-affinity siderophores. PGP microbes which produce siderophores with high-affinity colonize efficiently in the rhizosphere (Kloepper et al. 1980). The role of microbial-origin (including actinobacteria) siderophores in phytopathogens suppression are reported widely (Yamanaka et al. 2005; Barona-Gomez et al. 2006; Sontag et al. 2006; Macagnan et al. 2008; D'Onofrio et al. 2010; Johnson et al. 2013).

2.3.5 Role in Nutrient Cycles

Soil microbes, plant growth-promoting microbes in particular, are the key propellers of biogeochemical cycles on nutrients including N, C, P, and S (Bloem et al. 1997) and of which C and N cycle are most important.

2.3.5.1 Carbon Cycle

It is well known that microbes transfer the C primarily for their own survival. For instance, under aerobic conditions, i.e., in rhizosphere soil and oxic layers of wetland systems, aerobic methane-oxidizing bacteria (MOB) play the role in C cycle (Chistoserdova et al. 2005), whereas, under waterlogged anaerobic soils, hydrogenotrophic archaea and methanogenic bacteria play the role (Trumbore 2006). Microbial C is found maximum in forest soils compared to any other soils. Typically, microbial C is found a minimum of 100–1000 μg g − 1 in well aerated soils and a maximum of 500–10,000 μg g − 1 in undisturbed forest soils with the intermittent values in ecosystems such as semiarid tropics (SAT) and grasslands (Kandeler et al. 2005). Microbial biomass C is found about 1–6% of total organic C in rhizosphere soil with an indirect relationship for increasing soil depth. Formation of soil organic matter (OM), a major fraction containing soil organic carbon (OC), is aided by the decomposition process through various hydrolytic enzymes such as cellulase, protease, amylase, chitinase, glucosidase, and phenoloxidase. These hydrolytic enzymes convert the macromolecules into low molecular weight micromolecules for the ready assimilation of microbes (Burns and Dick 2002).

2.3.5.2 Nitrogen Cycle

The first step in the N cycle is assimilation, i.e., biological N_2 fixation (BNF). It is aided by a group of diazotrophic bacteria such as rhizobia, *Frankia*, *Azotobacter*, cyanobacteria, and green sulfur bacteria and of which the first two (rhizobia and *Frankia*) occur through the symbiotic process while the last three through the nonsymbiotic process (Thamdrup 2012). The N fixed, in the form of ammonium, during the BNF process, is further dissimilated by two-step microbial process, nitrification (the aerobic oxidation of ammonium to nitrite and nitrate) and denitrification (the anaerobic reduction of nitrate to N_2 through nitrite, nitric oxide, and nitrous oxide) (Simon 2002). Nitrification is done by two different sets of microbial groups: (1) ammonia-oxidizing bacteria (AOB) such as *Nitrosomonas*, *Nitrosospira*, and *Nitrosococcus*, which transform ammonia to nitrite using ammonia monooxygenase, and (2) nitrite-oxidizing bacteria (NOB) such as *Nitrobacter* and *Nitrococcus*, which transform nitrite to nitrate using nitrite oxidoreductase (Vaccari et al. 2006).

2.4 Indirect Plant Growth-Promoting Mechanisms

It refers to the use of PGP agents for managing the deleterious effects of biotic stresses (such as insect pests and pathogens) to improve the overall health of the plant. Such PGP microbes are also referred as biocontrol agents (BCAs) which

employ several mechanisms to alleviate the insect pest and pathogen effects. Some of the important mechanisms are as follows.

2.4.1 Competition

Root exudates of plants play an important role in determining the specific group of microorganisms living in its vicinity. For instance, flavonoids and phenolic compounds in the rhizosphere were reported to influence plant symbiosis with beneficial rhizobacteria (Palaniyandi et al. 2013). Amino acids, organic acids, vitamins, and sugars were reported to serve as important nutrients for microbes (Dakora and Phillips 2002). Antagonism through competition for available nutrients is one form of the biocontrol mechanism used by the beneficial microbes to outlive pathogens and suppression of disease (Palaniyandi et al. 2013). The production of hydrolytic enzymes, siderophores, antibiotics, and volatile compounds are some of the other mechanisms exerted by PGP microbes (Agbessi et al. 2003; Macagnan et al. 2008; Wan et al. 2008).

2.4.2 Cell Wall-Degrading Enzymes

The cell wall of insect pests and fungal pathogens of plants contain polymers such as lipids, glucan, chitin, cellulose, and proteins. PGP microbes are known for their ability to produce cell wall-degrading enzymes. These enzymes disrupt the cell wall components of insect pests and pathogens which results in cell lysis. PGP microbes use this as one of the mechanism to manage plant pathogens and insect pests. PGP microbes are widely reported to produce these hydrolytic enzymes such as peroxidase, chitinase, glucanase, and protease (Gupta et al. 1995; Chater et al. 2010).

2.4.3 Antibiosis

It is one the major biocontrol mechanisms of PGP microbes in nature. The diffusible compounds produced by BCA and/or PGP bacteria are known to inhibit the rhizospheric plant pathogens. A broad spectrum of antibiotics such as polyenes, macrolides, aminoglycosides, nucleosides, and benzoquinones were reported to be produced by PGP microbes. Actinobacteria are the leading producer of antibiotics. For instance, the total number of microbial bioactive molecules (as of year 2012) was about 33,500 and of which 13,700 (41%) were produced by actinobacteria (Berdy 2012). Of these, 1800 metabolites showed antibiosis against pathogenic fungi (Berdy 2005). Antibiotics produced by actinobacteria are listed in Table 2.2.

2.4.3.1 Induction of Systemic Resistance

Host plant resistance is the best strategy to manage plant pathogens and insect pests of crops. Induced resistance in plants is elicited by interaction with an external factor

Table 2.2 List of actinobacteria capable of producing antibiotics

Actinobacterial species	Antibiotic	References
Streptomyces sp., *S. alboniger*, *S. padanus*	Alnumycin, coronamycins, fungichromin, goadsporin, kakadumycins, pamamycin-607, rhodomycin	Shockman and Waksman (1951), Kondo et al. (1987), Bieber et al. (1998), Onaka et al. (2001), Castillo et al. (2003), Shih et al. (2003) and Ezra et al. (2004)
Actinoplanes teichomyceticus	Teichomycins, teicoplanin	Parenti et al. (1978) and Somma et al. (1984)
Actinoplanes friuliensis sp. nov. *II.*	Friulimicins	Vertesy et al. (2000)
Actinoplanes ianthinogenes N. sp.	Purpuromycin	Coronelli et al. (1974)
Actinoplanes	Lipiarmycin	Coronelli et al. (1975)
A. utahensis	Echinocandin	Boeck et al. (1989)
Actinomadura sp.	Cationomycin, chandrananimycins, oxanthromicin	Nakamura et al. (1981), Patel et al. (1984) and Maskey et al. (2003)
Actinomadura spiralis	Pyralomicins	Kawamura et al. (1995)
Microbispora sp.	Cochinmicins, glucosylquestiomycin	Igarashi et al. (1998) and Lam et al. (1992)
Microbispora aerata	Microbiaeratin	Ivanova et al. (2007)
Micromonospora lomaivitiensis	Lomaiviticins A and B	He et al. (2001)
Micromonospora inyoensis	Sisomicin	Reimann et al. (1974)
Micromonospora carbonacea	Everninomicin	Weinstein et al. (1964)
Micromonospora echinospora subsp. *armeniaca* subsp. nov.	Clostomicins	Omura et al. (1986)
Nocardiopsis	New thiopeptide antibiotic	Engelhardt et al. (2010)
Nocardia sp. *I.*	Nocathiacins	Li et al. (2003)
Nocardia mediterranei subsp. *kanglensis*	Chemomicin A	Sun et al. (2007)

Source: Swarnalakshmi et al. (2016)

such as PGP microbes or even with a metabolite. In plants, two types of nonspecific defense systems are widely reported. These are PGP microbe-induced systemic resistance (ISR) and pathogen-induced systemic acquired resistance (SAR) (Schuhegger et al. 2006). Hoffland et al. (1995) demonstrated ISR for the first time in radish. In ISR, plants are treated with PGP microbes providing protection from pest and pathogen attack (Alstrom 1991; Walters et al. 2013). ISR is regulated by salicylic acid, ethylene, and jasmonic acid (De Meyer et al. 1999; Verhagen et al.

Table 2.3 List of antagonistic actinomycetes suppressing plant pathogens

Diseases	Pathogen	Antagonistic strain	References
Root rot of lupine	*P. cinnamomi*	*M. carbonacea*	El-Tarabily (2003)
Root rot of turfgrass	*P. infestans*	*S. violaceusniger* strain YCED-9	Trejo-Estrada et al. (1998)
Root rot of wheat	*P. infestans*	*S. olivaceoviridis*	Aldesuquy et al. (1998)
Lupin root rot	*P. tabacinum*	*A. missouriensis*	El-Tarabily (2003)
Lupin root rot	*F. oxysporum*	*S. halstedii*AJ-7	Joo (2005)
Wood rot	*P. chrysosporium*	*S. violaceusniger* XL-2	Shekhar et al. (2006)
Wood rot	*P. placenta*	*S. violaceusniger* XL-2	Shekhar et al. (2006)
Wood rot	*C. versicolor*	*S. violaceusniger* XL-2	Shekhar et al. (2006)
Wood rot	*G. trabeum*	*S. violaceusniger* XL-2	Shekhar et al. (2006)
Damping-off	*P. aphanidermatum*	*A. campanulatus*	El-Tarabily et al. (2009)
Crown rot	*P. aphanidermatum*	*M. chalcea*	El-Tarabily et al. (2009)
Damping off chickpea	*P. aphanidermatum*	*S. rubrolavendulae* S4	Loliam et al. (2013)
Damping off chickpea	*F. oxysporum*	*Streptomyces* sp.	Ashokvardhan et al. (2014)
Lupin root rot	*R. solani*	*S. vinaceusdrappus*	Yandigeri et al. (2015)

Source: Arasu et al. (2016)

2004). Representative reports on microbes inhibiting plant pathogens and insect pests are listed in Tables 2.3 and 2.4.

2.5 PGP Research at ICRISAT

PGP microbial research was at a peak in ICRISAT in the 1980s and 1990s but mostly on BNF. BNF plays an important role in sustaining productivity of soils in the semiarid tropics (Wani et al. 1995). It was reported to benefit succeeding cereal crops, such as wheat, rice, sorghum, and maize with a relative yield increase of up to 350% in different cropping systems. Besides calculating the amount of fixed N by legumes during the determination on legume fixed N in different cropping systems, it was important to consider the overall N balance of the cropping system. ICRISAT had gathered such information using chickpea (CP), pigeon pea (PP), cowpea (C), sorghum (S), safflower (SF), fallow (F), and mung bean (M) on several cropping system involving S/PP-S + SF, S + CP-S + SF, C/PP-S + SF, S + SF-S + SF, F + S-F + S, F + CP-F + S, and M + S-M + S (/ intercrop; + sequential crop; - rotation) (Wani et al. 1994). It was also suggested that significant contributions observed in those cropping systems were not only due to legume fixed N but also due to its N sparing effect, the break-crop effect, and soil microbial activity. It is

Table 2.4 Lists of microbial compounds with insecticidal and larvicidal properties

Source	Compound	Activity	References
Streptomyces nanchangensisNS3226	Nanchangmycin	Insecticidal	Sun et al. (2002)
Streptomyces sp. CP1130	Tartrolone C	Insecticidal	Lewer et al. (2003)
Streptomyces galbus	Ethyl acetate extract	Pesticidal	Jo et al. (2003)
Streptomyces sp.173	Fermented broth	Insecticidal	Xiong et al. (2004)
Metarrhizium sp. FKI-1079	Hydroxyfungerins A & B	Insecticidal	Uchida et al. (2005)
Streptomyces qinlingensis sp. nov.	Fermented broth	Insecticidal	Zhi-Qin et al. (2007)
Streptomyces sp.4138	Staurosporine	Insecticidal	Xiao-Ming et al. (2008)
Streptomyces sp. KN-0647	Quinomycin A	Insecticidal	Liu et al. (2008)
Streptomyces sp. ERI-04	Curde extract	Antifeedant	Valanarasu et al. (2010)
Streptomyces microflavus	Crude extract	Larvicidal	El-Bendary et al. (2010)
Saccharomonospora sp. (LK-1), *Streptomyces roseiscleroticus* (LK-2), &*Streptomyces gedanensis* (LK-3)	Crude extract	Larvicidal	Karthik et al. (2011)
Streptomyces sp. CMU-MH021	Fervenulin	Nematocidal	Ruanpanun et al. (2011)
Streptomyces microflavus neau3	Macrocyclic lactone	Insecticidal	Wang et al. (2011a)
Serratia marcescens NMCC46	Prodiogisin	Larvicidal	Patil et al. (2011)
Streptomyces avermitilis NEAU1069	Doramectin congeners, 1–4	Acaricidal & insecticidal	Wang et al. (2011b)
Streptomyces sp.	2-Hydroxy-3,5,6-trimethyloctan-4-one	Larvicidal	Deepika et al. (2011)
Chromobacterium violaceum ESBV 4400	Violacein	Larvicidal & pupicidal	Baskar and Ignacimuthu (2012)
Streptomyces sp.,VITSVK5	5-(2,4-Dimethylbenzyl) pyrrolidin-2-one (DMBPO)	Larvicidal	Saurav et al. (2011)
Saccharopolyspora pogona	Butenylspinosyn	Insecticides	Lewer et al. (2009)

Source: Vijayabharathi et al. (2014b)

important to identify high nodulating genotypes for developing an efficient variety. ICRISAT had done series of research in identifying high nodulating genotypes in all of its mandate legumes including chickpea, pigeon pea, and groundnut (Nigam et al. 1985; Rupela et al. 1995; Rupela and Johansen 1995). On the other hand, identifying non-nodulating legumes as a reference for quantifying BNF is a key feature in BNF research, and ICRISAT had also identified non-nodulating variants in all of its mandate crops including chickpea (Rupela 1992), pigeon pea (Rupela and Johansen 1995), and groundnut (Nambiar et al. 1986). Such selection, when developed, should be used not only in crossing programs as sources of high BNF genes but should also be developed as cultivars after evaluation.

ICRISAT had also contributed significantly to identifying effective rhizobial strains from chickpea, pigeon pea, and groundnut (Rupela et al. 1991). It also observed the effect of soil depth, cropping pattern, and season in influencing the soil rhizobial counts and identified that chickpea rhizobial counts have maintained even after cropping with pigeon pea, groundnut, and maize but not on rice where the 100-fold decrease was noticed (Rupela et al. 1987). ICRISAT always shared its rhizobial germplasm to researchers around the world. One set of the rhizobial collection at ICRISAT was transferred to Indian Agricultural Research Institute (IARI), New Delhi, India, and another set to University of Queensland, Australia, and CSIRO, Australia. The collection comprises 800 rhizobia strains nodulating chickpea, groundnut, and pigeon pea (Rupela 1997).

In addition to the legume-rhizobia symbiosis, nonsymbiotic N fixation has also been studied mainly on pearl millet and sorghum. Wani (1986) reported genotypic variation in germplasm lines of sorghum and pearl millet for rhizospheric nitrogenase activity. In these studies, of the 184 pearl millet germplasm tested, 18 lines enhanced nitrogenase activity (> 460 nmol C_2H_4 h^{-1} 15 cm diam $core^{-1}$) in the 0–15 cm rhizosphere soils, while 2 lines, J1407 and Gam 73, were reported be to consistently active over several seasons. Similarly, of the 334 sorghum germplasm, 28 lines enhanced nitrogenase activity (>460 nmol C_2H_4 h^{-1} 15 cm diam $core^{-1}$) (Wani 1986). Dart and Wani (1982) observed the effects of inoculation of *Azotobacter* and *Azospirillum* on the grain weight (increased up to 22%) and dry matter (increased up to 29%) of sorghum. Besides all these significant contributions on BNF research, ICRISAT had to freeze BNF research in the mid-1990s due to lack of policy support and funding opportunities. In later years, ICRISAT research focus has been diverted toward the use of low-cost biological inputs in influencing crop sustainability. Long-term experiments were conducted for a period of 9 years in ICRISAT with a new approach in calculating sustainability index with the consideration of nutrient, biological, microbial, and crop indices and demonstrated that the low-cost biological inputs obtained the highest sustainability index than the conventional systems (Hameeda et al. 2006).

ICRISAT and a few private sector biopesticide manufacturing companies initiated the ICRISAT-Private Sector Biopesticide Research Consortium (BRC) in January 2005, which was later renamed as Bio-products Research Consortium to include PGPR and biofertilizers (Rupela et al. 2005). Eleven biopesticide/biofertilizer companies joined the consortium as its founding members. The overall

goal of BRC was to make quality biopesticides and other bio-products to the farming community at an affordable price. ICRISAT had a collection of >1500 microbial germplasm (many with PGPR and biopesticidal properties), a few on-the-shelf technologies [such as *Helicoverpa* nuclear polyhedrosis virus (HNPV) and a few proven biopesticidal microbial strains], fermentation technologies, small-scale fermenters, and expertise in policy issues related to biopesticide testing and registration. On the other hand, the biopesticide/biofertilizer companies had medium- to large-scale capacity factories to manufacture bio-products and also the needed market linkages with a network of agro-dealers. The BRC Phase I was implemented with good success (2005–2007), and Phase II was started in 2008. Unfortunately, only 3 out of 11 companies continued their membership.

In recent times, ICRISAT research is focused more toward the usage of PGP microbes and their secondary metabolites for crop production and protection of our mandate crops. ICRISAT has isolated and identified more than 1500 PGP microorganisms including bacteria and actinobacteria, isolated from vermicompost and rhizospheric soils of rice and chickpea. Of the 1500, 59 PGP bacteria (mostly *Bacillus* spp. and *Pseudomonas* spp.) and actinobacteria (mostly *Streptomyces* spp.) were documented for their PGP traits, evaluated under field conditions (Gopalakrishnan et al. 2014a). Some of the promising PGP bacteria including *Pseudomonas monteilii*, *P. plecoglossicida*, *Brevibacterium altitudinis*, *B. antiquum*, *Enterobacter ludwigii*, and *Acinetobacter tandoii*, isolated from system of rice intensification fields, were documented for their PGP traits under field conditions on rice. These bacteria enhanced root weight, root length and volume, tiller numbers, panicle numbers, stover yield, and grain yield (Gopalakrishnan et al. 2012). Actinobacteria such as *Streptomyces* sp., *S. caviscabies*, *S. globisporus* subsp. *caucasicus*, and *S. griseorubens* were also demonstrated for their PGP performance on rice (Gopalakrishnan et al. 2014b). A PGP diazotrophic bacterium *P. geniculata* IC-76, isolated from chickpea nodules, enhanced plant growth and agronomic traits including nodule weight, pod weight, and seed weight (Gopalakrishnan et al. 2015) under field conditions on chickpea.

Besides increasing plant growth and yield traits, they also significantly enhanced rhizospheric available phosphorus (13–44%), total nitrogen (8–82%), and organic carbon (OC; 17–39%). Production of hydrolytic enzymes, including chitinase, cellulase, protease, and lipase, by these bacteria and actinobacteria (Table 2.5), is an additional evidence for the increased soil OC and total nitrogen contents (Gopalakrishnan et al. 2014a, b). Soil health indicators such as microbial biomass nitrogen (MBN; 7–321%), microbial biomass carbon (MBC; 23–48%), and dehydrogenase activity (14–278%) were also found to enhance on inoculated plots over the uninoculated control plots on chickpea (Gopalakrishnan et al. 2015), rice (Gopalakrishnan et al. 2012, 2013, 2014b), and sorghum (unpublished). Figures 2.1, 2.2, and 2.3 illustrate the results of field trials of PGP bacteria/actinobacteria on enhancing soil health traits.

Apart from their plant growth and yield promotion and soil health traits, PGP bacteria were also found to have antagonistic traits and act as biocontrol agents. PGP bacteria including *B. antiquum*, *P. plecoglossicida*, *E. ludwigii*, *B. altitudinis*,

Table 2.5 Extracellular enzyme profile identified for PGP bacteria and actinomycetes

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

Source: Gopalakrishnan et al. (2014a)

A. tandoii, and *P. monteilii* and actinobacteria including *S. setonii*, *Streptomyces* sp., *S. tsusimaensis*, *S. africanus*, and *S. caviscabies* were found to have antagonistic activity against soilborne plant pathogens including *Macrophomina phaseolina* (causes charcoal rot in sorghum) and *Fusarium oxysporum* f. sp. *ciceri* (causes wilt in chickpea) under both greenhouse and field conditions (Gopalakrishnan et al. 2011a, b). These PGP bacteria/actinobacteria were also found to produce hydrolytic enzymes (in the context of biocontrol) including chitinase and β-1,3-glucanase (Gopalakrishnan et al. 2014a). In another study, 15 *Streptomyces* spp. were found to have broad-spectrum insecticidal activities against lepidopteran insect pests including *Spodoptera litura*, *Helicoverpa armigera*, and *Chilo partellus* (Vijayabharathi et al. 2014a). Two insecticidal metabolites, N-(1-(2,2-dimethyl-5-undecyl-1,3-dioxolan-4-yr)-2-hydroxyethyl)stearamide and cyclo(Trp-Phe), have been isolated and purified from *Streptomyces* sp. (Sathya et al. 2016a; Gopalakrishnan et al. 2016a).

In the context of formulations, ICRISAT is using peat-based formulation for groundnut, pigeon pea, and chickpea rhizobial inoculants. Quality of microbial inoculants can be improved only if good carrier is used for maintaining and multiplying. In order to find whether peat can be used as suitable carrier material for rhizobial inoculant, a total of 16 rhizobia (six rhizobia specific for chickpea and five each rhizobia specific for pigeon pea and groundnut) were inoculated on sterilized peat and allowed to multiply at 28 ± 2 °C for 15 days. At the end

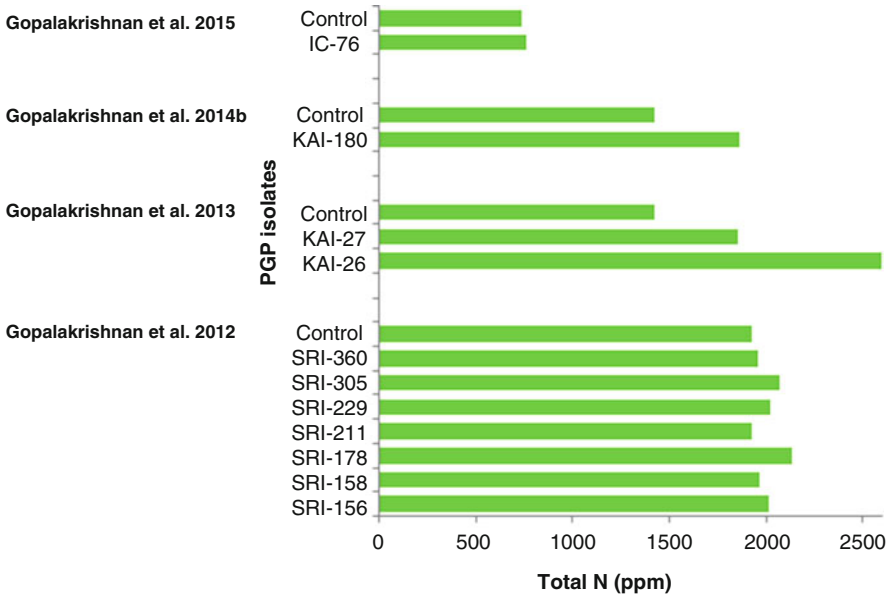

Fig. 2.1 Effect of PGP bacteria and actinomycetes on soil total N under field conditions of chickpea and rice cultivation. Control indicates the treatment groups without any PGP bacterial inoculation. (Source: Sathya et al. 2016b)

incubation and at 1-month interval for 10 months, peat-based inoculants were tested for survival and longevity of rhizobia. The rhizobia were enumerated as colony-forming units (CFU). The results indicated that all 16 rhizobia survived and maintained purity (at least 10^8 CFU/ml) up to 9 months in peat-based carrier materials (Table 2.6).

2.6 Commercialization

Voluminous data and information on better understanding of commercialization are available for various formulation technologies and longevity and efficacy of the PGP bacteria and/or rhizobia. However, still the quality of PGP bacteria/rhizobia available in the market worldwide is of suboptimal or poor quality. Development of rhizobia as inoculants for leguminous crops is the most valuable contributions ever made by science to agriculture, as BNF has been demonstrated to reduce N fertilizer use (Fred et al. 1932). Good quality PGP bacteria/rhizobia are available in the European Union market for a range of crop species (Guthrie 1896; Perret et al. 2000). However, in Asian countries, still the PGP bacteria/rhizobia inoculant technology is underdeveloped/slowly developing, and the major reasons are discussed here.

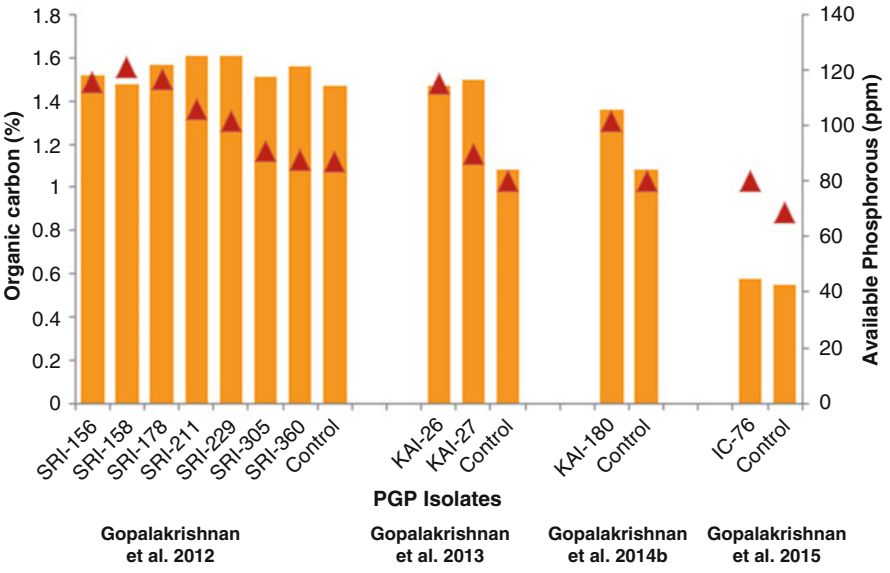

Fig. 2.2 Effect of PGP bacteria and actinomycetes on soil organic carbon and available phosphorous under field conditions of chickpea and rice cultivation. Solid bars ( ) are the % organic carbon on the left axis, and solid triangles ( ) are the available phosphorous (ppm) on the right axis. Control indicates the treatment groups without any PGP bacterial inoculation. (Source: Sathya et al. 2016b)

2.7 Inoculant Selection and Development

The effective PGP microbial strain selection is the core for developing an inoculant. A set of must-have desirable and essential traits required for selection of inoculant strains including competence with native microbial flora, host specificity, and genetic stability are available (Brockwell et al. 1995). Asian countries including China, India, Myanmar, and Nepal have been reported with a vast diversity of nodulating rhizobia for many leguminous crops including chickpea, pigeon pea, and groundnut (Adhikari et al. 2012; Ansari et al. 2014; Htwe et al. 2015; Jiao et al. 2015). Diversity analysis of rhizobia under hostile environments such as soils with alkalinity, acidity, and micronutrient deficiency was also reported (Biate et al. 2014; Mishra et al. 2014; Unno et al. 2015; Singh et al. 2016). Biogeographic and phylogenetic diversity of rhizobia across the world are available through two genome sequencing reports (Reeve et al. 2015). According to this report, among the 107 rhizobial strains, only 7 were from Asian origin. This suggests that further exploration and characterization of rhizobial biodiversity in Asian countries needs to be done in order to get good quality inoculant.

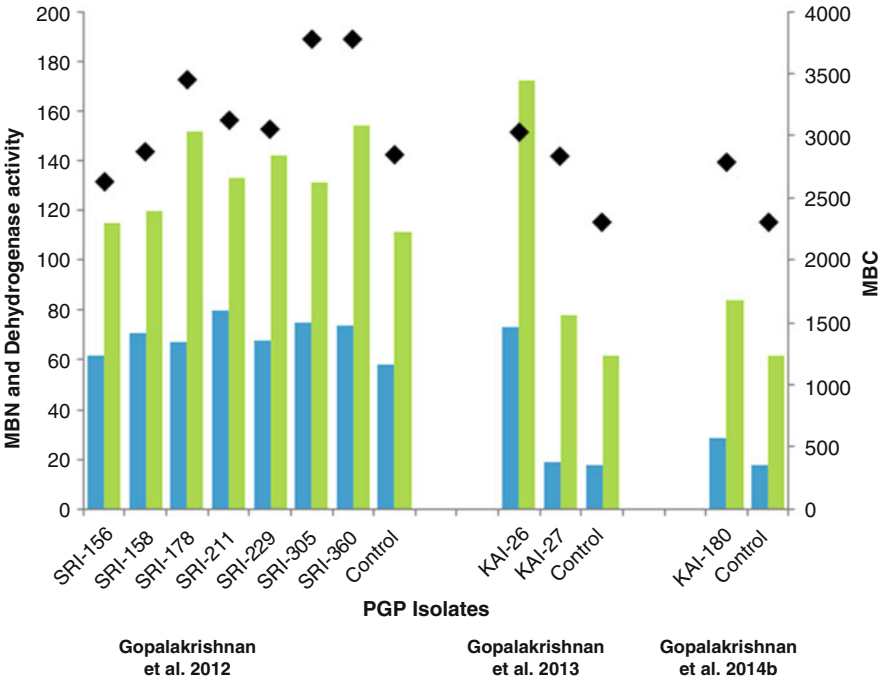

Fig. 2.3 Effect of PGP bacteria and actinomycetes on soil health indicators during field trials of rice cultivation. Solid bars (;) are the microbial biomass nitrogen (μg g^{-1} soil) and dehydrogenase activity (μg TPF g^{-1} soil 24 h^{-}) on the left axis, and solid diamond ( ) is the microbial biomass carbon (μg g^{-1} soil) on the right axis. Control indicates the treatment groups without any PGP bacterial inoculation. (Source: Sathya et al. 2016b)

2.8 Conclusions

PGP microbe possesses a broad spectrum of benefits including plant growth and yield, defense against diseases, and survival under stress conditions. This chapter illustrates the potential of PGP bacteria and highlights its importance in plant growth induction, defense pathways, and resistance spectrum available against various stresses on many crops. However, the extent of success in realizing the benefits of PGP tends to diminish as it moves from laboratory to greenhouse and to fields, which reflects the scarcity of research on the beneficial effects of PGP microbes under field conditions. Therefore, the generation of comprehensive knowledge on screening strategies and intense selection of best strain for rhizosphere competence and survival is the need of the hour to enhance the field-level successes.

Table 2.6 Viability and longevity of 16 rhizobia in peat formulations over 10 months

Rhizobial isolates	Colony-forming units (CFU/ml) at different months (values are mean of 3 replications)										
	0	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
Chickpea rhizobia											
IC-53	8.5×10^9	8×10^9	6.5×10^9	2.7×10^9	1.2×10^9	2.9×10^8	2.8×10^8	2.8×10^8	2.5×10^8	2×10^8	1.1×10^8
IC-59	3.3×10^9	2.3×10^9	2.2×10^9	1.7×10^9	2.3×10^8	1.5×10^8	1.3×10^8	1.2×10^8	1×10^8	3×10^7	2.0×10^7
IC-76	4.6×10^9	3.5×10^9	2.3×10^9	2×10^9	1.7×10^9	4.2×10^8	4×10^8	3.9×10^8	3.7×10^8	2.65×10^8	1.2×10^8
IC-2002	16×10^9	12×10^9	6.6×10^9	4.3×10^9	1.5×10^9	2.5×10^8	2.2×10^8	2×10^8	1.7×10^8	0.9×10^8	2.3×10^7
IC-2018	7.5×10^9	7.2×10^9	5.6×10^9	4.2×10^9	16×10^8	4.7×10^8	4.2×10^8	3.6×10^8	3.2×10^8	1.8×10^8	1.3×10^8
IC-2099	4.4×10^9	3.8×10^9	2.4×10^9	2.1×10^9	7×10^8	2.1×10^8	2×10^8	1.7×10^8	1.5×10^8	9×10^7	7.0×10^7
Pigeon pea rhizobia											
IC-3195	16×10^9	8.7×10^9	3.4×10^9	2.5×10^9	1.9×10^8	1.1×10^8	9×10^7	8×10^7	4×10^7	1×10^7	4×10^6
IC-4059	8.6×10^9	7.5×10^9	5.8×10^9	3.6×10^9	1×10^9	5.2×10^8	4.9×10^8	3.4×10^8	2.3×10^8	1.1×10^8	4.1×10^7
IC-4060	18×10^9	17×10^9	7.6×10^9	4.1×10^9	1×10^9	4.5×10^8	4.3×10^8	4.1×10^8	3.1×10^8	1.3×10^8	7×10^7
IC-4061	15×10^9	11×10^9	9.7×10^9	4.2×10^9	1.7×10^9	4.4×10^8	4.2×10^8	4.1×10^8	3.6×10^8	2.2×10^8	1.1×10^8
IC-4062	7.7×10^9	6.3×10^9	2.2×10^9	1.9×10^9	2.3×10^8	3.4×10^8	2.6×10^8	2.1×10^8	1.4×10^8	1×10^8	6×10^7
Groundnut rhizobia											
IC-7001	5.2×10^9	4.8×10^9	4×10^9	2×10^9	1.9×10^8	1.2×10^8	2.3×10^8	2.210^8	2×10^8	1×10^8	2.4×10^7
IC-7017	7.6×10^9	6.6×10^9	3×10^9	2.1×10^9	2.2×10^8	1.7×10^8	1.6×10^8	1.3×10^8	1.1×10^8	7×10^8	3.3×10^7
IC-7029	8.2×10^9	6.8×10^9	5.2×10^9	3.6×10^9	2.0×10^9	5.8×10^8	5.5×10^8	5.2×10^8	4.8×10^8	1.3×10^8	1.5×10^7
IC-7100	6.1×10^9	8.2×10^9	6.3×10^9	3.6×10^9	1.7×10^9	3.7×10^8	3.2×10^8	3×10^8	2.7×10^8	1.3×10^8	7×10^7
IC-7113	8.1×10^9	7.5×10^9	5.4×10^9	4.5×10^9	2.1×10^9	5.5×10^8	5.1×10^8	4.5×10^8	3.7×10^8	1.9×10^8	1.2×10^8

Source: Gopalakrishnan et al. (2016b)

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The Biological Method of Increasing Seed Germination and Productivity of Grain Crops

3

Irina Smirnova and Amankeldy Sadanov

Contents

Abstract

Cereals such as wheat, barley, and oat are the main strategically important crops cultivated in Kazakhstan. At the same time, more than 23–30% of grain crops were lost in the fields in 2014–2015 due to low seed germination and various diseases. The main tasks of this research were to develop a biological method to enhance seed germination and protect root rots caused by fungal pathogens. From the rhizosphere of grain crops were isolated more than 50 isolates of cellulolytic bacteria. After the testing, two strains which showed no phytotoxicity and possessing growth-stimulating ability were selected. The mixture of strains when applied more actively promoted seed germination and increased seedling growth compared to single strain application. The increase of seed germination was up to 80–92%. Treatment with the association of the strains significantly suppressed root rot diseases caused by *Fusarium*, *Alternaria*, and *Bipolaris* in cereals. Molecular genetic characterization of PGPR strains used was shown to belong to the genera of *Bacillus* and *Cellulomonas* and species *B. cytaseus* and *C. flavigena*. Mode of action studies for seed germination by these strains was

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showed due to the synthesis of cellulase enzymes. The efficacy of these strains to enhance plant growth of grain crops was shown to be associated with the synthesis of biologically active substances such as B-group vitamins and amino acids. It was also established that the strain *B. cytaseus* VKPM B-4441 could fix molecular nitrogen from the atmosphere (62.5×10^{-5} N_2/ml/h). The tested strains could colonize the hard seed coat and then began to synthesize cellulase enzymes to partially degrade the seed coat to form microcracks. The seed coats then become less firm and allow the increase of water transport and dissolved mineral substances and nutrients to the seed embryo. We hypothesize that the enhanced seed germination and stimulated seedling growth are due to the process of "the biological scarification." Seeds treated with these strains prior to seeding significantly increased their germination up to 80–89% compared to control (61–65%). The root rot rating was decreased 7.3 times compared to control. The yield of grain increased in the range of 2.6–3.2 centner/ha compared with the control. On the basis of this association of strains, the new biological preparation "Batsirin A" was developed. When use biopreparation enhanced seed germination and seedling growth and protected against root rots caused by fungal pathogens.

Keywords
Cellulolytic bacteria · Seed germinations · Antagonistic activity · Nitrogen-fixing ability · Biosynthesis B-group vitamins

3.1 Introduction

The major food and fodder plants around the world are grain crops (wheat, barley, and oats). They are grown in almost 70 countries and provide bread and fodder for about half of the world's population (Vetrov 2017). Its production leads other crops, such as rice, maize, and potatoes. China has the largest land area devoted to grain production, followed closely by the United States, India, and the Russian Federation. Kazakhstan and Canada, ranking fifth and sixth, produce grain crops on about half the area of the top four countries (Curtis 2017).

Grain crops such as wheat, barley, and oats are the main strategically important agricultural crops cultivated in Kazakhstan. According to the Ministry of Agriculture, in 2016, the sown areas under all agricultural crops reached 21.7 million hectares, of which grain crops occupied 15.2 million hectares, including wheat grown on 12.2 million hectares (Aytuganov 2016). This demonstrates the high importance of these crops for an agro-industrial complex of Kazakhstan. At the same time, more than 23–30% of grain crops were lost in the fields of Kazakhstan in 2014–2015. This is caused by two main reasons: low seed germination and yield losses due to various diseases.

As a result of the economic crisis, many Kazakhstan farms are buying poor-quality seed grain with low germination capacity which leads to a significant decline in the yield of grain crops. At present, enhancing the germination of grain crops is an

urgent problem in Kazakhstan. The second reason for the yield decline is that there are the diseases of grain crops caused by the fungi belonging to the genera *Fusarium*, *Helminthosporium*, and *Alternaria*. In 2014–2015, due to diseases caused by these fungi, more than 30% of grain yields were lost on the fields of the Republic. Without solving the problems of enhancement in germination and protection from phytopathogenic microorganisms, it is impossible to improve the effectiveness and stability of grain production.

The use of chemical methods and products for protecting and enhancing the germination of agricultural crops has a number of negative consequences: formation of the stable phytopathogenic races, reduction in the number of beneficial microorganisms in microbiocenoses, and accumulation of toxic substances in the soil (Reddy et al. 2009; Fan et al. 2012; Lu et al. 2017; Pertot et al. 2017).

An alternative approach involves the development of biological methods using microbial preparations. These biopreparations are based on highly active strains of microorganisms (Novikova et al. 2003; Labutova 2011). In this regard, the use of cellulolytic bacteria is the most promising trend (Suo et al. 2011). Their physiological and biochemical properties, including high growth rate, unpretentiousness to the nutrition sources, and the simplicity of the cultivation, ensure high processability in the biomass production. In addition, due to their biological characteristics, such as the population stability and ability to synthesize antifungal metabolites, they are active fungal antagonists.

Previously, we have developed a bacteria-based biological method for increasing the germination of melilot (Smirnova et al. 2012, 2015, 2016). The method consists in the application of cellulolytic bacteria synthesizing cellulase enzymes that partially degrade the hard coat of melilot seeds.

The aim of this research was to develop a biological method for increasing seed germination and protecting grain crops against root rots caused by fungal phytopathogens.

3.2 Materials and Methods

3.2.1 Isolation of Cellulolytic Bacteria

Cellulolytic bacteria isolated from the rhizosphere of grain crops served as objects of the study. These bacteria were isolated from soils of agricultural lands in the north of Kazakhstan. This region is the major grain-producing area.

For isolated cellulolytic bacteria, the liquid Hutchinson's medium was used. Wheat straw served as a source of cellulose. To prepare an enrichment culture, a certain amount of soil from the rhizosphere was placed in a liquid medium. Cultivation was carried out for 5–7 days on a shaker at a speed of 180 rpm and a temperature of 28–30 °C. Further, the organized Hutchinson's medium without a carbon source was poured into Petri dishes. A circle of a certain diameter was cut out of the filter paper and placed on the bottom of the dish. The filter was tightly pressed against the medium with sterilized tweezers. The 1:10 and 1:100 dilutions of the enrichment

culture were prepared. 2 ml of suspension was taken from these dilutions and applied to filter paper, evenly spreading over the entire surface. The closed Petri dishes were placed into a thermostat and kept for 12–14 days at a temperature of 28–30 °C. The microorganisms were selected from the holes formed on the filter paper by the loop. They were plated in Petri dishes with the agarized Hutchinson's medium containing 2% water-soluble cellulose (Na-carboxymethyl cellulose). The pure bacterial cultures were obtained.

The resulting cultures were grown on the liquid Hutchinson's and Gould-Dexter's media and solid Hutchinson's and MPA media. The temperature for culturing bacteria was 28–30 °C. The cultures were maintained in nutrient agar slants and regularly subcultured (Egorov 2006; Emtsev and Mishustin 2005).

3.2.2 Studies on Germinations Seeds and Growth-Promoting Activity of Cellulolytic Bacteria Under Model Laboratory Conditions

To study the ability to increase the seed germination and their growth-stimulating activity, the bacteria were grown in liquid Hutchinson's medium under shaking conditions at a speed of 180 rpm and a temperature of 28 °C for 5–7 days. Seeds of wheat, barley, and oats recommended for cultivation in Kazakhstan were used in the experiments. Seeds of cereals before sowing were inoculated with a bacterial suspension at a concentration of 10^6–10^7 cells/ml, for 2 h at 23 °C. The treated seeds were sown in the 450 ml growth vessels. The duration of the experiments was 30 days. The non-treated seeds served as controls. Vermiculite was used as a substrate for plant growth. To feed the seedlings, Knop's liquid medium was used. Prior to the experiment, the substrate and Knop's solution were sterilized; the plants were watered with the sterile tap water. All the experiments were carried out in seven replicates. The plant biometric parameters, such as the length of stems and roots, were measured after 30 days of growing (Posypanov 2007; Stepanov et al. 2012).

The model laboratory experiments on the effect of bacteria on the germination and development of the grain crop seedlings were carried out in a climatic chamber (Memmert HPP 750 Constant Climate Chamber, Germany). The humidity, illumination, and temperature values in the chamber corresponded to the average parameters in the spring period.

3.2.3 Studies on Antagonistic Activity of Cellulolytic Bacteria Against Phytopathogenic Fungi

To study the antagonistic activity of bacteria, the phytopathogenic fungi *Alternaria alternata*, *Bipolaris sorokiniana*, *Fusarium solani*, and *F. oxysporum* var. *orthoceras*, causing alternariosis and root rot grain crops, were used as test organisms. We have isolated phytopathogens from the damaged plants in the fields

of the northern areas of Kazakhstan 2013–2015. Czapek's and PDA media were used for the cultivation of phytopathogenic fungi.

The antagonistic activity was determined by measuring the growth inhibition zones of phytopathogenic fungi (Egorov 2005). The fungi were inoculated by the pour plate technique in a medium, melted and cooled to 40 °C, and poured into Petri dishes. After solidification of the agar, blocks were cut out of the medium. A suspension of bacterial cells with a certain concentration was poured into the resulting wells. The plates were incubated at a temperature of 28–30 °C in a thermostat for 6 or more days. The data were presented as the growth inhibition zones of phytopathogenic fungi.

3.3 Identification of Cellulolytic Bacteria

To determine the taxonomic position of cellulolytic bacteria, the classical microbiological methods based on studying the culture-morphological and biochemical characteristics and properties of bacteria (Holt et al. 1997) and molecular genetic techniques were used. The Sanger sequencing method was used to confirm the taxonomic position of cellulolytic bacteria (Kwan Soo Ko et al. 2004; Kazartsev 2013). Genomic DNA was isolated from the examined strains using the PureLink® Genomic DNA Kits (Invitrogen, USA). The DNA concentration in the samples was determined with the Qubit Fluorometer (Invitrogen, USA) using the scale for dsDNA HS. Sequencing of the bacterial 16S rRNA gene was performed on the automated sequencer 3500 DNA Analyzer (Applied Biosystems, USA) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's protocol (BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol Applied Biosystems USA). Identification was carried out by analyzing sequences of the 16S rRNA gene fragment using universal primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3′) and 806R (5'--GGACTACCAGGGTATCTAAT-3′). The reaction mixture (30 μl) contained 3 μl of the 10x reaction buffer (Fermentas), 2.5 mM of $MgCl_2$, 0.2 mM of each deoxyribonucleoside triphosphate (dNTP), 10 pmol of each primer, and one unit of Maxima Hot Start Taq DNA Polymerase (Fermentas). PCR has performed in the Mastercycler proS (Eppendorf) thermal cycler. The reaction was started by incubating the mixture at 95 °C for 7 minutes, followed by 30 cycles consisting of 95 °C for 30 seconds, 55 °C for 40 seconds, and 72 °C for 1 minute. The final elongation was carried out at 72 °C for 10 minutes. The amplified product was separated in the1.5% agarose gel; the bands were stained with ethidium bromide and visualized in a UV transilluminator. The 1 × TAE buffer was used as a running buffer. The PCR product was purified using the CleanSweep™ PCR Purification Reagent (Thermo Fisher Scientific, USA). Sequencing of fragments of the 16S rRNA gene from bacteria was performed on an automatic sequencer 3500 DNA Analyzer (Applied Biosystems, USA) using the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's protocol (BigDye® Terminator v3.1 Cycle Sequencing Kit Protocol Applied Biosystems USA).

3.3.1 Studies of Cellulolytic Activity of Bacteria and Composition of Cellulase Complex

The cellulase activity of bacteria was determined in a solid medium with 0.1% Na-CMC and expressed in unit/ml (Tolchenov et al. 2009). The Mandels-Weber method was used to quantify the cellulase activity (Mandels and Weber 1969). The composition of the bacterial cellulase complex was established in the A. Bach Institute of Biochemistry, Russian Academy of Sciences (Moscow, Russia).

3.3.2 Study of the Nitrogen-Fixing Ability, Biosynthesis B-Group Vitamins, and Composition of Amino Acids of Cellulolytic Bacteria

The nitrogen fixing of bacteria was evaluated using the Agilent technology 6890B Gas Chromatograph, USA (Wang et al. 1994). B-group vitamins were determined by microbiological methods (Tolysbaev and Bisenbayev 1996). The quantitative and qualitative composition of free amino acids was established using the AAA 400 Amino Acid Analyzer (INGOS, the Czech Republic) according to the protocol attached to the user's guide. The bacterial biomass was measured nephelometrically with spectrophotometer PD-303 ("Apel", Japan) in optical density units (RODU), calculated per absolute dry mass (a.d.m.) using the calibration curve, and expressed in g/1000 mL.

3.4 Statistical Analysis

The experiments were carried out in 5–7 replicates. Statistical significance of the obtained results was determined by the Student's t-test for the confidence probability with $p < 0.01$ (Urbach 2005).

3.5 Results

From the rhizosphere of grain crops, more than 50 isolates of cellulolytic bacteria were isolated. Of these, 23 cultures were selected, presumably with the ability to increase the germination of grain seeds. We checked the phytotoxicity of these cultures to the grain crop seedlings. After the testing, six cultures which showed no phytotoxicity and possessing growth-stimulating ability were selected.

For selecting the most active bacterial strains, the model experiments for studying their effect on seed germination and grain growth were carried out under laboratory conditions in a climatic chamber. Seeds of wheat, barley, and oats were used in the experiments. Seeds were inoculated with bacterial suspensions at a concentration of 10^6–10^7 cells/ml. The duration of the experiments was 30 days. The non-treated seeds served as controls. The data obtained in the study are presented in Table 3.1.

Table 3.1 Effect of cellulolytic bacteria strains on the germination and growth of cereals

Strains	Germination (%)	Shoot length (cm)	Root length (cm)
Wheat			
Control	66.1 ± 1.1	15.9 ± 0.1	9.3 ± 0.1
150	75.1 ± 1.3	17.4 ± 0.1	10.3 ± 0.1
121 N	82.3 ± 1.9	17.7 ± 0.1	11.2 ± 0.1
60(5)4	80.4 ± 1.8	17.2 ± 0.2	10.1 ± 0.1
21AS	90.1 ± 2.,1	18.2 ± 0.3	13.2 ± 0.1
60CS	88.3 ± 2.2	18.4 ± 0.2	13.1 ± 0.2
160	76.3 ± 1.2	17.5 ± 0.1	11.0 ± 0.1
Barley			
Control	60.2 ± 1.3	16.2 ± 0.1	10.8 ± 0.1
150	65.1 ± 1.3	17.4 ± 0.1	12.3 ± 0.1
121 N	69.3 ± 1.1	16.9 ± 0.1	12.2 ± 0.1
60(5)4	65.4 ± 1.3	17.6 ± 0.2	13.1 ± 0.1
21AS	78.0 ± 1.3	18.2 ± 0.2	14.9 ± 0.2
60CS	78.2 ± 1.4	18.3 ± 0.2	15.1 ± 0.1
160	70.3 ± 1.0	17.1 ± 0.1	12.0 ± 0.1
Oats			
Control	58.7 ± 1.1	15.3 ± 0.3	7.6 ± 0.1
150	65.1 ± 1.1	17.4 ± 0.1	8.8 ± 0.1
121 N	75.3 ± 1.0	15.9 ± 0.1	8.7 ± 0.1
60(5)4	73,4 ± 1.3	16.2 ± 0.2	8.1 ± 0.1
21AS	76.0 ± 1.3	17.4 ± 0.1	9.6 ± 0.2
60CS	75.3 ± 1.2	17,2 ± 0.1	9.8 ± 0.1
160	72.3 ± 1.1	16.0 ± 0.2	8.5 ± 0.3

It was established that all strains increased the germination of grain seeds by 8–36% (depending on the strain) and increased the growth of seedlings. The length of stems and roots increased by 4–19% and 6–42%, respectively, as compared with the control. Of the six strains studied, two bacterial strains 21AS and 60CS were selected which most actively improved seed germination and growth of grain crop seedlings.

We have examined the biocompatibility of these strains. It was established that the strains are not antagonists, but positively affect each other. At their joint cultivation, the rate of biomass accumulation is 28–31% higher than that of monocultures. It was also shown that the joint use of strains significantly enhances the effectiveness of their impact on grain crops. Inoculation of seeds with the strain association improved germination to 80–92% and increased the length of stem and roots by 24.5% and 45.3%, respectively, as compared to the use of monocultures.

The selected strains were examined for their antagonistic activity against phytopathogenic fungi. The following fungi that cause root rots in grain crops were used as test cultures: *Alternaria alternata*, *A. tenuis*, *Bipolaris sorokiniana*, *Fusarium solani*, *F. oxysporum*, and *F. oxysporum* var.*orthoceras* (Table 3.2).

Table 3.2 Antagonistic activity of bacteria against phytopathogenic fungi

Fungi	The diameter of the zone of inhibition (mm)		
	Strain21AS	Strain60CS	Association
A. alternate 28M	48.4 ± 2.1	45.3 ± 2.1	51.5 ± 2.2
A. tenuis 64S	39.1 ± 1.3	36.2 ± 1.3	42.4 ± 1.2
B. sorokiniana ET	26.2 ± 2.5	21.6 ± 2.5	40.1 ± 2.4
F. solani C11	48.5 ± 2.2	41.5 ± 2.2	50.3 ± 2.0
F. oxysporum M2	29.1 ± 1.4	23.3 ± 1.2	32.3 ± 1.5
F. oxysporum var. *orthoceras 18S*	13.3 ± 1.0	11.1 ± 1.1	17.8 ± 1.2

Values are means of five replications

Table 3.2 shows that cellulolytic bacteria were potent inhibitors of fungal phytopathogens. The strains of bacteria most strongly inhibited the growth of *A. alternate*, *A. tenuis*, and *F. solani* (diameter of the inhibition zone of 48–36 mm), somewhat less of *B. sorokiniana* (21–26 mm) and *F. oxysporum* (23–29 mm), and much less of *F. oxysporum* var. *orthoceras* (up to 13 mm). The data in Table 3.2 shows that the bacterial association inhibited the growth of phytopathogenic fungi more actively than that of monocultures. The zone ingibition of the fungi *F. oxysporum* var. *orthoceras* for association was 17.8 mm, for strains 21 AS and 60CS – 11.1 and 13.3 mm, respectively.

To identify strains of cellulolytic bacteria, their cultural-morphological and biochemical characteristics and properties were studied. It was found that the strain 21AS belongs to the genus *Bacillus* and the strain 60CS to the genus *Cellulomonas*. The sequencing according to the procedure described in the Materials and Methods section was performed to confirm the taxonomic position of cellulolytic bacteria. The resulting nucleotide sequences were added to the BLAST program and compared to the existing database. In this case, a similarity was found to the bacteria belonging to the genera *Bacillus* and *Cellulomonas*. Phylogenetic analysis for species identification of the strains belonging to the genera *Bacillus* and *Cellulomonas* was carried out by comparing them with 16S rRNA gene sequences of related bacterial strains from the NCBI database. Phylogenetic trees were constructed with the MEGA 6.0 software, using the neighbor-joining clustering method for calculating genetic distances (Fig. 3.1).

The results of phylogenetic analysis of the 16 s rRNA gene showed that the strains *Bacillus halodurans* C-125 and *Paenibacillus amylolytic* NBRC 15957 (Fig. 3.1a) were phylogenetically the closest to the strain *Bacillus cytaseus* 21AS. In the dendrogram, the strain 60CS was assigned to the genus *Cellulomonas* and defined as *C. flavigena* (Fig. 3.1b). Comparison of the 16S rRNA gene nucleotide sequences against the reference strain *Cellulomonas flavigena* DSM 20109 from the NCBI database showed a 99% homology.

The strains were deposited with the All-Russian Collection of Industrial Microorganisms (Russia, Moscow). They were assigned the following numbers: *Bacillus cytaseus* VKPM V-4441 and *Cellulomonas flavigena* VKPM V-4465.

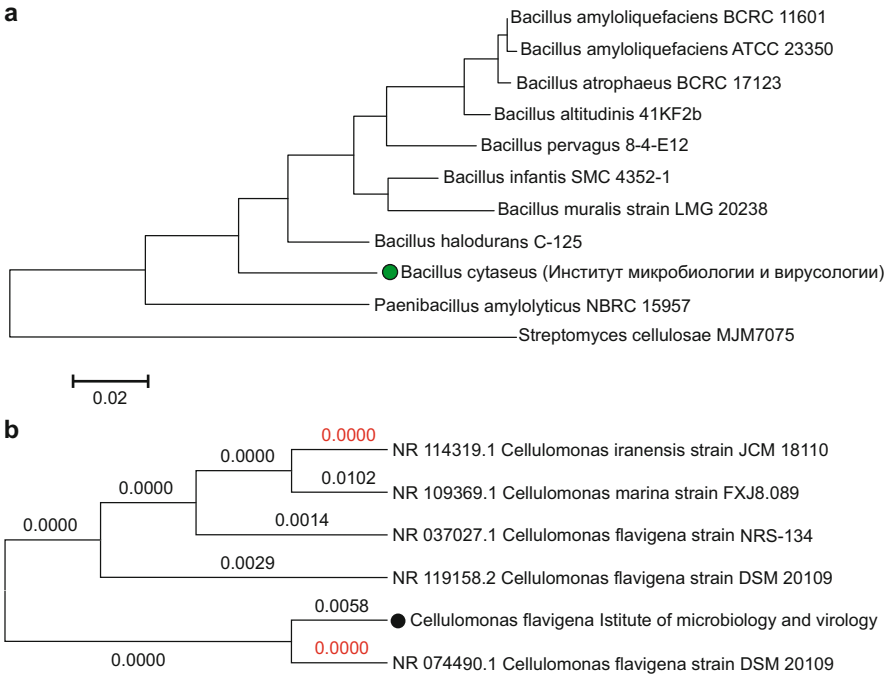

Fig. 3.1 Phylogenetic trees of genus *Bacillus* (**a**) and genus *Cellulomonas* (**b**)

The studies on the composition of the bacterial cellulase enzyme complex showed that the cellulase complex of *B. cytaseus* 21AS consisted of three enzymes: endo-1.4-β-glucanases, cellobiohydrolases, and β-glucosidases. The cellulase complex of *C. flavigena* 60CS contained two enzymes: endo-1.4-β-glucanase and cellobiohydrolase.

A detailed study showed that the association effectively increased seed germination by synthesizing cellulase enzymes. The ability of strains to actively stimulate plant growth is due to the fact that they synthesize biologically active substances, such as B-group vitamins, amino acids, and probably gibberellins. In addition, the strain *B. cytaseus* 21AS fixes the molecular nitrogen of the atmosphere, which creates additional nitrogen nutrition for plants (Table 3.3.).

The mechanism of action of the strains has been examined: after inoculation of the seeds, the bacteria colonize the hard seed coat, begin to grow, using cellulose of the hard seed coats as a carbon source; for this purpose, cellulase enzymes are synthesized that partially degrade the coat. Microholes and microcracks are formed on the seed coat. The seed coats become less strong, and through it, the transport of water and mineral and nutrient substances dissolved in it to the seed germ increases. This leads to an increase in germination and stimulation of seedling growth. We have named this process "biological scarification." The changes in the coating structure are clearly visible on micrographs of the wheat seed coats under the effect of the association of cellulolytic bacteria (Fig. 3.2).

Table 3.3 Biosynthesis of B-group vitamins and nitrogenase and cellulase activity by strains cellulolytic bacteria and association

Variants	Vitamins (mkm/g a.d.m.*)				Nitrogenase activity (mkmolC_2H_4/ml/h)	Cellulase activity (unit/ml)
	B_1	B_3	B_6	Pp		
Strain 21AS	18.4 ± 0,2	19.8 ± 0.2	14.6 ± 0.2	170.4 ± 2.2	3.87 ± 0.01	4.7 ± 0.01
Strain 60CS	15.2 ± 0,2	0	12.6 ± 0.2	168.6 ± 2.2	–	3.8 ± 0.01
Association	21.6 ± 0,2	43.8 ± 0.2	21.4 ± 0.2	235.0 ± 2.2	6.25 ± 0,02	5.3 ± 0.02

Values are means of five replications; a.d.m.* – absolutely dry mass

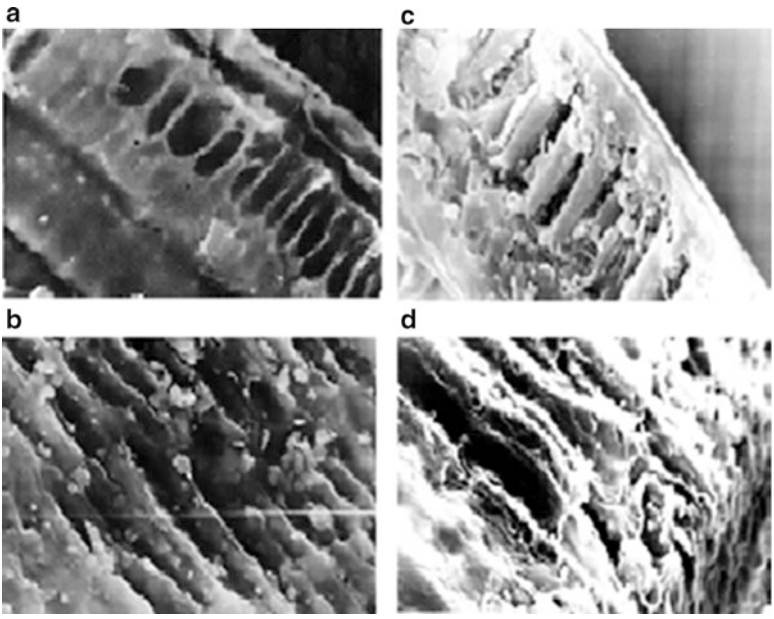

Fig. 3.2 Electron micrographs of hard coat seeds of wheat (1) and barley (2): a,b – control (without inoculation), c,d – after inoculation seeds

To study the effectiveness of the association under production conditions, the field trials of the bacterial association have been conducted in the north of Kazakhstan (Kostanay region) for 2 years. Spring wheat and barley seeds were used in the experiments. Seeds were subjected to pre-sowing treatment with the bacterial association. The non-treated seeds were used as the control. The agricultural chemical Biosil served as a reference (application rate of 0.05 L/t). The agrotechnology, generally accepted for this agricultural zone, was used in the experiments. Field trials have confirmed the high effectiveness of the bacterial association (Table 3.4).

The data in Table 3.4 show that the pre-sowing inoculation of grain crop seeds increased their germination up to 80–89% (61–65% in the control). The number of plants affected by root rot decreased 2.4–7.3 times. The grain crop yield increased by 2.6–3.2 centner/ha as compared to the control.

3.6 Discussion

The chemicalization of agriculture causes a decrease in immunity of agricultural crops, the emergence of resistant forms of phytopathogenic microorganisms, disturbance of soil microflora, and reduction of soil fertility. All this leads to a deterioration in food quality and adversely affects human health.

Table 3.4 Effect of inoculation seeds by the association of cellulolytic bacteria on germination and grain yield of wheat and barley

Variants	Germination (%)	Root rots (%) by growth phases		Grain yield (c/ha)
		Tillering	Ripeness	
Wheat				
Control	65.4	3.6	21.5	18.3
Biosil	80.2	2.3	14.5	20.7
Association	89.3	1.5	9.0	21.5
Barley				
Control	61.2	12.9	65.8	11.3
Biosil	83.8	10.5	12.6	16.8
Association	88.9	5.2	8.8	17.9

Values are means of eight replications

The problems of agriculture are solved in different ways. Some farmers improve the chemical plant protection products, continuing to have a disastrous effect on the environment, while others develop biological farming methods based on the use of preparations containing natural microorganisms (Bessonova and Mereshchenko 2014; Heusinkveld et al. 2016; Curutiu et al. 2017).

Our studies are related to the application of PGPRs in order to control phytopathogens and increase seed germination of agricultural crops. PGPRs can reduce the use of chemicals, improve yields, lower the production costs, and make the final products environmentally clean (Beneduzi et al. 2012; Ahemada and Kibretb 2014; Wang et al. 2014).

To increase seed germination, various methods are used in agriculture, including physical, chemical, and mechanical (Ning et al. 2014; Zin et al. 2016; Kataria et al. 2017; Ruttanaruangboworn et al. 2017). Scarification is the most commonly used method, that is, a mechanical violation of the seed coat integrity. This method often causes damage to the seed embryos, which leads to their infection with pathogens and a decrease in germination. In addition, scarification is an energy-intensive process and requires significant energy and labor costs (Behlyarova 2009; Chaodumrikul et al. 2016; Wagner et al. 2017).

We have developed a biological method for increasing the germination of cereals. The method makes it possible simultaneously to increase the germination of seeds and to protect the cereals from root rot. The method is based on the application of the association of cellulolytic bacteria. The use of cellulolytic bacteria in our studies is explained by the fact that the selected strains of the association have a wide range of desirable properties for use in agro-biotechnology:

- Are able to carry out "biological scarification" of seeds – partially destroy the hard seed coat, thereby increasing the access for water and nutrition to the seed embryo.

- Inhibit the growth of phytopathogenic fungi, reduce the damaging effects of root rots on grain crops seven times, and improve the phytosanitary situation in the soil.
- Stimulate the growth of plants due to the synthesis of biologically active substances (B-group vitamins, free amino acids, and probably gibberellins).
- Fix atmospheric nitrogen and increase its availability to the root of plants.
- Closely interact with plants and form an "associative symbiosis."
- Since cellulose is the bulk of plant residues in the soil, the size of this bacterial group is more significant as compared with other soil groups of microorganisms. Cellulolytic bacteria are frequently detected in the rhizosphere of agricultural crops, and bacteria with useful properties for agriculture can be found among them (Rasmussen et al. 2002; Naplekova 2010; Talia et al. 2012; Hong-Sheng Wu et al. 2017).

Bacteria are developing on the cellulose-containing substrates in the soil, destroy them, and improve the physicochemical properties of the soil and its structural state. The soil is supplied with organic matter, and the humus formation is intensified. All these processes have a positive effect on soil fertility.

The use of biological preparations based on this group of bacteria is environmentally safe, because it is based on the interaction of organisms in nature and does not lead to a disturbance of the biological equilibrium in the soil. Cellulolytic bacteria are widely distributed in nature, being nontoxic and nonpathogenic to humans, warm-blooded animals, and bees. Cellulolytic bacteria themselves as a natural factor are involved in improving soil fertility. The production created on their basis will not serve as a source of environmental pollution.

In conclusion, a biological method for increasing seed germination and protecting grain crops has been developed on the basis of the association of cellulolytic bacteria. The method enables to increase the germination to 80–89%, reduce the number of plants affected by root rot 7.3 times, and improve the yields of grain crops by 2.6–3.2 centner/ha. On the basis of this method, we have developed a new biological preparation "Phytobatsirin" to increase seed germination and their protection against root rots. Patents of the Republic of Kazakhstan have been obtained for the association of cellulolytic bacterial strains and method of producing the biopreparation in industrial conditions. Currently, the biopreparation is produced in the factory at the Institute of Microbiology and Virology and successfully used by the farming enterprises.

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4 Development and Formulation of Beneficial Rhizobacteria Consortia to Improve Soil Health and Agricultural Practice Sustainability in Indonesia

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Contents

Abstract

Since the green revolution, the inorganic fertilizers and other agrochemical products have been used intensively to boost the agricultural productivity. Thus, these practices increased the food production significantly but, at the same time, caused the severe degradation of soil health. Currently, about 70%

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of paddy soils and 90% of the dry land ecosystem have been exhausted and severely degraded as indicated mainly by the low organic content (<1.5%). These chemical inputs also slowly change the nutrient supplying capacity, making the soils even more infertile. Nutrient deficiency has become a major limiting factor in food production. Beneficial microorganisms in the rhizosphere and soil mediate nutrient cycle, enhance nutrient mobilization, and facilitate the uptake, leading to increased root growth, biomass, and yield of plants. Based on the condition, we conducted field experiments to investigate the effect of beneficial rhizobacteria consortia to improve soil health, fertilizer use efficiency, and crop productivity in various Indonesian soil ecosystems. The consortia of beneficial rhizobacteria were formulated with organic-based carriers. Consortia of *Bradyrhizobium japonicum*, *Pseudomonas* sp., and *Bacillus* sp., given at the rate of 200–400 g ha^{-1} on soybean; *Azotobacter* sp., *Azospirillum* sp., *Pseudomonas* sp., *Bacillus* sp., and *Acinetobacter* sp., at 400–600 g ha^{-1} on rice; and *Azotobacter* sp., *Azospirillum* sp., *Pseudomonas* sp., *Bacillus* sp., *Streptomyces* sp., and *Trichoderma* sp., at 800–1200 g ha^{-1} on corn, were evaluated. Our results showed that soybean inoculants could increase the soybean grain yield and reduce the inorganic N fertilizers by 50–75%; rice inoculants increased the grain yield and reduced the inorganic N and P by 25%, and corn inoculants increased the corn grain yield and reduced the inorganic N and P fertilizers by 25%. The combination of rhizobacterial inoculant and 2–5 t straw compost (ameliorant) increased the soil organic carbon and rice productivity and reduced inorganic NPK fertilizer by 25–50%. Beneficial rhizobacteria consortia composition with organic-based carriers has a great commercial potential to improve soil health, crop productivity, and sustainability of agricultural practices.

Keywords
Rhizobacteria · PGPR · Biofertilizers · Sustainable agriculture · Soil health

4.1 Introduction

The main challenge of Indonesian agriculture is to boost crop productivity and fulfilling the agricultural product demand to a rapidly growing population. The intensive uses of inorganic fertilizers and other agrochemical products since green revolution adaptation increased agricultural production but also caused land degradation and environmental problem. Indonesian food crop production and productivity by adopting a technology package comprising improved high-yielding varieties of rice, irrigation or controlled water supply, improved moisture utilization, fertilizers and pesticides, and associated management skills have been increased sharply from 2.5 tons ha^{-1} in the 1960–1970s to 5–6 ton ha^{-1} 2015 (Simarmata et al. 2011; Panuju et al. 2013).

There is a strong indication that high use of inorganic fertilizers has reached a leveling off (plateau) in productivity increment. The average growth in rice productivity from 1970 to 1984 was about 3.7% per year and in the period of 1985–2008 has decreased to 0.95% per year. It seems this problem also occurred in another Asian country (Pinstrup et al. 1985). Most of agricultural soils either wetland or

dryland ecosystems in Indonesia have been degraded and exhausted due to intensive cultivation and overexploitation. Recent data revealed that about 70% of paddy soils have low organic carbon (less than 1.5%) and about 90% of dry land fell into sick soil category (low organic carbon and high acidity) (Simarmata et al. 2011, 2017). Consequently, the need for organic fertilizers and other organic ameliorants became crucial in order to improve soil's health and quality (Simarmata et al. 2011; Sudjana et al. 2017). The status of essential plant nutrient of most agricultural lands such as macro- and micronutrients is low, especially nitrogen, phosphorus, and potassium. Furthermore, the burden of land has increased in order to support the intensive agriculture. Intensive farming practices have virtually mined nutrients from soil; when fertilizers are applied to the soil, not only nitrogen, phosphorus, and potassium from the fertilizer were taken by the plant but also proportionately increased levels of micronutrients from the soil, including zinc, iron, and copper. Over time, the soil becomes deficient in these micronutrients. Continuous heavy application of chemical fertilizers may effect environmental pollution and degradation of soil quality and soil health. In addition, inorganic fertilizer is no longer affordable. The price of inorganic fertilizer continues to increase along with the fuel price (Choudhury and Kennedy 2004; Apriyantono 2008; Aggani 2013; Purwanto et al. 2016).

One of the efforts that can be done to increase the nutrient availability is through the usage of beneficial rhizobacterial as one of the plant nutrient suppliers in the form of biofertilizer. Generally, biofertilizers make use of soil microorganisms to increase the availability and uptake of essential mineral nutrients for plants. Most commonly it is defined as follows: (1) Biofertilizer is a substance which contains living microorganisms which, when applied to seeds, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey 2003). (2) Biofertilizers are natural fertilizers which contain a large population of specific or a group of beneficial microorganisms for enhancing the productivity of soil either by fixing atmospheric nitrogen, by solubilizing soil phosphorus, or by stimulating plant growth through synthesis of growth-promoting substances or latent cells that activate the biological process to form a fertilizer compound or make available the unavailable form of elements or to facilitate nutrient availability for plants (Gupta and Rog 2004; Singh and Purohit 2011; Begum et al. 2011; Bhattacharjee and Dey 2014).

Under the scope of this terminology, biofertilizers will include (1) nitrogen-fixing bacteria, (2) phosphate-solubilizing microorganisms, (3) cellulolytic microorganisms (decomposer), (4) plant growth-promoting rhizobacteria (PGPR), and (5) mycorrhizal fungi (Saha and Biswas 2009; Reddy et al. 2000; Singh and Purohit 2011; Simarmata 2013; Malusa et al. 2016). Biofertilizers are low-cost, renewable sources of plant nutrients that can act as inorganic fertilizer supplements and has definite advantage over inorganic fertilizers:

(a) Provide not only the main nutrient but also certain growth-promoting substances like hormones, vitamins, amino acids, etc., while chemical fertilizers provide only a certain nutrient. Biofertilizers supply the nutrients continuously throughout the entire period of crop growth in the field under favorable conditions, while chemical fertilizers only replace or provide nutrients in a certain time.

(b) Eco-friendly and renewable. It has a residual effect on subsequent crops, and it improves the soil quality and plant health.
(c) Highly efficient. Biofertilizers have about ≥90% of fertilizer efficiency (losses due to leaching and fixation are negligible), while chemical fertilizers have an efficiency of only about 30–50%, 25–40%, and 30–50% for N, P, and K fertilizers, respectively.
(d) Has nearly no negative impact on environment (environmentally friendly fertilizers) (Singh and Purohit 2011; Simarmata 2013; Simarmata et al. 2017; Ghany et al. 2013).

4.2 Importance of Rhizobacteria

Diverse soil bacteria which live and intensively colonize the plant roots (rhizosphere) are known as rhizobacteria and may form the intense relationship with the plant's roots (Singh and Purohit 2011; Simarmata 2013; Shokati and Poudineh 2017). The term rhizobacteria comes from the Greek word "rhiza" which means root. They are often referred to as plant growth-promoting rhizobacteria (PGPR) (Hiltner 1904; Kloepper and Schroth 1978; Vessey 2003; Vijay et al. 2017). Rhizobacteria play an important role in enhancing plant growth, have different key functions in the soil ecosystem, and have been proven to be beneficial for soil health, plant health, and crop productivity. They are involved in chemical transformation and essential nutrient cycle, conversion of non-useable compound to usable or available, solubilization of mineral nutrients, decomposition of organic matter, soil structure formation and improving the soil health or quality, producing numerous plant growth regulators, degrading organic pollutants, stimulation of root growth, biocontrol of soil and seed-borne plant pathogens (bio-protector) and promoting and contributing to plant growth and development (Simarmata 2013; Gupta et al. 2015; Shokati and Poudineh 2017).

The mode of action of rhizobacteria to improve soil health and promote plant growth includes protecting and improving abiotic stress tolerance of plant; producing secondary metabolites such as antibiotics, siderophores, and phytohormones (auxin, gibberellin, and cytokinin) or plant growth regulators; increasing the nutrient availability; fixation of atmospheric nitrogen; solubilizing the insoluble phosphate; and producing biologically active substances.

Since the 1970s, biofertilizers containing inoculants of symbiotic nitrogen-fixing bacteria have been widely used in Indonesia to substitute inorganic fertilizers for soybean cultivation, containing *Bradyrhizobium japonicum* as an active microorganism (Simarmata 2013). Since the last three decades, the formulation of various biofertilizers had been developed progressively either as single, dual, or consortia of inoculants. The significance of biofertilizers has a wide effect on growth or crop production (Gupta and Rog 2004; Reddy et al. 2009; Ellafi et al. 2010; Begum et al. 2011; Malusà et al. 2016). The average increment ranges between 0 and 50 %, but under proper application and condition, it can increase the yield by about 25% and

Table 4.1 Active ingredients of formulated solid biofertilizers in an organic-based carrier (cocopeat and compost)

Formula	Active ingredient	Population ($cfug^{-1}$)
Soybean	*Bradyrhizobium japonicum*, *Pseudomonas* sp. *Bacillus* sp.	$\geq 10^8$–10^9
Rice	*Azotobacter* sp., *Azospirillum* sp., *Pseudomonas* sp., *Bacillus* sp., and *Acinetobacter* sp.	$\geq 10^8$–10^9
Corn	*Azotobacter* sp., *Azospirillum* sp., *Pseudomonas* sp., *Bacillus* sp., and *Streptomyces*	$\geq 10^8$–10^9
	Trichoderma sp.	$\geq 10^7$

reduce the application of inorganic fertilizers until 25–50% for nitrogen and about 25% for phosphor nutrient (Simarmata 2013; Ghany et al. 2013). In addition, biofertilizers can improve soil health and provide protection against drought and some soil-borne diseases (Malusa et al. 2016; Singh and Purohit 2011). In this chapter, the application of beneficial rhizobacteria formula as biofertilizer consortia inoculant to improve the soil and the health and productivity of food crops (soybean, rice, and corn) in Indonesia was studied.

4.3 Formulation and Development

The experiments to investigate the effectiveness of different biofertilizer inoculant formulas on the yield of different crops were conducted at a different location in Indonesia. Biofertilizer consortia with organic-based carrier for soybean, rice, and corn were developed (Table 4.1). The selected superior bacterial or fungal isolates were obtained from the culture collection of the Soil Biology Laboratory, Faculty of Agriculture, Universitas Padjadjaran, Indonesia.

4.4 Field Assessment in Food Crops

4.4.1 Soybean

The experiments to investigate the effect of biofertilizer consortia inoculant for soybean were conducted on Ultisols (clay, pH = 4.8, org-C = 1.62%, N = 0.18%, CEC = 18.81 cmol kg^{-1}) and Inceptisols (silty loam, pH = 5.9, org-C = 2.16, N = 0.43%, CEC = 30.4 cmol kg^{-1}). The experiment was arranged as randomized block design, which consists of seven treatments (P0 = control, P1 = 45 kg N, P2 = 200 g CBI, P3 = 200 g CBI + 45 kg N, P4 = 200 g CBI + 11.25 kg N, P5 = 200 g CBI + 22.5 kg N, P6 = 200 g CBI + 37.5 kg N); three replicates were conducted in each treatment. The inoculum was applied as a seed treatment. Soybean seed was moistened with water and mixed carefully with 200 g of consortia of biofertilizer inoculants (CBI) (5 g kg^{-1} of seed). Soybean was planted two seeds per hill 40 cm × 15 cm planting space in plots. The standard or recommended fertilizer

(45 kg N, 36 kg P_20_5, and 60 kg K_2O) or nitrogen treatment fertilizer was applied 7 days after planting in the hole about 10–15 cm apart from the plant.

4.4.2 Rice

The experiment to investigate the effect of different biofertilizer formulas on rice yield was conducted in the dry season of 2013 at The Indonesian Rice Research Center in Sukamandi, West Java, Indonesia. Randomized block design consisted of 15 treatments: control, NPK fertilizer in various dosage, and eight selected superior biofertilizer formulas were used. Three replicates were conducted for each treatment. The treatments were P0 = control, P_1 = 100% of NPK recommended dosage (112.5 kg N, 45 kg P_20_5, and 48 kg K_20), P_2 = 50% NPK recommended dosage, P3 = 50% NPK recommended dosage + organic fertilizers, P4 = 60% NPK recommended dosage, and P5 = 600 g consortia of CBI + 75% of NPK recommended dosage. Two single rice seedlings (15 days old) were planted as twin seedling, 5 cm apart with the cross section of 30 × 30 cm in 20 m^2 plots. Two hundred grams of CBI was applied for seed treatment and 400 g for field application. Ten grams of CBI was mixed carefully with 1 kg pre-soaked in water of rice seed, and 400 g inoculant was mixed with 40 kg of compost and distributed evenly on the rice plots before seedling transplantation. Half of the organic N and K fertilizers were applied 10 days after transplantation (DAT) and 42 DAT, while all phosphate fertilizers were applied at 10 DAT. The regular water management as water-saving technology was adopted (Simarmata et al. 2011; Antralina et al. 2015). Weeds and pest were removed carefully from the plant in planting duration. Rice was harvested at 100 DAT.

4.4.3 Corn

The experiment to investigate the effect of different biofertilizer formulas on corn was conducted April–August 2014 in Bantompo district of South Sulawesi. The randomized block design consisted of 16 treatments and provided with three replications was used as an experimental design. The treatments were P0 = control, P_1 = 100% of NPK recommended dosage (225 kg N, 36 kg P_2O_5, and 60 kg K_2O), P_2 = 50% NPK recommended dosage, P3 = 75% of NPK, and P4 = 1000 g consortia of biofertilizer inoculant (CBI) + 75% of NP + 60 kg of K_20 recommended dosage. A single corn seed (a hybrid of Bima URI-1) was sown at planting distance 75 × 20 cm in plots (6 × 4 m). Two tons of compost fertilizers per hectare was applied evenly on the surface of the soil of all plots before planting. The applied dosage of CBI was 1000 g ha^{-1} and applied as seed treatments (400 g) and field application (600 g). Ten g of biofertilizer inoculant was mixed with 1 kg of corn seed for seed treatment, and 600 g was mixed with 60 kg of compost and applied into the planting hole and followed by sowing. The 50% N, 100% P, and 50 K dosage were

applied at 10 days after planting (DAP). The remaining 50% were applied at 30 DAP. Fertilizer was applied 5–7 cm from the crop side.

4.5 The Effectiveness of Rhizobacteria Inoculant

The effectiveness of rhizobacteria inoculant (single and consortia) can be obtained by measuring the plant growth or crop productivity. The results for some food crops are as follows.

4.5.1 Soybean

The grain yield of soybean either on Ultisols or Inceptisols was significantly influenced by the application of CBI (Table 4.2). Application of 200 gram of CBI and 22.5 kg N (50% of recommended N dosage) resulted in 1.49 t ha^{-1} and 1.70 ha^{-1} of grain yield on Ultisols and Inceptisols, respectively. These results were not different from the fertilized plots with 45 kg N ha^{-1}.

It is an indication that the application of CBI for soybean can reduce the rate of inorganic N fertilizers by 50–75%. The similar result was obtained (Table 4.3). Moreover, the obtained yield of fertilized plots with 45 kg N and inoculated with CBI was not significantly different compared to plots received 22.5 kg N, and 200 g of CBI indicated that the high rate of N fertilizer resulted in a negative impact on the nitrogen-fixing activity and nodulation. Plants use the available N in soils (Sharma et al. 2004; Singh and Purohit 2011; Simarmata 2013). In addition, the application of *Bacillus* sp. and *Pseudomonas* sp. as coinoculant plays an important role in improving the P availability and in producing the plant growth promotor (Kloepper 1993; Vessey 2003; Srivastava 2002; Reddy et al. 2000; Hayat et al. 2010).

Table 4.2 Effect of consortia of biofertilizer inoculants (CBI) on grain yield of soybean on Jatinangor Ultisols and Inceptisols

	Ultisols		Inceptisols	
Treatments (Dosage ha^{-1})	Grain yield	Increment (%)	Grain yield	Increment (%)
P_0 = control (unfertilized)	0.97a	–	1.14 a	–
P_1 = 45 kg N (100% of N dose)	1.29 bcd	33.0	1.64 bc	43.9
P_2 = 200 g CBI	1.11 ab	14.4	1.20 a	5.3
P_3 = 200 g CBI + 45 kg N	1.29 bc	33.0	1.57 bc	37.7
P_4 = 200 g CBI + 11.25 kg N	1.21 cd	24.7	1.40 ab	22.8
P_5 = 200 g CBI + 22.5 kg N	1.40 cd	44.3	1.70 c	49.1
P_6 = 200 g CBI + 37.5 kg N	1.49 d	53.4	1.79 c	57.0

Note: The mean value followed by the same letter within the same column is not significantly different ($p = 0.05$)

Table 4.3 The effectiveness of CBI compared to inorganic fertilizers in improving the fertilizer efficiency and soybean grain yield

Treatments (kg ha^{-1})	Pods (pods/plant)	Nodules (nod./plant)	Yield (g/plant)	Increment (%)
P_o = control	9.00 b	10.00 ab	2.26 bc	–
P_1 = 45 N + 36 P_2O_5 + 60 K_2O	13.25 a	22.00 a	4.39 ab	94.2
P_2 = 22.5 N + 18 P_2O_5 + 30 K_2O	10.50 ab	9.50 ab	4.24 ab	87.6
P_3 = 22.5 N+ 27 P_2O_5+ 60 K_2O	12.75 ab	21.50 a	4.38 ab	93.8
P_4 = CBI + 22.5 N + 27 P_2O_5 + 60 K_2O	14.75 a	20.50 a	5.29 a	234.0

Note: The mean value followed by the same letter within the same column was not significantly different (p = 0.05)

4.6 Rice Grain Yield

Application 600 g $ha^{-1}$of CBI and 75% of recommended NPK dosage (P5 = 84.4 kg N + 33.75 kg P_2O_5 + 27 kg K_20) resulted in 5.89 t ha^{-1} of rice grain yield (Table 4.4). This result was not significantly different from the plot received 100% NPK dosage (P1). It is a strong indication that CBI can be applied to increase the rice grain yield and fertilizer efficiency by reducing the application of inorganic fertilizers about 25%. The contribution of CBI in reducing the dosage of inorganic fertilizers of N and P fertilizers depends on the effectiveness of nitrogen-fixing bacteria and phosphate-solubilizing bacteria in the rhizosphere. The reservoir of nitrogen in air is unlimited, while phosphate depends on the deposit in soils, and potassium depends on immobilized K in organic matter.

Consequently, recycling of plant waste such as composted straw will increase the availability of P and K significantly. The application of CBI and 2–5 composted straw combined with water-saving technology known as "SOBARI" (system of aerobic rice intensification) increased the rice grain yield by 30–70% (Table 4.5).

4.7 Corn Grain Yield

The growth and the grain yield of corn were significantly affected by the application of inorganic and CBI (Table 4.6). The application of 800 g ha^{-1} of CBI + 168.75 N + 27 kg P_2O_5 + 60 kg K_2O(P4) increased significantly the corn grain yield and efficiency of inorganic fertilizers.

Compared to the plots which received 100% dosage of NPK fertilizers (P1), the corn grain yield increased by about 12.2%, and the N and P fertilizer requirement reduced by 25%. It was confirmed that the biofertilizer consortia were able to adapt and increase the nitrogen and phosphate availability. Bacterial strains in the biofertilizer consortium also produces plant growth promoting phytohormone such as auxin and gibberellic acid (Niranjan et al. 2005; Reddy et al. 2009; Sharma et al. 2004; Maksimov et al. 2011; Simarmata 2013).

Table 4.4 The effectiveness of CBI to improve fertilizer efficiency and rice productivity at ICRR in Sukamandi, West Java, Indonesia

Treatments (dose kg ha^{-1})	Rice Grain Yield	
	(ton ha^{-1})	Increment (%)
P_0 = control	3.57 b	–
P_1 = 112,5 N + 45 P_2O_5 + 48 K_2O	6.10 a	70.1
P_2 = 5.625 N + 22.5 P_2O_5 + 24 K_2O	5.17 ab	44.8
P_3 = 67.5 N + 27 P_2O_5 + 28.8 K_2O	4.86 ab	36.6
P_4 = 84.4 N + 33.75 P_2O_5 + 35 K_2O	5.40 ab	51.3
P_5 = 600 g CBI + 84.4 N + 33.75 P_2O_5 + 27 K_2O	5.89 a	65.0

Note: These treatments are extracted from 15 treatments. The mean value followed by the same letter within the same column is not significantly different (p = 0.05)

Table 4.5 Rice productivity in response to biofertilizer application combined with a system of aerobic rice intensification (SOBARI) at different locations in Lampung (Yuriansyah et al. 2012)

Responds	SOBARI Technology			Control (farmer)
	Pesawaran	B. Lampung	Batanghari	
Plant height (cm)	104.5	92.1	94	89.4
Number tiller	56.9	53.2	62.3	28.9
Productive tiller	33.8	31.4	32.4	15.9
Rice grain yield (t ha^{-1})	10.2	7.0	7.8	6.0
Increment	70%	16,6%	30%	–

Table 4.6 The effectiveness of CBI in improving the fertilizer efficiency and productivity of hybrid corn BIMA URI

Treatment (kg ha^{-1})	Plant height (cm)	Cob length (cm)	Yield (t ha^{-1})	Increment (%)
P_o = control	135.3 c	13.1 e	5.5 f	–
P_1 = 225 N + 36 P_2O_5 + 60 K_2O	175.3 ab	18.4 ab	9.3 bc	67.3
P_2 = 112.5 N + 18 P_2O_5 + 30 K_2O	181.7 ab	17.8 bcd	7.8 de	40.1
P_3 = 168.75 + 27 36 P_2O_5 + 45 K_2O	186.0 ab	17.7 bcd	9.7 abc	75.0
P_4 = 800 g of BIC + 168.75 N + 27 kg $P_2$05 + 60 kg K_2O	189.0 ab	18.9 ab	10.1 a	81.2

The mean value followed by the same letter within the same column is not significantly different (p = 0.05)

4.8 Conclusion

Beneficial rhizobacteria as biofertilizers are low-cost, renewable, and environmentally friendly fertilizers which can be applied to facilitate nutrient availability and improve and remediate soil health to increase the productivity of agricultural crops and to reduce the application of inorganic fertilizers in sustainable ways.

Consortia of beneficial rhizobacteria as biofertilizer inoculants could increase the soybean grain yield and reduce the inorganic N fertilizers by 50–75%; rice inoculants increased the grain yield and reduced the inorganic N and P by 25%, and corn inoculants increased the corn grain yield and reduced the inorganic N and P fertilizers by 25%. The combination of rhizobacterial inoculant (RI) and 2–5 t straw compost (ameliorant) increased the soil organic carbon and rice productivity and reduced inorganic NPK fertilizer by 25–50%. Beneficial rhizobacteria consortia formulas with organic-based carriers have a great potential to improve soil health, crops productivity, and sustainability of practices in Indonesia.

Intensive researches are needed to improve the quality of biofertilizers, such as superior isolates of biofertilizers, formulation of inoculant consortia, carrier composition, biofertilizer retention in soils, quality control, and biofertilizer regulation. The development of appropriate technology for mass production of biofertilizers is needed to widen the access of this fertilizer in a reasonable price for farmers.

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5 Biochemical Characterization of Microbials and Their Effects on the Growth and Yield of Multiplier Onion (*Allium ascalonicum* L.) in Northwestern Philippines

Dionisio S. Bucao, Romeo H. Yapit Jr., and Maura Luisa S. Gabriel

Contents

Abstract

Onion is a highly nutrient-responsive crop. Conventional methods of fertilization have undoubtedly helped in improving both bulb yield and quality. But lately, routine management practices in the Philippines appear to be incapable of maintaining yields over the long term. The steady depletion of native soil fertility and the occurrence of multiple nutrient deficiencies in onion fields have led to the use of microbials in combination with fertilizers for sustainable onion production. Biofertilization methods played an essential role in plant nutritional requirements since biofertilizers were reported to enhance crop productivity through improving plant nutrition, enhancement of nutrient availability, nitrogen fixation, phosphate solubilization, and plant hormone production. Three microbial strains with biofertilizer potentials were chosen to evaluate for growth promotion and yield of multiplier onion (Australian variety). A field experiment was designed in a strip plot design comprising of fertilizer source and microbials. Data obtained were analyzed using analysis of variance, and mean values were subject to Honestly Significant Difference Test at $P = 0.05$. Two of the three microbials used in this experiment were identified as *Bacillus subtilis* and *Paenibacillus polymyxa* through their biochemical characterization. The results of field

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experiment showed that onion treated with *P. polymyxa* significantly produced the longer laminar length of onion seedlings at 45 days after treatment (33.24 cm) compared to untreated control (28.44 cm). Also, onion treated with *B. subtilis* strains 1 (32.02 cm) and 2 (31.31 cm) showed a significant increase in laminar length compared to control. The treated onion seedlings with microbials grown under conventional practice produced larger bulbs. The yield of harvested green onion and dry bulbs was higher in microbial-treated onion plots compared to the untreated (18.16 t ha^{-1} versus 10.99 t ha^{-1}). The higher percentage of marketable yield (97.07%) of green onion has resulted in microbial-treated plots. Our results suggest that treatments supplying inorganic fertilizers and microbials or organic fertilizer and microbials in combination generated a significant bulb yield response over the control.

Keywords
Allium ascalonicum L. · Growth promotion · Inorganic fertilizers · Organic fertilizer and *Bacillus subtilis* · *Paenibacillus polymyxa* fertilizers

5.1 Introduction

Multiplier onion (*Allium ascalonicum* L.) is widely grown in Ilocos Region, Philippines particularly in the provinces of Ilocos Norte and Ilocos Sur which share about 94% of the country's production with an average yield of 9 t ha^{-1} (BAS 2013). Conventional methods of growing the crop have undoubtedly helped in improving both bulb yield and quality. Fertilizer application rate is about 271-56-146 kg N-P_2O_5-K_2O ha^{-1} (DA-RFO -1 2011). However, lately, routine management practices in the Philippines appear to be incapable of maintaining yields over the long term as testified by the farmers themselves. With the intensive cropping practices and continuous application of high amounts of synthetic fertilizers, soil degradation in the region has been observed (Gumtang et al. 1999). The steady depletion of native soil fertility, the occurrence of multiple nutrient deficiencies in onion fields, and the increasing prices of synthetic fertilizers have led to the use of microbials in combination with fertilizers for sustainable onion production.

The role of plant growth-promoting rhizobacteria (PGPR) in crop production cannot be denied. PGPR was introduced by Kloepper and Schroth (1978) which is defined by Schroth and Hancock (1982) as the bacteria that colonize roots effectively or "rhizobacteria." Root colonization is a process whereby microorganisms penetrate and multiply throughout the growing root and exude subtances which are rich in carbohydrates and amino acids (Kloepper et al. 1989). Rhizobacteria are commonly isolated in the plant rhizosphere, a narrow zone surrounding closely the roots (Sivasakthi et al. 2013) which are characterized by intense microbial activities which are being stimulated by the root exudates, the chemicals secreted into the soil by roots. The mechanism involved in PGPR-mediated plant growth promotion is directly by the production of plant growth regulators, i.e., auxins, cytokinins, and gibberellins, and facilitation of the uptake of nutrients, i.e., nitrogen fixation and

phosphorus solubilization (Lazarovits and Nowak 1997). PGPR enhances soil fertility by increasing the amount of available nitrogen and phosphorus and other plant nutrient and synthesizing several different phytohormones that can act to enhance various stages of plant growth (Haque and Dave 2005).

Biofertilization methods played an essential role in plant nutritional requirements since biofertilizers were reported to enhance crop productivity through improving plant nutrition, enhancement of nutrient availability, nitrogen fixation, phosphate solubilization, and plant hormone production (Kim et al. 2011).

Initially, 15 microbial isolates were characterized morphologically, but only 6 were screened for plant growth-promoting activities (Bucao and de Leon 2014). This paved for the identification and evaluation of the three promising microbial isolates. Thus, this study aims to biochemically characterize the three promising microbials and determine their effectiveness in comparison with inorganic and organic fertilizer and their combinations on the growth and yield of multiplier onion under field condition.

5.2 Materials and Methods

5.2.1 Biochemical Characterization

The three promising microbial isolates were submitted to the Nueva Vizcaya State University DNA-Based Indexing Laboratory for biochemical characterization using plate techniques.

5.2.2 Efficacy Trial

The study was conducted in farmer's field in Sinait, Ilocos Sur. The area has a type II climate characterized by distinct wet (May–October) and dry (November–April) seasons. The soil in the area is San Manuel clay loam with slightly alkaline (8.04), very low organic matter (0.90%), low phosphorus (5.70 ppm), and sufficient potassium (276.69 ppm). The area is almost flat and with good drainage.

The study was laid out in a strip plot design with three replications under farmer's field. The horizontal factor was fertilizer source while the potential biofertilizers as the vertical factor. Specifically, the elements of the study were as follows:

Horizontal factor (biofertilizer)

S_0 – control (no biofertilizer)
S_1 – *B. subtilis* 1
S_2 – *B. subtilis* 2
S_3 – *P. polymyxa*

Vertical factor (fertilizer source)

F_0 – control (no fertilizer)
F_1 – inorganic fertilizer (90-60-60 kg $NP_2O_5K_2O$ ha^{-1})
F_2 – organic fertilizer (5 t ha^{-1})

5.2.3 Cultural Management Practices

5.2.3.1 Land Preparation

The experimental area was prepared thoroughly by plowing twice using a tractor before planting. The area was cleaned by manually removing the weeds and plant debris. Thirty-six experimental plots measuring 2 m × 4 m were laid out following the strip plot design with 1 m distance between replications and 0.75 m between plots. Plots were raised to 20 cm to avoid waterlogging due to rainfall.

5.2.3.2 Preparation of Planting Materials

Australian variety of multiplier onion was used in the study. Seed materials were purchased from an accredited grower. The bulblets were cleaned, and small- to medium-size bulblets were selected for planting materials. The bulblets were thoroughly washed with water to remove any storage treatments applied, spread out on a plastic screen, and air-dried for a day before planting.

5.2.3.3 Planting and Mulching

Plots were mulched with about 3 cm-thick rice straw. Planting was done by holding the bulb between the thumb and forefinger and pressing it downward. About 50% to 75% of the bulb was set into the soil. A string marked 20 cm apart was used as planting guide to obtain a uniform spacing of 20 cm × 20 cm.

5.2.3.4 Application of Potential Biofertilizer

The three potential biofertilizers were inoculated on the multiplier onion bulbs before planting. About 250 ml of cultured bacteria were mixed with the same volume of distilled water and inoculated on 5 kg multiplier onion bulbs. The same amount was applied as foliar on the treatments at 15 and 30 days after planting (DAP).

5.2.3.5 Irrigation

The experimental area was irrigated to saturate condition a day before planting. Irrigation water was applied as needed.

5.2.3.6 Weeding

Manual weeding was done at 15 and 30 DAP.

Table 5.1 Doses of fertilizer application based from the recommended rate

Treatment	Method of application	Time of application	Rate
Control	None	None	None
Inorganic RR (90-60-60)			
14-14-14	Broadcast	Before planting	60-60-60 kg-$NP_2O_5K_2O$ ha^{-1}
21-0-0	Topdress	30 days after planting	30 kg-N ha^{-1}
Organic fertilizer	Broadcast	Before planting	5 t ha^{-1}

5.2.3.7 Fertilizer Application

The time and rate of fertilizer application based from the recommended rate are summarized in Table 5.1.

5.2.3.8 Harvesting

Harvesting was undertaken when the plants reach its maturity. For the green onion, harvesting follows as bulbs reach 1–3 cm diameter or when it reaches 40–50 (DAP). Sample plants were pulled manually from each plot. Harvesting was done when bulbs were developed fully or the neck tissue of the multiplier onions becomes soft.

5.2.3.9 Statistical Analysis

Data were collated and were subjected to analysis of variance (ANOVA) using Statistical Tool for Agricultural Research (STAR ver. 2, International Rice Research Institute) and mean values were subject to Honest Significant Difference Test at $P = 0.05$.

5.3 Results and Discussion

5.3.1 Biochemical Characterization

Results of the biochemical test revealed that the three promising microbial isolates were Gram-positive (Table 5.2). Their colony color ranged from creamy white to dirty white. Nitrate reduction test was positive in all of the three strains. The result suggests that these microbial isolates can perform nitrification on nitrate and nitrite to produce molecular nitrogen. Reduction of either nitrate or nitrite provides energy for the growth of the bacteria in the absence of oxygen, with nitrate reduction to nitrite via nitrate reductase contributing more significantly to proton-motive force or energy production than nitrite reduction (Berks et al. 1995; Zumft 1997).

Meanwhile, urease test showed negative reaction, which indicates that the isolates cannot degrade urea via enzyme urease. Moreover, the citrate utilization test showed a positive response showing that the microbial strains can utilize citrate as their sole

Table 5.2 Biochemical characteristics of the three promising PGPB

	Microbial isolates		
Biochemical characterization	Isolate 1	Isolate 2	Isolate 3
Gram staining	+	+	+
Colony color	Creamy white	Creamy white	Dirty white
Nitrate reduction	+	+	+
Urease	+	+	+
Citrate utilization	+	+	+
Catalase	+	+	+
Acid processed from			
Sucrose	–	–	–
Galactose	–	–	–
Mannitol	+	+	+
Xylose	+	+	+
Lactose	–	–	+
Glucose	+	+	+
Mannose	+	+	+
Cellobiose	–	–	+
Fructose	–	–	+
Maltose	+	+	+
Trehalose	+	+	+
Identification	*Bacillus subtilis*	*Bacillus subtilis*	*Paenibacillus polymyxa*

carbon source. They use the enzyme citrate or citrate permease to transport the citrate into the cell. These bacteria also convert the ammonium dihydrogen phosphate to ammonia and ammonium hydroxide, which creates an alkaline environment in the medium.

The three microbial isolates also showed a positive reaction to the catalase test, which manifests that the bacteria are either aerobic or facultative anaerobes and could convert hydrogen peroxide into water and oxygen. Further, the starch hydrolysis test showed a positive reaction to the three microbial isolates, which means that they can produce certain exoenzymes, including a-amylase and oligo-1,6-glucosidase to degrade the starch into subunits for the organisms to utilize. On the other hand, sucrose and galactose tests showed negative reaction by the bacteria. However, mannitol test showed a positive reaction indicating that the bacteria are motile and serve as an agent to improve the fermentation. The positive reaction of the bacteria to xylose test suggests that they belong to the genus *Bacillus* (Sharma et al. 2013). Only one isolate showed a positive reaction to lactose, cellobiose, and fructose tests. This isolate can break down the cellobiose which is characterized by a disaccharide derived from the condensation of two glucose molecules which are linked in a bond. Based on the test conducted, isolates 1 and 2 are both *Bacillus subtilis*, while isolate 3 is *Paenibacillus polymyxa*.

Reports revealed that *Bacillus* species showed yield increase in rice (Sudha et al. 1999), barley (Çakmakçı et al. 2001), wheat (de Freitas 2000; Çakmakçı et al. 2007), maize (Pal 1998), sugar beet (Çakmakçı et al. 1999), and sugarcane. Similarly, *Bacillus* strains increased total bacteria and the PSB population and root and shoot dry weight, as well as total N and P uptake by plants (Canbolat et al. 2006). Plant growth responses were variable and dependent upon the inoculant strain, soil organic matter content, growing stage, harvest date, and growth parameters evaluated (Çakmakçı et al. 2006). On the contrary, *Paenibacillus polymyxa* has a range of reported properties, including nitrogen fixation (Coelho et al. 2003; Çakmakçı et al. 2006), phosphorus solubilization (de Freitas et al. 1997), antibiotic production (Rosado and Seldin 1993), cytokinin production (Timmusk et al. 1999), and increased root and shoot growth (Sudha et al. 1999).

5.3.2 Days to Emergence

Table 5.3 shows the effect of biofertilizers and fertilizer source on the emergence of multiplier onion. Control plots emerged the earliest with only 6 days after planting but comparable to plots applied with *P. polymyxa* which emerged 7 days after planting. Plots applied with *B. subtilis* 2 and *B. subtilis* 1 appeared the longest with almost 8 and 9 days after planting. This is due to the characteristics of rhizobacteria that require time to colonize the roots of plants. No significant differences were observed regardless of fertilizer source as well as the interaction between the two factors. This conformed to the study of Corpuz (2013) that the

Table 5.3 Days to the emergence of multiplier onion as affected by rhizobacteria strains and fertilizer sources

Treatment	Days to emergence
Biofertilizer (A)	**
No biofertilizer	6.89 a
B. subtilis 1	8.56 c
B. subtilis 2	8.22 bc
P. polymyxa	7.22 ab
Fertilizer source (B)	**ns**
No fertilizer	7.67
Inorganic	7.75
Organic	7.75
A × B	**ns**
CVa (%)	13.82
CVb (%)	15.11
CVc (%)	9.41

Means marked with the same letter within each column are not significantly different at 5% level using Honestly Significant Difference (HSD) Test
CV coefficient of variation; *ns* not significant
**Significant at 1% level

Table 5.4 Plant emergence (%) of multiplier onion at 7, 14, and 21 DAP as affected by biofertilizer and fertilizer source and their interactions

Treatment	Plant emergence (%)		
	7 DAP	14 DAP	21 DAP
Biofertilizer (A)	**	**ns**	**ns**
Control	77.33 a	94.72	97.56
B. subtilis 1	52.50 b	91.11	98.50
B. subtilis 2	56.06 b	92.89	98.83
P. polymyxa	67.94 a	94.94	98.94
Fertilizer source (B)	**ns**	**ns**	**ns**
Control	63.50	92.04	98.21
Inorganic	62.92	94.88	98.33
Organic	63.96	93.33	98.83
A × B	**ns**	**ns**	**ns**
CVa (%)	21.61	5.10	0.77
CVb (%)	25.77	8.09	0.64
CVc (%)	12.92	3.39	1.09

Means marked with the same letter within each column are not significantly different at 5% level using Honestly Significant Difference (HSD) Test
CV coefficient of variation, *ns* not significant
**Significant at 1% level

application of different fertilizer materials did not affect the emergence of multiplier onion.

5.3.3 Percent Plant Emergence

Percent plant emergence of multiplier onion *(*var. Australian*)* taken at 7 DAP was significantly affected by biofertilizer applied but not of the fertilizer source and their interaction as presented in Table 5.4. Results reveal that plots with no biofertilizer garnered the highest percent plant emergence (77.33%) at 7 DAP but comparable to *P. polymyxa* (67.94%). Lowest plant emergence was observed on plots applied with *B. subtilis 1* (52.50%) but comparatively related to *B. subtilis* 2 (56.06%). However, at 14 and 21 DAP, the percent plant emergence of multiplier onion did not differ significantly by the application of biofertilizer, fertilizer source, and their interaction with almost 95% and 99%, respectively.

5.3.4 Laminar Length

Laminar length of multiplier onion was significantly affected by biofertilizer and fertilizer source but not their interaction at 15, 30, and 45 DAP as presented in Table 5.5. At 15 DAP, plots applied with *P. polymyxa* produced the most extended laminar length with 21.62 cm followed by those applied with *B. subtilis* 1 (20.83 cm) and *B. subtilis* 2 (20.64 cm), and no biofertilizer plots produced the shortest with

Table 5.5 Laminar length of multiplier onion at 15, 30, and 45 DAP as affected by biofertilizer, fertilizer source, and their interaction

Treatment	Laminar length (cm)		
	15 DAP	30 DAP	45 DAP
Biofertilizer (A)	**	*	**
Control	19.99 c	23.83 b	28.44 b
B. subtilis 1	20.83 b	24.60 ab	31.31 a
B. subtilis 2	20.64 b	24.36 b	32.02 a
P. polymyxa	21.62 a	25.62 a	33.24 a
Fertilizer source (B)	**	*	*
Control	20.22 b	23.84 b	29.43 b
Inorganic	21.38 a	25.45 a	32.39 a
Organic	20.72 b	24.52 b	31.95 a
A × B	ns	ns	ns
CVa (%)	3.43	5.13	5.18
CVb (%)	3.24	2.69	3.27
CVc (%)	2.24	3.37	5.05

Means marked with the same letter within each column are not significantly different at 5% level using Honestly Significant Difference (HSD) Test
CV coefficient of variation, *ns* not significant
*Significant at 5% level; **Significant at 1% level

19.99 cm. Regarding fertilizer source, plots applied with inorganic fertilizer produced the most extended laminar length with 21.38 cm as compared to those applied with organic fertilizer and no fertilizer at all with 20.72 cm and 20.22 cm, respectively. The same pattern was observed at 30 DAP. However, at 45 DAP, plots with biofertilizer produced a similar laminar length from 31.31 cm to 33.24 cm but significantly different to plots with no biofertilizer with only 28.44 cm. The result affirms with Glick and Lee et al. that plant growth-promoting rhizobacteria (PGPR) was reported to promote plant growth by producing and modulating the level of plant hormones. Furthermore, Haque and Dave (2005) described PGPR that enhances soil fertility by increasing the amount of available nitrogen and phosphorus and other plant nutrients and synthesizing several different phytohormones that can act to enhance various stages of plant growth. Phytohormones are responsible for the proper growth and development of plants, and any deviation from the normal hormonal level is often an indication of plant stress.

On the other hand, regarding fertilizer source, plots applied with inorganic and organic fertilizers have a similar laminar length of 32.39 cm and 31.95 cm, respectively. No-fertilizer plots produced the shortest laminar length with only 29.43 cm.

5.3.5 Plant Vigor

The vigor of multiplier onion both at 30 and 45 DAP is significantly influenced by the biofertilizer and fertilizer source but not their interaction (Table 5.6). At 30 DAP, plots applied with *P. polymyxa* produced vigorous plants (3.73). Moderately

Table 5.6 Plant vigor of multiplier onion at 30 and 45 DAP as affected by biofertilizer, fertilizer source, and their interaction

Treatment	Vigor rating	
	30 DAP	45 DAP
Biofertilizer (A)	*	**
Control	3.27 b	3.58 b
B. subtilis 1	3.40 b	3.56 b
B. subtilis 2	3.40 b	3.63 b
P. polymyxa	3.73 a	3.92 a
Fertilizer source (B)	**	**
Control	3.27 b	3.44 b
Inorganic	3.57 a	3.82 a
Organic	3.52 a	3.75 a
A × B	**ns**	**ns**
CVa (%)	6.48	5.02
CVb (%)	9.30	5.97
CVc (%)	5.72	4.22

Means marked with the same letter within each column are not significantly different at 5% level using Honestly Significant Difference (HSD) Test
CV coefficient of variation, *ns* not significant
*Significant at 5% level; **Significant at 1% level

vigorous plants were observed in plots with no biofertilizer (3.27) and those applied with *B. subtilis* 1 (3.40) and *B. subtilis* 2 (3.40) as shown in Table 5.7. The result indicates that PGPR through nitrogen fixation could be able to convert gaseous nitrogen (N_2) to ammonia (NH_3) making it an available nutrient to the host plant which can support and enhance plant growth (Lazarovits and Nowak 1997). The result of the study reveals that *P. polymyxa* could fix nitrogen more efficiently than *B. subtilis* which contributed in producing vigorous plants. Regarding fertilizer source particularly at 30 DAP, plots applied with inorganic and organic fertilizers significantly produced vigorous plants with 3.57 and 3.52, respectively, as compared to the control which produced moderately vigorous plants (3.27). At 45 DAT, the same trend was observed in which plots applied with fertilizer are more vigorous than those with no fertilizer.

5.3.6 Pest Damaged

Pest monitoring was carried out at 30 DAP for downy mildew and at 45 DAP for onion leaf twister (Table 5.7). At 30 DAP, plots applied with *P. polymyxa* were the least damaged by downy mildew with only 16.78% while as compared to *B. subtilis* 2 (18.44%), *B. subtilis* 1 (18.22%), and no biofertilizer (18%). At 45 DAP, both biofertilizer and fertilizer source treatments significantly influenced the percent damage caused by onion leaf twister. Least damaged plots were noted from those applied with *P. polymyxa* with only 22% as compared to those plots applied with *B. subtilis* 2 (26.11%), no biofertilizer (28.78%), and *B. subtilis* 1 (32.67%). Results

Table 5.7 Pest damaged (%) of multiplier onion at 30 and 45 DAP as affected by biofertilizer, fertilizer source, and their interaction

	Pest damaged (%)	
	30 DAP	45 DAP
Treatment	Downy mildew	Onion leaf twister
Potential biofertilizer (A)	*	**
Control	18.00 ab	28.78 b
B. subtilis 1	18.22 a	32.67 a
B. subtilis 2	18.44 a	26.11 c
P. polymyxa	16.78 c	22.00 d
Fertilizer sources (B)	**Ns**	*
Control	17.67	26.75 b
Inorganic	18.08	28.75 a
Organic	17.83	26.67 b
A × B	**Ns**	**ns**
CVa (%)	13.19	45.12
CVb (%)	4.51	4.75
CVc (%)	5.28	5.39

Means marked with the same letter within each column are not significantly different at 5% level using Honestly Significant Difference (HSD) Test
CV coefficient of variation, *ns* not significant
*Significant at 5% level; **Significant at 1% level

imply that *P. polymyxa*-treated plants have the lowest occurrence of diseases. Result suggests that it has antagonist property which is in consonance to the study of Lazarovits and Nowak (1997). PGPR minimizes or controls the harmful effects of plant pathogens on plants by the production of inhibitory substances (antibiotics, antifungal metabolites, iron-chelating siderophores, cell wall-degrading enzymes, and competition for sites on roots) or increases the natural resistance of the host (induced systemic resistance). On the other hand, plots applied with inorganic fertilizer have the highest damaged observed with 28.75% compared to plots with no fertilizer (26.75%) and with organic fertilizer plots (26.67%).

5.3.7 Days to Maturity

Influence of biofertilizer showed a significant variation on days to maturity of both green and dry bulb multiplier onions (Table 5.8). Plots applied with *P. polymyxa* have the longest day to mature (47.78 days) but comparable to plots applied with *B. subtilis* 2 (47.67 days). The earliest to mature were noted on plots without strain (46.78 days) followed by plots applied with *B. subtilis* 1 which mature within 47.22 days.

Fertilizer source shows a significant variation on days to maturity of green onion. Plots applied with inorganic fertilizer have the longest day to mature (47.67 days), whereas control plots and those applied with organic fertilizer matured earlier with

Table 5.8 Days to maturity of multiplier onion as affected by biofertilizer, fertilizer source, and their interaction

Treatment	Days to maturity	
	Green	Bulb (dry)
Potential biofertilizer(A)	**	**
Control	46.78 c	57.00 c
B. subtilis 1	47.22 b	58.22 b
B. subtilis 2	47.67 ab	58.67 ab
P. polymyxa	47.78 a	58.78 a
Fertilizer sources (B)	*	ns
Control	47.25 b	58.08
Inorganic	47.67 a	58.42
Organic	47.17 b	58.00
A × B	ns	ns
CVa (%)	1.10	0.79
CVb (%)	0.82	0.49
CVc (%)	0.67	0.54

Means marked with the same letter within each column are not significantly different at 5% level using Honestly Significant Difference (HSD) Test
CV coefficient of variation, *ns* not significant
*Significant at 5% level; **Significant at 1% level

47.25 days and 47.17 days, respectively. The results confirmed to the study of Corpuz (2013) that application of fertilizer with a higher amount of nutrients (inorganic) takes green multiplier onion longer days to mature. Interaction effect of biofertilizer and fertilizer source showed no significant differences in the maturity of both green and bulb multiplier onions.

The variation of days to maturity of dry bulb multiplier onion, on the other hand, was caused by the application of biofertilizer and not of the fertilizer source and their interaction. Plots applied with *P. polymyxa* had the longest to mature with 58.78 days but comparable to those applied with *B. subtilis* 2 with 58.67 days. The control plots had the earliest to mature with 57 days followed by plots applied with *B. subtilis* 1 with 58.22 days.

5.3.8 Number of Bulblets

The result of the analysis of variance showed no significant differences in biofertilizer, fertilizer source, and their interaction on the number of bulblets of shallot onion as presented in Table 5.9. The result could be due to varietal characteristics that application of treatments would not significantly affect the number of bulblets.

5.3.9 Bulblet Diameter

Bulblet diameter was affected by the factors biofertilizer and fertilizer source as well as their interaction (Table 5.9). As shown in Fig. 5.1, interaction indicates that application of *P. polymyxa* whether solely applied alone (2.86 cm) or in combination with either inorganic (3.03 cm) or organic fertilizers (2.96 cm) produced the biggest. However, this was comparable to those applied with *B. subtilis* 2 with 2.78 cm,

Table 5.9 The number of bulblets and bulblet diameter (cm) of the multiplier onion as affected by biofertilizer, fertilizer source, and their interaction

Treatment	No. of bulblets	Bulblet diameter (cm)	
		Green	Bulb (dry)
Biofertilizer (A)	ns	**	**
Control	5.57	2.75 c	2.52 c
B. subtilis 1	6.54	2.96 b	2.76 b
B. subtilis 2	5.37	3.12 a	2.92 a
P. polymyxa	5.67	3.13 a	2.95 a
Fertilizer source (B)	ns	**	**
No fertilizer	5.23	2.90 b	2.70 b
Inorganic	6.04	3.05 a	2.85 a
Organic	6.08	3.03 a	2.81 a
A × B	ns	ns	*
CVa (%)	26.36	9.69	10.14
CVb (%)	19.46	2.36	2.27
CVc (%)	17.61	2.12	1.60

Means marked with the same letter within each column are not significantly different at 5% level using Honestly Significant Difference (HSD) Test
CV coefficient of variation, *ns* not significant
*Significant at 5% level; **Significant at 1% level

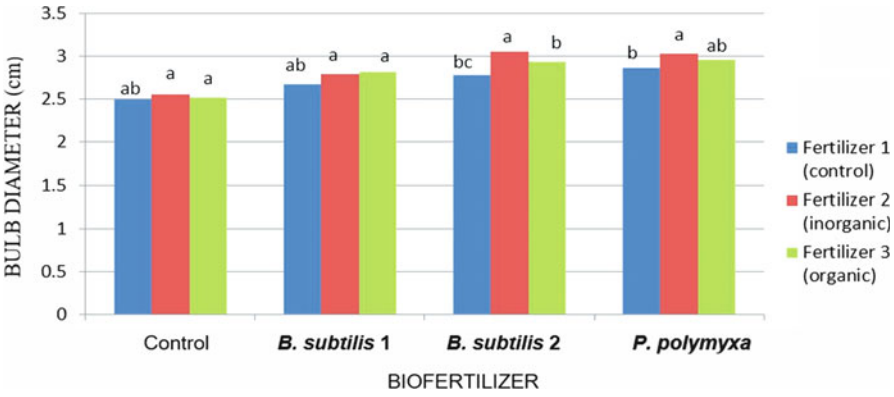

Fig. 5.1 The interaction effect between biofertilizer and fertilizer sources on the bulb diameter (cm) of multiplier onion

Table 5.10 Percent marketable plants/bulbs of multiplier onion as affected by biofertilizer, fertilizer source, and their interaction

Treatment	Percentage marketable crop	
	Green	Bulb (dry)
Biofertilizer (A)	*	*
Control	92.26 b	89.03 b
B. subtilis 1	91.54 b	89.50 b
B. subtilis 2	94.47 ab	86.95 c
P. polymyxa	97.07 a	92.72 a
Fertilizer source (B)	**ns**	**ns**
Control	94.04	89.84
Inorganic	93.11	89.44
Organic	94.35	89.37
A × B	**ns**	**ns**
CVa (%)	1.90	4.94
CVb (%)	1.74	4.04
CVc (%)	3.14	5.79

Means marked with the same letter within each column are not significantly different at 5% level using Honestly Significant Difference (HSD) Test
CV coefficient of variation, *ns* not significant
*Significant at 5% level; **Significant at 1% level

3.05 cm, and 2.93 cm, respectively. Plots with no biofertilizer and the combination of inorganic and organic fertilizers produced the smallest diameter with 2.49 cm, 2.55 cm, and 2.52 cm, respectively.

5.3.10 Percent Marketable Yield

The percent marketable yield of green multiplier onion was significantly affected by the biofertilizer treatment but not by the fertilizer source and their interaction (Table 5.10). Plots applied with *P. polymyxa* produced the highest percent marketable plant with 97.07% which is significantly higher than plots applied with *B. subtilis* 2 (94.47%), *B. subtilis* 1 (91.54%), and no biofertilizer (92.26%). Fertilizer sources and its interaction with the biofertilizer showed no significant differences.

Like the green onion, biofertilizer treatment greatly influenced the percent marketable yield of dry bulb multiplier onion. *P. polymyxa* treatment gave the highest percent marketable bulbs with 92.72%. No biofertilizer and *B. subtilis* 1 treatments gave comparable percent marketable bulbs with 89.03% and 89.50%, respectively. *B. subtilis* 2 treatment gave the lowest marketable bulbs. The results imply that the application of *P. polymyxa* produced higher dry matter yield compared to the other treatments particularly to *B. subtilis* 2. Fertilizer source treatment did not show significant variability on the percent marketable bulbs.

Table 5.11 Yield per hectare (t ha^{-1}) of multiplier onion as affected by biofertilizer, fertilizer source, and their interaction

Treatment	Yield per hectare (tha^{-1})	
	Green	Bulb (dry)
Biofertilizer (A)	**	**
Control	14.21 bc	10.01 b
B. subtilis 1	13.02 c	9.25 c
B. subtilis 2	15.84 ab	10.74 a
P. polymyxa	18.16 a	10.99 a
Fertilizer source (B)	**ns**	*
No fertilizer	14.47	9.73 b
Inorganic	15.84	10.77 a
Organic	15.61	10.28 ab
A × B	**ns**	**ns**
CVa (%)	10.28	10.46
CVb (%)	4.68	4.21
CVc (%)	6.37	2.56

Means marked with the same letter within each column are not significantly different at 5% level using Honestly Significant Difference (HSD) Test
CV coefficient of variation, *ns* not significant
*Significant at 5% level; **Significant at 1% level

5.3.11 Yield (t ha^{-1})

Yield per hectare of green and dry bulb shallot onion was significantly influenced only by the biofertilizer treatment (Table 5.11). Results revealed that plots applied with *P. polymyxa* were able to produce 18.16 t ha^{-1} green onion but comparable to those applied with *B. subtilis* 2 that yielded 15.84 t ha^{-1}. Moreover, plots without biofertilizer and those applied with *B. subtilis* 1 have comparable yields with 14.21 t ha^{-1} and 13.02 t ha^{-1}, respectively. Regarding dry bulb onion, a similar pattern was noted. Plots with *P. polymyxa* yielded 10.99 ha^{-1} and were comparable to plots applied with *B. subtilis* 2 yielding about 10.74 t ha^{-1}. The lowest yield was found on plots applied with *B. subtilis* 1 with only 9.25 t ha^{-1}. Control plots produced significantly higher yield (10.01 t ha^{-1}) than plots with *B. subtilis* 1. Whereas in the fertilizer source treatment, plots applied with inorganic fertilizer produced the highest yield (10.77 t ha^{-1}), which was comparable to plots applied with organic fertilizer (10.28 t ha^{-1}). The control plots produced a similar yield to the organic plots with 9.73 t ha^{-1} but significantly different to plots applied with inorganic fertilizer.

5.4 Conclusions

Biochemical characterization showed that isolates 1 and 2 are identified as *Bacillus subtilis* and *Paenibacillus polymyxa* for isolate 3. The kind of biofertilizer and fertilizer source as applied on multiplier onion can slightly delay emergence,

increase laminar length by 1–4 cm, be more vigorous, delay maturity by 1 day, and increase the bulblet diameter by 0.30–0.45 cm and yield. Under field condition and using Australian variety of multiplier onion, application of *P. polymyxa* alone as biofertilizer could be possible, but better results are obtained if combined with organic or inorganic fertilizers. Application of *P. polymyxa* on multiplier onion could suppress the occurrence of pest and diseases because it induces systemic resistance to the plant as revealed in the percent damage caused by insects and diseases as compared to inorganic fertilizer.

5.5 Recommendation

Molecular characterization should be made to confirm the identification of the promising bacterial isolates before they could be packaged as biofertilizer and be used in support of the organic agriculture program of the government particularly the Department of Agriculture.

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Evaluation of the Side Effects of Nitrification-Inhibiting Agrochemicals in Soils

6

Ferisman Tindaon and Gero Benckiser

Contents

Abstract

Selected bioassays were used to assess the side effects of agricultural chemicals on microbial activities under laboratory conditions using loamy clay, loam, sandy soil, and the nitrification inhibitors (NI) 3,4-dimethyl pyrazole phosphate (DMPP), 4-chlor-methyl pyrazole (ClMP), and dicyandiamide (DCD). The effect of NI on general microbial activity was assessed at concentrations from 1 to 1000 times the recommended application rate. Also, dehydrogenase (DHA) and dimethyl sulfoxide reductase activity (DRA) on nitrogenase activity (NA) was evaluated. The potential denitrification capacity (PDC), representing the nitrogen cycle specific to soil microbial processes, was examined in incubation experiments in the presence of DMPP, ClMP, and DCD. DHA was estimated spectrophotometrically, and DRA, PDC, and NA gas were quantified using gas chromatography. The morphological changes of a nitrifying bacterial consortium in the presence of the recommended DMPP field application rate and a tenfold-higher concentration were observed under transmission electron microscopy. Inhibition in the presence

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of increasing NI concentrations was calculated as no effect level (NOEL), effective dose ED_{10} (10% inhibition), and ED_{50} (50% inhibition). Dose–response curves expressing the effectiveness of inhibition were most distinct in sandy soils. Most NI-sensitive reacted PDC > DRA > DHA > NA and ClMP added to the three differently structured test soils influenced nontarget microbial process activity at a higher level. Our results clearly indicated that evaluation of agrochemical side effects with soil enzymes is a reliable, sensitive, reproducible, and suitable method to investigate interference with soil microbial activity.

Keywords
Dehydrogenase · Dimethyl sulfoxide reductase · Nitrogenase · Microbial activity

6.1 Introduction

Nitrogen cycling starts with the introduction of photosynthesis or crude oil-coupled biological and technical N_2 fixation (BNF, TNF). The form NH_4^+ is converted by nitrifying bacteria and archaea into NO_3^-, and the denitrification process returns NO_3^- as N_2 and in small but increasing amounts of N_2O to the atmospheric N pool from which NH_4^+ originated (Benckiser et al. 2016). The key regulator in N cycling, in e^- donator–acceptor balancing, is the manifold controlled nitrification process (Benckiser 2017). Nitrification is controlled by the availability of NH_4^+, O_2, and CO_2, derived from organic matter degradation. Then, the nitrifying bacteria and archaea activity is inhibited by compounds formed during organic matter degradation, or by nitrification-inhibiting organic compound-stabilized nitrogen fertilizer granules that farmers spread during sowing (McCarty and Bremner 1989; McCarty 1999; Wu et al. 2012; Benckiser et al. 2016). A subdivision of the O_2-dependent NH_4^+-derived e^- and H^+ flows towards electron transport phosphorylation to gain energy or towards CO_2 reduction to form the bacterial/archaeal biomass that controls the nitrification process additionally. Finally, NH_4^+ oxidation is carried out in two steps with moderate energy gains. The first oxidation step, from NH_4^+ to NO_2^-, is carried out by the nitroso-group, and the second oxidation step, from NO_2^- to NO_3^-, by the nitro-group. Less energy is gained by the nitro-group than by the nitroso-group, which must make available NO_2^- oxidize quickly to NO_3^- for energy-gaining functions, and that avoids toxic nitrite accumulation in ecosystems (Benckiser et al. 2016; Benckiser 2017). A delayed formation of less mobile NH_4^+ into mobile NO_3^- ions keeps nitrate leaching and biological denitrification (BDI), itself controllable by procyanidin compounds, in their natural ranges (Azam et al. 2001; Bardon et al. 2016). The capability of nitrifying bacteria and archaea to reduce NO_2^- with NH_4^+ to N_2 and to denitrify allows additional energy conservation, further nitrite accumulation, and NO_3^- leaching, a minimizing strategy (Kleineidam et al. 2011; Benckiser et al. 2016).

Environmental pedoturbation and management practices, such as tillage, drizzle application of pesticides, or the spreading of marketed nitrification inhibitor (NI)-stabilized nitrogen fertilizer granules with organic compounds may cause soil quality changes by affecting the microbial soil enzyme activity (Thom et al. 1997; Weiske et al.

2001; Tindaon et al. 2011, 2012). Marketed in NI-stabilized nitrogen fertilizer granules, 3,4-dimethyl pyrazole phosphate (DMPP) or dicyandiamide (DCD) may affect nontarget soil activities, and thus there is a need to develop methods assessing the field recalcitrance and side-effecting potential of pesticides or NI (Thom et al. 1997; Tindaon et al. 2011, 2012; Benckiser et al. 2013). Dehydrogenase (DHA), which transfers H^+ and e^- metabolically, is an intercellular enzyme that describes the overall soil microbiological activity. DHA was employed inter alia to describe microbiological activity in Mediterranean forest soils (Quilchano and Maranon 2002). Besides DHA, dimethylsulfoxide (DMSO) reductase activity (DRA) is traceable in many soil microbes and is seen as another possibility to describe overall microbiological activity in soils. The DRA transfers electrons onto the acceptor DMSO, which is reduced to gaseous dimethyl sulfide (DMS) that can easily be quantified with a gas chromatograph (Alef and Kleiner 1989; Alef 1995). N-cycle-concerned activity parameters are in addition to the manifold controlled nitrification the soil bacterial and archaeal nitrogenase activity (NA), introducing N_2 in the form of NH_4^+, and the potential NO_3^--, NO-, and N_2O-reductase capacities (PDC), returning the NO_3^- ions formed by nitrification as NO, N_2O, and N_2 into the atmosphere.

Total bacteria and archaea numbers, counted under the microscope or after cell growth on selective media as colonies on plates, tend to underestimate the microbial presence and related activity as inter alia molecular biological techniques exhibit (Kisand and Wikner 2003). It is assumed until now that only 1–10% of the present soil bacteria and archaea can be grown on artificial media, and thus more appropriate for evaluating NI side effects in actual soil situations seem to be such enzymatic methods as DHA, DRA, NA, and PDC (Alef and Kleiner 1989; Alef 1995; Allison et al. 2008; Araujo et al. 2009; Baldrian 2009; Ferreira et al. 2013; Santric et al. 2014; Zannatta et al. 2007). The marketed DCD or DMPP, spread as NI-stabilized N fertilizers, hold N in the root zone until the mature plants can begin forming NO_3^-. NI application reduces nitrate leaching reduction by about 60% and N_2O emissions by as much as 70%, and increases crop yields by more than 20% (Pasda et al. 2001; Weiske et al. 2001; Sahrawat 2004; Douma et al. 2005; Di and Cameron 2004, 2005, 2006; Di et al. 2007; Fillery 2007; Moir et al. 2007; Singh and Verma 2007). Side effects of nitrification-retarding chemicals such as DCD or DMPP cannot be excluded but are little studied. We report a standard laboratory test method developed on an enzyme basis for evaluating the toxic effects of chemicals, especially of crop-protecting pesticides, fertilizers, or soil microorganisms controlling agrochemicals. The International Organization for Economic Cooperation and Development (OECD), ISO, the United States Environmental Protection Agency (USEPA 1984), or the Biological Association for Agriculture and Forestry (Biologische Bundesanstalt für Land- und Forstwirtschaft, BBA) (Malkomes 1997a, b), on which the investigations reported here are based, have side-effect research as a focus. We studied the environmental and agronomy aspects in field plots in the NI DMPP, DCD mode of action (Weiske et al. 2001) and in the laboratory under use of a field soil spectrum; DHA, DRA, NA, and PDC test the potential nontarget side effects of the marketed NI DMPP and DCD and the unmarketed ClMP (Cl-substituent).

Table 6.1 Physicochemical properties of the differently textured model soils

Parameters	Type of soil		
	Silty clay	Silt	Loamy sand
C_{total} (%)	1.35	1.30	0.70
C_{H2O} (%)	0.40	0.55	0.27
N_{total} (%)	0.15	0.15	0.08
C/N	10	9	9
pH $_{H2O}$	6.30	7.00	7.00
pH $_{KCl}$	6.00	5.50	6.40
Fraction (%)			
Clay	51	24	6
Loam	41	46	19
Sand	8	30	75

6.2 Materials and Methods

6.2.1 Soils and Nitrification Inhibitors Used

Carefully homogenized, sieved (<2 mm), and air-dried soil samples of a loamy sand (BASF Agricultural Center, "Limburgerhof"), a loam and a loamy clay (Experimental Station of the Department of Agronomy and Plant Protection, Justus Liebig University (JLU), Giessen, Germany) (0–20 cm) were physicochemically analyzed by standard methods (Schlichting et al. 1995). To the three differently textured soils with the properties shown in Table 6.1, ammonium sulfate-formulated DMPP, ClMP, and DCD (purity 99.9%, 99.7%, and 96%, respectively) were added in the concentration 0 (control) and in concentrations 5, 10, 25, 50, 100, 250, 500, 750, and 1000 times higher than the recommended field rate of 0.36 (DMPP), 0.25 (ClMP), and 10 μg g^{-1} dry field soil (DCD), calculated for 90 kg N ha^{-1}. The NI application rates were experimentally found by marketing companies (DMPP and CLMP, BASF SE, Ludwigshafen, Germany; DCD, SKW Trostberg AG, Trostberg, Germany).

6.3 Model Experiments

6.3.1 DMPP Effect on a Nitrifying Consortium

For an idea how a nitrifying enrichment culture from the loamy clay soil (Agronomy Experimental Station, Justus Liebig University, Giessen, Germany), which had no previous DMPP contact, may perform at the recommended field application rate and a tenfold higher concentration, 20 g clayey loam topsoil was weighed in 500-ml Pyrex bottles to enrich stepwise (three transfers) the nitrifying consortium; 4 ml nitrifying culture enrichment solution was added to 46 ml nitrification broth and incubated for 75 days in the presence of 0 (control), the recommended rate, and a tenfold higher DMPP field application rate (for experimental details, see Benckiser

et al. 2013). pH, NH_4^+, NO_2^-, and NO_3^- were routinely measured, and at the end of the experiment morphological bacterial changes were documented with a 80 kV transmission electron microscope (TEM; Philips EM300).

6.3.2 Effects of DMPP, ClMP, and DCD on DHA, DRA, Potential Denitrification Capacity (PDC), and Nitrogenase Activity (PNA)

The designated model experiments are described in detail by Tindaon et al. (2012). Briefly, dehydrogenase activity (DHA) was assayed in 50-ml test tubes with 2.5 g air-dried soil samples (five replicates) plus 2.5 ml alternative electron acceptor 2-(4-iodophenyl-3-(4-nitrophenyl)-5-phenyltetrazolium-chloride (INT)-Tris buffer solution according to the INT method (ISO 23753-2:2005; Von Mersi and Schinner 1991; Alef and Nannipieri 1995). The contents of the rubber stopper-sealed tubes were carefully mixed, and because of the light sensitivity of INT were incubated in semi-darkness together with control tubes without NI (4 h, 25 °C). The DHA-dependent formed iodophenyl-3-(4-nitrophenyl)-5-phenyltetrazolium formazan (INF) was subsequently extracted with 10 ml tetrahydro furane (Merck, Darmstadt) by overhead shaking (1 h). The extinction in the homogenized, filtered INF extracts was spectrophotometrically measured against the blank (ZEISS PM2-DL; 436 nm) and expressed in μg INF g^{-1} dry soil h^{-1}.

NI side effects on DRA were quantified in 4.8-ml small flasks (five replicates) containing 0.5 g soil, samples, 0.125 ml 6.6% DMSO solution (alternative electron acceptor) or water (blank), and varying NI amounts. During subsequent incubation (3 h, 25 °C), gaseous dimethyl sulfide (DMS), formed by intracellular DMSO reduction, was quantified by gas chromatography (0.25-ml gas samples; Hayesep-column, 2.0 m; R 1/8, 80–100 mesh, 160°°C; FID, 220 °C, carrier gas N_2), and expressed in ng DMS g^{-1} dry soil h^{-1} (Fig. 6.1) (Alef and Kleiner 1989; Alef 1995).

The NI side effects on potential denitrification capacity (PDC) were measured in 2-l flasks containing 40 g soil (four replicates) to which 20 ml 1 mM glucose and 1 mM KNO_3 solution was added. The flask atmosphere was evacuated five times and replaced by nitrogen. For blocking N_2O reductase, acetylene (0.1 atm partial pressure) was added to obtain N_2O exclusively as the denitrification end product after incubation at 25 °C in the dark for 6 h. Flask headspace gas samples with forming N_2O were injected each hour into a gas chromatograph (Perkin Elmer 8500; ECD, 350 °C; 2.0 m Porapak Q, 1/8 80–100 mesh column, 50 °C; carrier gas N_2, 30 ml min^{-1}) and expressed in μg N_2O-N g^{-1} soil dry weight h^{-1} (Pell et al. 1998).

Nitrogenase activity (NA) was determined with the acetylene-ethylene method (Martensson 1993; Chalam et al. 1997), and the C_2H_4 gas was chromatographically analyzed (Perkin Elmer 8500; FID, 220 °C; 2.0 m Hayesep R 1/8, 80–100 mesh column, 160 °C; carrier gas, N_2, 35 ml N_2/min).

For describing dose–response relationships, nonlinear regression, analysis of variance (ANOVA), and Fisher's least significant difference pairwise comparison tests were applied (Stephenson et al. 2000) to denote the doses of the NI concerned

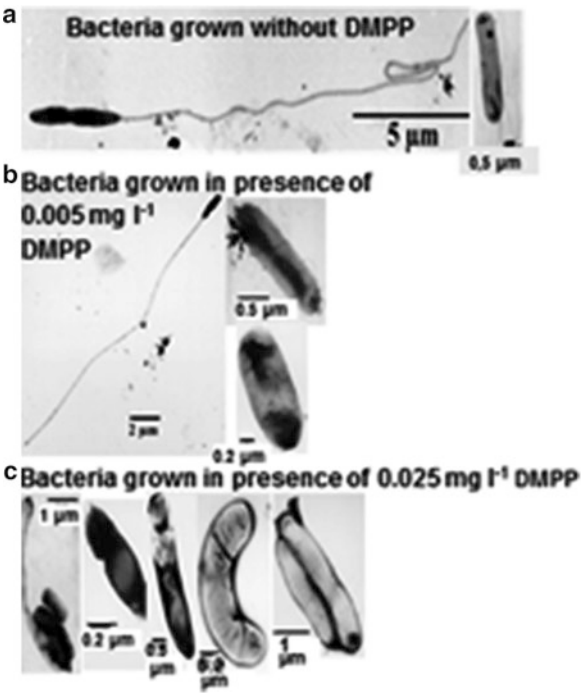

Fig. 6.1 Transmission electron micrographs (TEM) of bacteria found in a nitrifying enrichment culture from ammonium-N fertilizer-treated soil of a 3-year field experiment, classified as anallochtone brown earth, derived from river sediments with a clayey loam topsoil (Vega) (Weiske et al. 2001) after 75 days of incubation: (**a**) in absence of 3,4-dimethyl pyrazole phosphate (DMPP); (**b**) at recommended rate of field application; (**c**) at a tenfold higher DMPP concentration

causing 10% and 50% inhibition, respectively, of nitritation (ED_{10} and ED_{50} values). The highest dose at which no effect can be observed (NOEL, No Observed Effect Level) describes the critical concentration that does not cause adverse effects. NOEL, ED_{10}, and ED_{50} were calculated by the equation $Y = a/\{1 + \exp[-(X_t - X_0)/b]\}$, whereby Y equals the maximum response a divided by $1 + \exp[-(X_t - X_0)/b]$. X_0 and X_t are the log NI doses at the beginning and end of the experiment, and b is a constant describing the NI influence (USEPA 1984; Richter et al. 1996). Significant differences at the $P < 0.05$ level were obtained by using Sigma Plot and Sigma Stat Software (SPSS).

6.4 Results

6.4.1 DMPP Concentration-Dependent Morphological Changes

The TEM images (Fig. 6.1) show members of a nitrifying bacteria consortium enriched from the clayey loam soil (Weiske et al. 2001). Although the autotrophic nitrifying bacteria are grown in consortium in a medium in the absence and presence of both the recommended and the tenfold higher DMPP field application rate, it cannot definitely be excluded that there are no non-nitrifying bacteria among the consortium. At the recommended field DMPP application rate, cell sizes appeared

Table 6.2 Dehydrogenase- and dimethyl sulfoxide reduction activity in soil samples from controls (soil not treated with nitrification inhibitors used)

Soil type	Dehydrogenase activity[a] [μg (INF) g^{-1} dry soil, h^{-1}]	Dimethyl sulfoxide reduction activity[a] (ng DMSO g^{-1} dry soil, h^{-1})
Silty clay	431.6 ± 3.4	369.8 ± 2.5
Silt	274.2 ± 2.3	321.1 ± 2.6
Loamy sand	121.0 ± 0.9	96.5 ± 1.2

[a]Average of 5 replicates

slightly enlarged in comparison to the control, but the bacteria consortium after a 75-day incubation period when transferred to a fresh medium for nitrifying bacteria replicated and grew again. At the tenfold higher DMPP application rate, no growth renewal was possible. Because cell shaping through DMPP starving may have had a concerning role, and to achieve a further approach towards a safer NI side-effect evaluation, we started with experiments by using enzyme methods recommended by local and globally acting authorities. We tested the reliability of DHA, DRA, PDC, and PNA concerning side effects caused by the marketed NI DMPP, DCD, and the unmarketed ClMP by calculating NOEL, ED_{10}, and ED_{50} values at control and varying NI levels.

6.4.2 Dose–Response Relationships

Table 6.2 reports DHA and DRA estimates in the three investigated control soils, and Fig. 6.2 shows, by the equation $y = a/(1+\exp(-(X_t-X_a)/b))$, the calculated, semilogarithmic DMPP, ClMPP, DCD, and DHA dose–response relationship for the silty clay soil (Richter et al. 1996; for details see Tindaon et al. 2011). The DHA and DRA estimates in the control soils (Table 6.2) are comparable with DHA and DRA soil estimates by other investigators and appear as trustworthy for evaluating DMPP, ClMP, and DCD side effects on nontarget microbial activities in the three studied soil types (Alef and Kleiner 1989; Casida 1977; von Mersi and Schinner 1991; Friedel et al. 1994; Griebler and Slezak 2001; Obbard 2001). The semilogarithmically in percent (%) allegorized DMPP, ClMP, and DCD concentration-dependent side effects on DHA (Fig. 6.2) show that the less mobile NIs 3,4-dimethyl pyrazole phosphate (DMPP) and 4-chloromethyl pyrazole (ClMP) already affect the DHA at a significantly lower concentration than the more mobile dicyandiamide (DCD) in the three soil types studied.

In addition to Fig. 6.2, Table 6.3 compares the DMPP effect on calculated DHA, DRA, and PDC-derived NOEL, ED_{10}, and ED_{50} values in the three test soils (recommended dosage, 0.36 μg DMPP g^{-1} dry soil). For safety considerations, the estimated NOEL is divided by 10 (laboratory trials) and 100 (field trials). Assumingly, therefore, DHA, DRA, and PDC side effects were expressed first in the silty clay soil at a DMPP application rate approximately 50 times higher than the

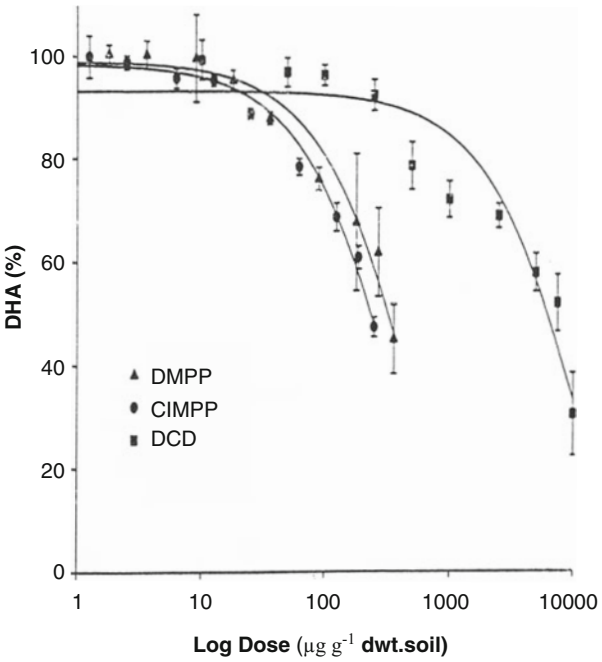

Fig. 6.2 DMPP, 4-chlormethyl pyrazole (ClMPP), and dicyandiamide (DCD) concentration-dependent side effects on dehydrogenase activity (DHA) (recommended field dosage per gram dry soil: 0.36 µg DMPP; 0.25 µg ClMP; 10 µg DCD)

Table 6.3 DMPP-dependent, nontarget microorganism activity showing NOEL, ED_{10}, and ED_{50} values estimated in the three test soils: clay loam, loam, and sandy loam[a]

Parameters	Soil Type	Eco-toxicological values for DMPP		
		NOEL	ED_{10}	ED_{50}
DHA	Clay	91	133	371
	Loam	30	72	312
	Sand	25	56	230
	Ø	49	87	304
DRA	Clay	47	101	365
	Loam	11	53	287
	Sand	5	44	266
	Ø	24	66	306
PDC	Clay	30	62	241
	Loam	17	35	193
	Sand	5	17	150
	Ø	17	38	195

[a]Average of 5 replicates

field-recommended rate. DMPP side effects were expressed by NOEL DHA, NOEL DRA, and NOEL PDC values of 9, 3, 2.5, or 4.7, 1.1, 0.5, or 3, 1.7, 0.5 µg g^{-1} soil, respectively, permitting a NOEL value-dependent ranking of clayey soil > loam soil > sandy soil.

In addition to Tables 6.3, 6.4 shows the side effects on DHA, DRA, and PDC expressed by NOEL, ED_{10}, and ED_{50} values through evolving ClMP in the three test soils (recommended dosage, 0.25 µg CLMP g^{-1} dry soil). From a 25-fold higher

Table 6.4 ClMP-dependent, nontarget microorganism activity related to NOEL, ED_{10}, and ED_{50} values estimated in the three test soils: clay loam, loam, and sandy loam[a]

Parameters	Soil Type	Eco-toxicological values for ClMP		
		NOEL	ED_{10}	ED_{50}
DHA	Clay	32	66	255
	Loam	28	58	229
	Sand	12	33	147
	Ø	24	52	210
DRA	Clay	27	55	215
	Loam	19	48	197
	Sand	4	15	133
	Ø	17	39	182
PDC	Clay	7	29	155
	Loam	6	12	105
	Sand	2	11	98
	Ø	5	17	119

[a]Average of 5 replicates

Table 6.5 DCD-dependent, nontarget microorganism activity concerning NOEL, ED_{10}, and ED_{50} values estimated in the three test soils: clay loam, loam, and sandy loam[a]

Parameters	Soil type	Ecotoxicological value for DCD		
		NOEL	ED_{10}	ED_{50}
DHA	Clay	844	1754	6940
	Loam	550	1126	5558
	Sand	167	809	4450
	Ø	520	1230	5649
DRA	Clay	395	1083	4965
	Loam	296	1102	4533
	Sand	112	402	2597
	Ø	268	862	4032
PDC	Clay	366	761	2438
	Loam	299	646	2049
	Sand	159	487	1724
	Ø	275	631	2070

[a]Average of DHA, DRA, and PDC

field application rate than the recommended 0.25 μg ClMP g^{-1} dry soil and thus earlier than DMPP (Table 6.3) as expressed by NOEL DHA, NOEL DRA, and NOEL PDC values of 3.8, 2.8, or 1.2 μg g^{-1}; 2.7, 1.0, or 0.4 μg g^{-1}; and 0.73, 0.6, or 0.2 μg g^{-1}, respectively, side effects were observed for ClMP DHA, DRA, and PDC in the clay loam, loam, and loamy sand soils (Table 6.4).

Table 6.5 shows for DCD that the NOEL DHA, DRA, and PDC values for the silty clay, loam, and loamy sand soil are 84.4, 55, and 16.7 μg DCD g^{-1} or 39.5, 29.6, and 11.2 μg DCD g^{-1} or 36.6, 29.9, and 15.92 μg DCD g^{-1}), respectively. These data show that first at a 250-fold higher DCD concentration than at the recommended field application rate of 10 μg DCD g^{-1} dry soil, side effects become observable and thus much later than in the presence of DMPP and CLMP.

6.5 Discussion

The data shown, obtained under the given test conditions, suggest at first that the recommended DMPP and DCD application rates seem to be saved. Yet, NI-stabilized fertilizer granules have little controlled spread. In soil property-dependent NI diffusion, soil crumb DMPP concentration may enrich by ten times and the soil microbial activity may be incurred or as the nitrifying bacteria consortium in the autotrophic culture solution experiment suffers severe damage if the field-recommended DMPP concentration is elevated tenfold (Figs. 6.1, 6.2; Tables 6.3, 6.4, and 6.5; Azam et al. 2001; Barth et al. 2001; Barth 2006, 2008). It is thus crucial to have methods available allowing us to check how and under which conditions the NI degrades and to inform us when the very diverse, nitrogen-dependently shaped soil community, majorly consisting of bacteria, archaea, protozoa, and fungi, is essentially suffering from pesticides or NI side effects (Thom et al. 1997; Malkomes 1997a, b; Weiske et al. 2001; Benckiser and Bamforth 2010; Benckiser et al. 2016; Benckiser 2017). Such information allows developing a more controlled fertilizer granule spreading by precision farming.

ClMP, although it exhibited the highest side-effect potential among the tested NI candidates, is seemingly safe at the recommended application rate. Soil property-dependent effects apparently first mean DCD, DMPP, and ClMP concentrations of a dose 100-fold higher than the recommended one affect the soil environment negatively as the NOEL values are showing, whereby NI side effects in sandy soils are observable earlier than in loamy or clayey soils (Tables 6.3, 6.4, and 6.5; Barth et al. 2001; Barth 2006, 2008). A sensitivity ranking of the investigated cell internal enzyme activity PDC > DRA > DHA against NI may be explained by protective cell NI contact mechanisms. The NI insensitivity of the nitrogenase is from an ecological aspect of interest. This observation may be explained in that the nitrogenase works under highly controlled compartment conditions, or that under the given experimental conditions the significance of the expressed C_2H_4 concentrations is not representative and affords studies in the legume nodule environment (Benckiser et al. 2016).

Until now only limited data are available for DMPP and DCD culture solutions and field soil observations in the presence of increasing NI concentrations. In culture solution experiments NI absorption, diffusion, and degradation phenomena are different and not always comparable with those in a clayey loam, loam, or sandy loam field soil (Azam et al. 2001; Weiske et al. 2001; Di and Cameron 2004; Sahrawat 2004; Ali et al. 2008; Barth 2006, 2008; Ali et al. 2008; Barth 2006, 2008; Mahmood et al. 2011; Benckiser et al. 2013). At 9, 6, and 250 μg DMPP, ClMP, DCD g^{-1} dry clay loam soil, respectively, or from a 25-fold higher DMPP or ClMP, or a 250-fold-higher DCD concentration than at the recommended field basic application rates, the NOEL-abrogated DHA starts to be affected. Apparently because of NI absorption, diffusion, and degradation phenomena, side effects on DHA set in more sandy soils are already at 3.6, 2.5, and 100 μg DMPP, ClMP, and DCD g^{-1} dry soil, respectively. The nontarget soil organisms protecting soil texture

indicate that triggering is possible for DMPP, ClMP, or DCD-dependent DHA, DRA, or PDC hazards that begin to be observable at application rates far above the recommended ones. In understanding how compounds such as DMPP, ClMP, and DCD inhibit the nitrification process, the syntrophic behaviour and activities of soil bacteria, archaea, protozoa, fungi in biofilms, soil microbial communities definitely are just at the beginning of research (Benckiser and Bamforth 2010; Bannert et al. 2011; Benckiser 2017). From DMPP it is reported that it indiscriminately binds on the membrane-bound protein complex, inclusively the AMO, whereas DCD seems to block the electron transport in the cytochromes during the conversion of NH_3 to hydroxylamine. On the other hand, we found that at the recommended field application rates DMPP and DCD have a nitrification-inhibiting potential that reduces nitrate availability and N_2O emissions by 26% to 49% and increases the crop yields by 20% (PICCMAT 2011). In spite of this control success, a cost–benefit analysis by the European Commission (EC) hesitates to recommend the use of NI-stabilized N fertilizers because they are still relatively expensive, the N-saving effectiveness is only insufficiently tested, and cereal maize crop yield improvements are not conclusively documented.

DCD-concerned 16S RNA-based studies by other researchers suggest no essential soil bacterial community structure and phyla changes at field-recommended NI concentration and substantiate our findings, at least partially. In the presence of NI potassium oxalate, an Uzbekistani study reports that the number of nitrifying and denitrifying bacteria decreased but the number of oligonitrophilic bacteria and cellulose-degrading activity increased, whereas after nitrapyrin application to an undisturbed semi-arid steppe, less organic matter decomposition was found. It was concluded that not the system N quantity but rather the ammonium versus nitrate ratio influences the carbon cycling of N species and finally ecosystem functioning (Egamberdiyeva et al. 2001; Muller et al. 2002; Austin et al. 2006; Di et al. 2007; Mahmood et al. 2005; O'Callaghan et al. 2010). DMPP field studies carried out by Weiske et al. (2001) by Barth (2006, 2008) tentatively confirm the view of Egamberdiyeva et al. (2001) and Austin et al. (2006) and show that soil microbial manipulation through NI-containing N fertilizers depends also on NI concentration, chemical structure, its gradual degradability, soil temperature, and organic carbon availability (Weiske et al. 2001; Sahrawat 2004; Benckiser et al. 2013). In the presence of DMPP, timely nitrification inhibition lasts because of slower degradability compared to DCD (Weiske et al. 2001; Di and Cameron 2004; Barth 2006, 2008). Yet, this seems only to be true for temperate environments, not for regions of high temperature (Ali et al. 2008; Mahmood et al. 2011).

In conclusion, more related research is needed. Biologists, ecologists, physicists, physicians, soil scientists, economists, and political scientists now have the analytical tools to detect single atoms, and with steadily increasing computer capacities available, are progressing in understanding the interdisciplinary nature of soils. In future, environmentally compatible used pesticides, including NI-stabilized N fertilizers, will increase the success of precision farming (Marco 2014).

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Cyanobacteria from *Sorghum bicolor*-Grown Fields of Ecopark at Cibinong Science Center-Botanic Gardens, Indonesia

7

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Contents

Abstract

Cyanobacteria (blue-green algae, BGA) are a group of microalgae that plays an essential role in the fixing of atmospheric nitrogen which is important for the soil fertility. BGA can be an economically and ecologically alternative solution for fertilizers in increasing the productivity of *Sorghum bicolor*. Isolation of these cyanobacteria from natural sources in pure form is an essential step for their efficient use as biofertilizer. The purpose of this study was to investigate cyanobacteria from *S. bicolor*-grown fields of Ecopark at Cibinong Science Center-Botanic Gardens, Indonesia, as a baseline data. The isolation of pure cultures was done by selecting a single colony from mixed cultures grown on selected media BG-11 and bold basal media, as different cyanobacterial strains can grow on different media. The same medium in liquid form was used for further purification and subculturing. The pure cultures were transferred to liquid media for further studies. From 20 soil sample cultures, 4 predominant isolates were identified on the basis of their morphological characteristics under light microscopy. Observations were made on heterocystous and non-heterocystous forms. The genera of *Nostoc* and *Anabaena* were found as the dominant heterocystous group, while the non-heterocystous group consisted of *Lyngbya* and *Oscillatoria* in the *S. bicolor*-grown fields.

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Keywords
Cyanobacteria · *Sorghum bicolor* · Biofertilizer · Plant growth promotion

7.1 Introduction

Cyanobacteria (blue-green algae, BGA) is a photoautotrophic prokaryote organism that has incipient nucleus types, hexagonal carboxysomes, loosely arranged thylakoids, gas vesicles which help maintain certain vertical positions in water to respond to physical and chemical properties, ribosomes, phycobilisomes, and also various storage granules consisting of glycogen, cyanophycin, or polyphosphate lipids (Singh et al. 2013). The BGA group name is given based on the color of the cell seen under a microscope. The color expression of their cells is caused by existing pigments such as chlorophyll-a, phycocyanin, and phycoerythrin. Many species have a golden brown or dark color, although sometimes red in cells around individual cells or throughout filaments and sheaths (Kondo and Yasuda 2003). BGA is a major producer in natural ecosystems (Field et al. 1998; Ohkouchi et al. 2006). BGA can be found in diverse habitats (Whitton 2000). They exhibit a wide distributional spectrum; they are ubiquitous under different soil, water, and agroclimatic condition, occurring in fresh and marine water, terrestrial environment, and growing wherever little light and moisture is available (Sao and Kritika 2015).

In farmlands, BGA plays a vital role in increasing the fertility of various ecological agricultures. These organisms can produce organic materials that can increase soil fertility (Mishra and Pabbi 2004). BGA excretes substances such as hormones, vitamins, amino acids, and organic acids that can increase plant growth and affect other organisms in many ways (Wilson 2006). The presence of BGA can also reduce erosion because it can stabilize the soil surface (Hu et al. 2004). Soil porosity, aggregation, and water holding capacity can increase with the presence of polysaccharides produced by several BGAs (Choudhary et al. 2007; Kaushik 2007). BGA can carry out two biological processes such as oxygen photosynthesis and nitrogen fixation, thus becoming the preferred biological fertilizer.

Song et al. (2005) reported that inoculation of agricultural land with algae could increase grain yield by 15–25%. BGA inoculation is reported to have beneficial effects on plants such as rice, corn, wheat, soybeans, oats, tomatoes, radishes, cotton, sugar cane, chili, peanuts, muskmelon, and lettuce (Thajuddin and Subramanian 2005; Maqubela et al. 2008; Karthikeyan et al. 2007). Svircev et al. (1997) also reported that plant growth was enhanced in the presence of BGA, even without organic N fertilizer application. Some species are widespread in agricultural land and are known to contribute significantly to their fertility, such as *Nostoc*, *Anabaena*, *Tolypothrix*, *Aulosira*, *Cylindrospermum*, *Scytonema*, *Westiellopsis*, and several other genera (Rao et al. 2008; Choudhary 2011). Although some BGAs have been widely studied, information in the diversity and ecological characteristics of BGA is very limited. No previous studies have been reported about cyanobacterial diversity from *S. bicolor* fields. In this study, we conducted an isolation and characterization

of cyanobacteria from S. *bicolor*-grown fields of Ecopark at Cibinong Science Center-Botanic Gardens, Bogor, Indonesia, using morphological observations, as the first step to their efficient use as a biofertilizer.

7.2 Materials and Methods

7.2.1 Collection of Soil Sample

Collection of soil samples was carried out at a depth of 0–5 cm on 20 different plots of *Sorghum bicolor*-grown fields of Ecopark at Cibinong Science Center-Botanic Gardens (CSC-BG), Bogor, Indonesia. Ecopark is built as an extension of Bogor Botanical Gardens that has a common purpose, namely, as a means of education, research, and ecotourism. Currently, Ecopark has a garden collection grouped by bioregion, covering Sumatera Bioregion, Java and Bali, Borneo, Lesser Sunda Island, Celebes, the Moluccas, and Papua. In addition, there is a lake known as Dora Lake. However, there is also empty land dominated by grass which has not been used and developed by the manager so that not all areas can be used by visitors. In previous research, we try converting *Imperata* grassland in CSC-BG into agriculture field by planting the *S. bicolor*.

7.2.2 Enriching of Soil Sample

Soil samples were placed on sterile Petri dishes which had contained sterile BG-11 media with a pH of 7.1 and bold basal media because different cyanobacterial strains could grow on different media. Incubation is carried out for 2 weeks by placing Petri dishes in the culture chamber at 25 °C and a light 12/12 h dark cycle. After colonization, for isolation and purification, loops are used to move parts of each colony to new plates with liquid media.

7.2.3 Isolation of Cyanobacteria by Capillary Micropipetting

Equipment such as silicon slope, sliding glass, microscope, and flat bottom 24-well plates including elongated Pasteur pipette with specific size and length of holes for the capillary micropipetting procedure prepared. The base of the Pasteur pipette is connected to the silicon slant. BG-11 medium with pH 7.1 and bold basal media were poured into 24-well plates. The presence and diversity of cyanobacteria on the plate were observed under a light microscope. Cyanobacteria that have been determined as the target of isolation are sucked using a sharp-tipped Pasteur pipette. The single-celled cyanobacteria were brewed and cultivated into one of the holes of 24-well plate containing the media. The observations were made every day and recorded accordingly. Repeated subculturings were performed until pure axenic cultures were obtained. Single cells from cyanobacteria were cultivated and selected for further studies.

7.2.4 Morphological Analyses

Morphological observations were carried out using an Olympus BX5 microscope fitted with software (Cell Sens Standart) and a digital camera. Observations were made on the shape and size and vegetative cell, presence and absence of sheaths, heterocysts, akinetes (if any), and the position of the axenic culture cyanobacterial branching pattern. Magnification used to capture cyanobacterial images is 100x. The strains were identified based on their morphological features and phenotypic characters following standard keys (Prescott 1951; Desikachary 1959; Wehr et al. 2002; Komárek and Anagnostidis 2005).

7.3 Results and Discussion

7.3.1 Morphological Analyses

Morphological characteristics studied were the cell shape and size, the heterocyst, the shape of the akinetes, and the presence or absence of a sheath. Four predominant cyanobacteria (blue-green algae, BGA) were obtained from the study sites. The genera of *Nostoc* and *Anabaena* were found as the dominant heterocystous group, while the non-heterocystous group consisted of *Lyngbya* and *Oscillatoria* in the *S. bicolor*-grown fields (Fig. 7.1 and Table 7.1).

***Nostoc* (Order: Nostocales; Family: Nostocaceae)**
Nostoc has a gelatin body consisting of many internal filaments which are packaged in a sheath or skin called trichomes. Each trichome being simple free and curved or circular in shape. The population looks denser toward the outer "skin" of the body. When gelatin colonies rupture, filaments or "trichomes" are in great abundance and

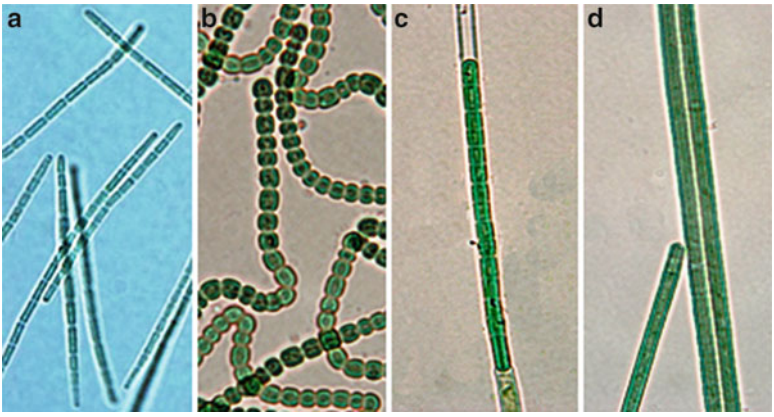

Fig. 7.1 Microscopy of cyanobacteria from *Sorghum bicolor* grown fields of Ecopark at Cibinong Science Center-Botanic Gardens: (**a**) *Nostoc*; (**b**) *Anabaena*; (**c**) *Lyngbya*; (**d**) *Oscillatoria*

Table 7.1 General morphological characteristics of the isolated cyanobacteria from *Sorghum bicolor*-grown fields of Ecopark at Cibinong Science Center-Botanic Gardens

Genus	Cell width (μm)	Cell length (μm)[a]	Sheath[a]	Motility[a]	Cellular shape	Other characteristic
Nostoc	2–8	NM	−	+	Cylindrical, spherical	Formation of thallus, heterocystous
Anabaena	2–20	NM	−	+	Cylindrical, ovoid	Filamentous, heterocystous
Lyngbya	4–15	20–80	+	−	Cylindrical, barrel shaped	Filaments occur within a firm sheath, non- heterocystous
Oscillatoria	2–15	35–65	+	+	Cylindrical	Transverse septa visible, non-heterocystous

[a]Meanings of symbols: NM, not measured; +, present; −, absent

cause a feeling of slimy. Vegetative reproduction will continue inside the colony until the moisture disappears. Then many cells produce blackish masses, forming spores. The majority of the cells seen do not have a nucleus, but a number of chromatin granules have the same size. Heterocyst cells occur in the filaments (subspherical and intercalary) which develop into larger ones with vegetative reproduction (fragmentation) and at which point they break into new colonies called hormones. Cells as a gelatinous body mature in a trichome change into akinetes, capable of forming new filaments.

***Anabaena* (Order: Nostocales; Family: Nostocaceae)**
Trichomes are untapered with conspicuous constrictions at cross-walls. Trichomes may be straight, curved, or helically (spirally) formed. The cells are cylindrical or ovoid (barrel-shaped) and not shorter than broad (or only slightly so). The terminal cells may be rounded, tapered, or conical in shape. Heterocysts are intercalary or terminal or both intercalary heterocysts are nearly spherical to cylindrical with rounded ends; terminal heterocysts are similar or sometimes conical. Akinetes are usually formed, and their position in trichomes differs with the species. A form of individual sheath is absent, but a soft mucilaginous covering is often present. Trichomes, when free of adhesive mucilage, are normally motile, and colonies are not formed. Reproduction is by fragmentation of "parental" trichomes into shorter trichomes indistinguishable in cell dimensions from the former trichome.

***Lyngbya* (Order: Oscillatoriales; Family: Oscillatoriaceae)**
Lyngbya is filamentous organisms that produce a distinct and persistent sheath. Thin sheaths can be seen with phase contrast optics when they go beyond trichome terminal cells. Trichomes of *Lyngbya* are usually non-motile within the sheath, but when placed on a new agar-solidified medium, the short section of the trichome

(hormogonia) sometimes moves slowly. Some strains produce many hormogonia which glide freely from the sheath and repr*oduc*e the new sheath. The terminal parts appear sheathless, with rapid growth extending the trichome out of the old casing, in some cases. All filament threads in liquid culture will unite because the sheaths of some strains are quite prominent and strong. In large-diameter species, laminated sheaths often occur.

***Oscillatoria* (Order: Oscillatoriales; Family: Oscillatoriaceae)**
Oscillatoria is a filamentous organism that can divide exclusively in one plane through binary fission. The trichomes are flexible or semirigid and straight to loosely to the tortuous apices. Transverse septa can be seen under a light microscope. Constructions can occur in cross-walls with total curvature never exceeding one-eighth of the diameter of a trichome. Generally, longitudinal walls are thinner than cross-wall septums. Fission of the cytoplasmic membrane that separates the new membrane of a daughter cell has a thinner peptidoglycan layer (cells may be much shorter than wide, appearing as a pile of disks), cells shorter than long. The trichome is motile and rotates from left or right in a scattered manner. The curved free end will oscillate when the trichome rotates if the terminal area does not come into contact with the substrate. When the trichome moves on a solid substrate, the sheath is barely visible. In some trichomes during periods of immobility in liquid cultures, the sheaths appear to accumulate in several trichomes.

Morphological, biochemical, and physiological properties that allow growth in a wide range of habitat can express the diversity of cyanobacteria. Its complex structure, unlike ordinary prokaryotes, can be their taxonomic differentiator based on phenotypic, mostly morphological properties. The diversity of heterotopic forms of cyanobacteria on agricultural land has been extensively studied (Choudhary 1999). The lower number of forms during the initial cultivation stage can be due to the effect of inhibiting high light intensity, while the loss of nutrients and low light intensity reaching the surface cause fewer forms in the later part.

Anabaena and *Nostoc* as nitrogen-fixing species were found in the of S. *bicolor*-grown fields of Ecopark, Cibinong Science Center-Botanic Gardens, Bogor, Indonesia, in this study. The same number was found in non-N-fixing species, namely, *Lyngbya* and *Oscillatoria*. Despite the application of periodic fertilizers, indications of N deficiency can be identified by the occurrence of N-fixing species in the soils (Nayak and Prasanna 2007). Enumeration of cyanobacteria during the mid-cultivation cycle of the farm fields can reveal the maximum diversity (Choudhary 2009). This study documented the biodiversity of cyanobacteria from S. *bicolor* in the fields of Ecopark, Cibinong Science Center-Botanic Gardens. According to Choudhary (2011), documentation regarding nitrogen-fixing cyanobacteria and their application in agricultural fields can be used for the management of nitrogen fertilizers at various stages of cultivation by making a supportive environment for nitrogen fixers for sustainable agriculture. Further studies are necessary to investigate the potential of cyanobacteria for soil fertility and productivity of crop plants such as S. *bicolor*.

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8 Stimulation of Seed Germination and Growth Parameters of Rice var. Sahbhagi by *Enterobacter cloacae* in the Presence of Ammonium Sulphate as Substitute of ACC

A. Sagar, P. K. Shukla, R. Z. Sayyed, and P. W. Ramteke

Contents

Abstract

Plant growth-promoting rhizobacteria (PGPR) with 1-aminocyclopropane-1-carboxylate deaminase (ACCD) activity facilitate the improvement of agricultural productivity and yield stability. The present work reports the efficiency of *Enterobacter cloacae* with ACCD activity isolated from an organic farm near SHUATS, Allahabad, India. This PGPR was screened for stimulation of seed germination and growth promotion of the rice var. Sahbhagi in the presence of

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ammonium sulphate as a substitute of 1-aminocyclopropane-1-carboxylate (ACC). Inoculation of rice var. Sahbhagi with the PGPR showed a significant ($p < 0.01$) increase in seed germination and enhancement in elongation of root and shoot compared to untreated control. Potential efficacy of *E. cloacae* (KP226569) to enhance the growth of rice will be discussed.

Keywords
Enterobacter cloacae (KP226569) · Rice var. Sahbhagi · Ammonium sulphate as a substitute of ACC

8.1 Introduction

Rice is a major grain crop and carbohydrate source, supplying the necessary daily calories for more than half the world's population. It has been predicted that the demand for rice in the world will increase from 560 million tons to 780 million tons by the year 2020. However, soil salinity is a serious issue confronting rice production (Ebrahimi et al. 2012; Bal et al. 2013).

Rhizobacteria beneficial to plant growth usually referred to as PGPR are capable to promote plant growth under abiotic stress. Bacterial isolates producing ACCD (E.C.4.1.99.4) enzyme are generally known to have longer roots and shoots (Glick 2014).

Rice var. Sahbhagi (IR74371-70-1-1) is a drought-tolerant rice variety which was released and notified in 2010 in India and subsequently in Nepal as 'Sukha Dhan 3' and in Bangladesh as 'BRRI Dhan 56'. Therefore, the present study was undertaken to examine the stimulation of seed germination and growth parameters of rice var. Sahbhagi by *E. cloacae* in the presence of ammonium sulphate as a substitute of ACC.

8.2 Material and Methods

8.2.1 Collection of Organism

The test organism *E. cloacae* were obtained from the culture collection of the Department of Biological Sciences, SHUATS, Allahabad, UP, India. This organism was isolated from the organic farm of SHUATS, Allahabad. The organism studied is characterized for the following plant growth-promoting (PGP) traits based on the standard procedures.

8.3 Production of Plant Growth-Promoting (PGP) Traits

8.3.1 Production of Ammonia

Bacterial isolate was screened for the production of ammonia in peptone water. The freshly grown culture was inoculated in 10 ml peptone water in each tube and incubated for 48–72 h, and after 2–3 days, Nessler's reagent (0.5 mL) was added to each tube. Development of brown to yellow colour was considered as a positive for ammonia production (Cappuccino and Sherman 1992). Qualitatively the isolates were designated with plus (+) sign from 1 to 3 depending on their efficiency to produce ammonia.

8.3.2 Production of Indole Acetic Acid (IAA)

Indole acetic acid (IAA) production was detected as described by Brick et al. (1991). Bacterial culture was grown in peptone water at 37 °C for 72 h. Fully fledged cultures were centrifuged at 3000 rpm for 30 min. The supernatant (2 mL) was mixed with two drops of orthophosphoric acid and 4 ml of Salkowski reagent (50 mL, 35% of perchloric acid, 1 mL 0.5 M $FeCl_3$ solution). Development of pink colour indicates IAA production.

8.3.3 Production of HCN

HCN was detected according to the method of (Bakker and Schippers 1987) king's medium and was amended with 4.4 g glycine l^{-1}, and bacteria were streaked on an agar plate. A Whatman filter paper no. 1 soaked in 2% sodium carbonate and 0.5% picric acid solution was placed at the top of the plate. Plates were sealed with parafilm and incubated at 37 °C for 4 days. Development of yellow to red on filter paper colour indicated the positive HCN production.

8.3.4 Production of Catalase

Bacterial culture was grown in nutrient agar medium for 18–24 h at 37 °C. The culture was placed on the clean slide with the help of loop and 2–3 drop mixed with H_2O_2 and observed for gas bubbles. If the organism gave gas bubbles, then it gives positive results (Schaad 1992).

8.3.5 ACC Deaminase Activity

ACCD activity was performed as described by Jacobson et al. (1994). The bacterial culture was grown in a test tube containing 100 ml of the liquid medium: KH_2PO_4

(2 g), K_2HPO_4 (0.5 g), $MgSO_4$ (0.2 g) and glucose (0.2 g). The medium was supplemented with 0.3 g ACC or 0.19 g $(NH_4)_2SO_4$ as an N source and incubated at 37 °C for 24–72 h. The appearance of bacterial growth indicated the ACC deaminase activity of the bacteria.

8.3.6 Phosphate Solubilisation

Phosphate solubilisation of isolate was evaluated from the ability to solubilize inorganic phosphate. Pikovskaya's agar medium containing calcium phosphate as the inorganic form of phosphate was used in the assay. A loopful of bacterial culture was streaked on the plates and kept incubated at 28 °C for 4–5 days. The appearance of a transparent halo zone around the bacterial colony indicated the phosphate-solubilizing activity of the bacteria (Nautiyal 1999).

8.3.7 Production of Siderophore

Siderophore production was tested using Chrome Azurol S (CAS) agar plates. The overnight culture was spot inoculated on CAS agar plate that was divided into equal sectors and incubated at 37 °C for 12 days. The appearance of orange halos around the colonies on the blue-coloured agar indicated siderophore production.

8.3.8 Production of Chitinase

Chitinase assay was performed as described by Das et al. (2010). Chitin plates were prepared with M9 agar medium amended with 1% (w/v) colloidal chitin. The plates were divided into equal sectors; spot inoculated with 10 ll of overnight grown culture and incubated at 37 °C for 24–96 h. Zone of clearance around bacterial colonies indicated chitinase production.

8.4 Salinity Tolerance

Salt tolerance of the organism was determined by inoculation in nutrient broth with different concentration of salt (05–20%) and incubated at 37 °C for 48–72 h. Growth in the medium was considered as tolerance to salt (Damodaran et al. 2013).

8.5 pH Tolerance

Tolerance to pH was determined by inoculation in nutrient broth with different pH (5–9) and incubated at 37 °C for 48–72 h. Growth in the medium was considered as tolerance to pH (Damodaran et al. 2013).

8.6 Seed Germination Test

Rice cv. Sahbhagi seeds were surface disinfected by immersion in 70% ethanol for 1 min and were then washed three times with sterile distilled water. Bacteria cultures were suspended in sterile distilled water, and disinfected seeds were transferred into a test tube and allowed to suspend for 30–35 min. Inoculated seeds were then subjected to germination using germination papers and irrigated with ACC. The petri plates were incubated at 25 °C for up to 9 days (Nandakumar et al. 2001). The per cent germination was calculated by the following formula. Control seeds without bacterial cultures were used for comparison.

$$\text{Seed Germination}\% = \frac{\text{No.of germinated seeds}}{\text{No.of total seeds}} \times 100$$

8.7 Root and Shoot Elongation

E. cloacae (KP226569) was subjected to inoculation of plants under the in vitro condition, in order to evaluate their influence on root and shoot elongation, which was measured at an interval of 3 days up to 9 days.

8.8 Results

8.8.1 Production of Plant Growth-Promoting (PGP) Traits

The PGP traits in *E. cloacae* are given in Table 8.1. The organism was positive for multiple PGP (MPGP) traits such as NH_3, HCN, ACCD and catalase, and the organism showed the ability of PS. SD and chitinase were not detected in the organism.

Table 8.1 Plant growth-promoting traits in *E. cloacae*

PGP traits	Positive/negative
ACC deaminase	**+++**
NH_3	+
HCN	+
SD	–
IAA(μg/ml)	9.0
PS	+
Catalase	+
Chitinase	–
Tolerance to salt %	20
Tolerance to pH	5–9

+ Positive, – negative

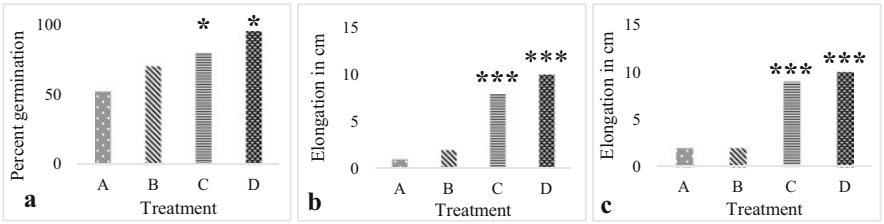

Fig. 8.1 Inoculation effect of *E. cloacae* on seed germination and growth parameters of rice var. Sahbhagi in the presence of ammonium sulphate as substitute of ACC. (**a**) Percent of seed germination, (**b**) elongation of root, (**c**) elongation of shoot. A = control, B = seeds treated with ammonium sulphate, C = seed inoculated with *E. cloacae*, D = seed treated with ammonium sulphate and inoculated with *E. cloacae*.*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

8.8.2 Seed Germination

*E. cloacae (*KP226569) induced a higher percentage (80%) of germination of seed as compared to untreated seeds (52%). Further enhancement (95%) of seed germination was observed in the presence of ammonium sulphate as a substitute of ACC compared to untreated. (Fig. 8.1a).

8.8.3 Root Elongation and Shoot Elongation

Highly significant ($p < 0.001$) enhancement was observed in elongation of the root of rice var. Sahbhagi by inoculation by *E. cloacae (*KP226569) in the presence of ammonium sulphate as a substitute of ACC compared to untreated (Fig.8.1b).

Similarly, *E. cloacae (*KP226569) induced highly significant ($p < 0.001$) shoot elongation in the presence of ammonium sulphate as a substitute of ACC compared to untreated. (Fig. 8.1c).

8.9 Discussion

E. cloacae showed multiple plant growth-promoting traits and additional traits such as tolerance to the high concentration of salt (NaCl) and pH. The organism used in the present study showed significantly improved seed germination and root and shoot elongation in the inoculated rice var. Sahbhagi. Similar observations were also noted by several workers (Belimov et al. 2007; El-Tarabily 2008; Qin et al. 2014; Han et al. 2015). Importantly the organism which was positive for ACC deaminase is a key indicator activity of PGPR (Glick 2014).

ACCD positive bacteria are known to resist the inhibitory effects of stress (Burd et al. 2000; Grichko and Glick 2001). PGPR attached to the surface of plant roots or seeds and may take up some of the ACC exuded by the plant and degrade it by the action of ACCD (Glick et al. 1998). ACCD enzyme also alleviates other

environmental stressors like flooding, heat, drought, metal contamination, organic pollutants, pathogens and insect infections (Glick et al. 2007; Tsavkelova et al. 2007) and also favours the K+/Na+ ratio and stimulates the production of osmolytes under stress conditions (Singh and Jha 2016). Sahbhagi is a known drought-tolerant rice variety; additionally, we observed its tolerance to salt by inoculation with *E. cloacae* (Unpublished data). This may yield an additional profit to farmers by cultivation of Sahbhagi as this variety is known for high straw yield as a good source of animal feed.

8.10 Conclusion

E. cloacae tolerant to salt (20%) and pH (5–9) bearing MPGP traits induced significant seed germination and elongation of root and shoot in rice var. Sahbhagi in the presence of ammonium sulphate as a substitute of ACC. The potential of this organism could be further explored for enhancement of rice production in soil with high concentration of salt and extreme pH which will be the developmental strategies for ecosystem friendly and sustainable agriculture.

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9 Impact of Biofertilizer on Crop Yield of Isabgol (*Plantago ovata*) and Senna (*Cassia alexandrina*)

Sangeeta Singh, Kamal Joshi, Sunil Choudhary, Rakesh Nagar, Bindu Nirwan, Neha Sharma, Kuldeep Sharma, Shiwani Bhatnagar, Diksha Bhola, and Ajit Varma

Contents

Abstract

Isabgol (*Plantago ovata* Forssk.) is an important cash crop in western part of Rajasthan during Rabi season, making India top ranked in its production. It is widely used in Ayurveda due to its laxative property. Senna (*Cassia alexandrina* Mill.) is perennial undershrubs whose leaves and seed both have medicinal importance. Due to the low level of soil health, inadequate agricultural input, the uncertainty of rain, and no other source of irrigation in Rajasthan, the introduction of biofertilizers to improve the productivity of both the medicinal crops was studied. The biofertilizers used were *Serendipita indica* a culturable arbuscular mycorrhiza, which is able to increase biomass and yield of crop plants and to induce local and systemic resistance to fungal diseases and tolerance to abiotic stress, and *Azotobacter* sp. a free-living nitrogen fixer, individually as well as in combination. The results showed that *S. indica* performed better than all the other treatments. The mean yield in Isabgol seed and husk, respectively, increased to 57% and 33% in *S. indica*-treated seeds followed by 36% and 14% in consortia of the fungus and bacterium and 23% and 4% in *Azotobacter* sp.-treated seeds as compared to control. Similarly, mean yield of Senna seeds was maximum in

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S. indica-treated seeds (39.69 g), followed by consortia (20.04 g) and *Azotobacter* (19.16 g) as compared to control (16.30 g).

Keywords
Cassia alexandrina · *Plantago ovata* · *Serendipita indica*

9.1 Introduction

Isabgol is an annual irrigated crop of rabi season which takes 4 months in the field to complete its cycle. India is the largest producer as well as exporter of Isabgol in the world (Jat et al. 2015). The main Isabgol-producing states in India are Gujarat and Rajasthan. Similarly, Senna is a valuable plant drug and used in Ayurveda for the treatment of constipation (Morales et al. 2009; Seethapathy et al. 2015). Due to the excessive use of chemical fertilizers and pesticides/fungicides, there has been the acute demand for sustainable production of crops by using organic methods. The soil health has been deteriorated resulting in low productivity of agricultural and horticultural plants, and now it is getting difficult to produce crops without using chemicals, which is beyond the reach of poor and marginal farmers of rural areas. Moreover, there is the harmful impact of synthetic chemicals. Such kind of problems can be overcome by using plant growth-promoting microorganism. These microbes are attaining importance worldwide. The beneficial microbes not only play an important role in increasing soil fertility but they also enhance growth and vigor of the plant and help in the value addition and productivity of economically important plants. Moreover, they also protect them from other harmful microbes. The production of plant growth hormones has been suggested as one of the mechanisms by which these biofertilizers stimulate growth. There are many species of fungus and bacteria which makes the nutrient in the soil available to plants. Arbuscular mycorrhiza colonizes the root of the diverse plants and extends the hyphae far away into the soil. Their main role is to mobilize phosphorus to the plant. *S. indica* is also one of the AM fungi which primarily supplies phosphate to the plant and also produces novel new plant growth hormone PYK-10. Similarly, the nitrogen supply is maintained by free-living nitrogen-fixing bacteria like *Azotobacter*, *Azospirillum*, and symbiotic nitrogen fixers like *Rhizobium*. The main aim is to develop a consortium of these microbes which are compatible and can help in rejuvenating the soil also along with providing nutrients to the plants. In this study, the effect of *S. indica* alone and in combination with *Azotobacter* was studied for Senna and Isabgol so that a package of practice for these species could be developed.

9.2 Material and Methods

9.2.1 Preparation of Liquid Cultures

The mass cultivation of *S.indica* was carried out in batch culture in shaking flasks. Special focus was given to minimize the expensive nutrients and to grow them exclusively on composite energy source Jaggery. The focus was on the excessive production of chlamydospores for better formulation, storage, and transfer to the field at room temperature compatible with *P. indica* nitrogen-fixing *Azotobacter* sp. which was also grown in batch culture. The CFU count of the bacteria was optimized to 10^9 mL^{-1}.

9.2.2 Preparation of Field Beds

A total of 12 beds having the size of 400 square meters were first flooded, and then the next day, the same was dug to soften the soil texture for easy seed germination. These fields were then labeled according to the treatment with three replications for each treatment.

9.2.3 Seed Treatment

Seeds of Isabgol and Senna were presoaked overnight in the batch cultures prepared as mentioned above. The viable seeds of these plants were treated separately with the liquid cultures of *S. indica*, *Azotobacter* sp., and their combination. In addition to this, some seeds of both plants were soaked only in-plane distilled water without any bioagent that was used as a control.

9.2.4 Seed Sowing and Experimental Design

The experimental design adopted in the present investigation was a randomized block design (RBD) with three replications for each treatment (four treatments). Therefore, the beds prepared above were distributed randomly for a different combination of treatment. The presoaked seeds that seem to be fully imbibed were then broadcasted in the beds as their respective treatment after which these were covered with a small layer of soil. These field beds were then left for 5–6 days without water for the proper germination of seedlings.

9.2.5 Maintenance of Beds and Data Collection

Once the seeds were germinated properly, the plots were watered periodically. Thinning was performed when the seedlings got an adequate length. Row-to-row

and plant-to-plant spacing for Senna was kept 45 × 30 cm, while for Isabgol, it was 30 × 5 cm. The crop was maintained for nearly 4 months, while for Senna, the duration was 6 months; thereafter, the yield data was recorded. The standard error of means and coefficient of variance was calculated for yield data as described by Gupta et al. (2001).

9.2.6 Results

In the present investigation, the mean yield in Isabgol (*Plantago ovata*) seed and husk, respectively, increased to 57% and 33% g/plant in *S. indica*-treated seeds followed by 36% and 14% in consortia of the fungus and bacterium and 23% and 4% in *Azotobacter*-treated seeds as compared to control. Similarly, mean yield of Senna seeds was maximum in *S. indica* -treated seeds (39.69 g), followed by consortia (20.04 g) and *Azotobacter* (19.16 g) as compared to control (16.30 g) (Fig. 9.1).

9.2.7 Discussion

Fertilizers are known to play an essential role in many crop productions in agricultural systems (Singh et al., 2012, but scientific research is little about the interactive effects of various fertilizers on yield and seed capabilities of medicinal plants. The effects of mycorrhizal fungi *S. indica* and free-living nitrogen fixer *Azotobacter* individually and in combination on yield component and seed capabilities of Isabgol (*Plantago ovate*) and Senna (*Cassia alexandrina* Mill.) were studied in the present investigation. The application of fertilizers is one of the primary methods for improving the availability of soil nutrients to plants. Fertilizing can change rates of plant growth, maturity time, size of plant parts, the phytochemical content of plants (Mevi-Schütz et al. 2003), and seed capabilities. The heavy use of chemical fertilizers has created a variety of economic, environmental, ecological, and social problems. Furthermore, the increasing costs of chemical inputs have left farmers helpless, resulting to decreasing seed quality of certain crops and resulting in the fall of commodity prices and consequently reducing farm income (Khadem et al. 2010; Tung and Fernandez 2007). In such situation, the present study can be proved as a milestone because in this investigation not only the global threat of chemical fertilizer use was prevented but also the methods used for the application of biofertilizers were also farmer friendly (cheap). The bioagents used in the study not only provide important soil nutrients such as nitrogen and phosphorous; additionally, using it as fertilizer can be an important disposal method, which was also suggested by many previous studies (Taheri et al. 2011; Madison et al. 1995). It was observed that *S. indica* has performed better for Senna as well as Isabgol in all the treatments. The germination percent was 98%; moreover, the flowering was also recorded 15 days earlier to the control in the case of *S. indica*-treated seeds of Senna and Isabgol. The fungus is able to increase biomass and yield of crop plants and to induce local and systemic resistance to fungal diseases, and tolerance to abiotic

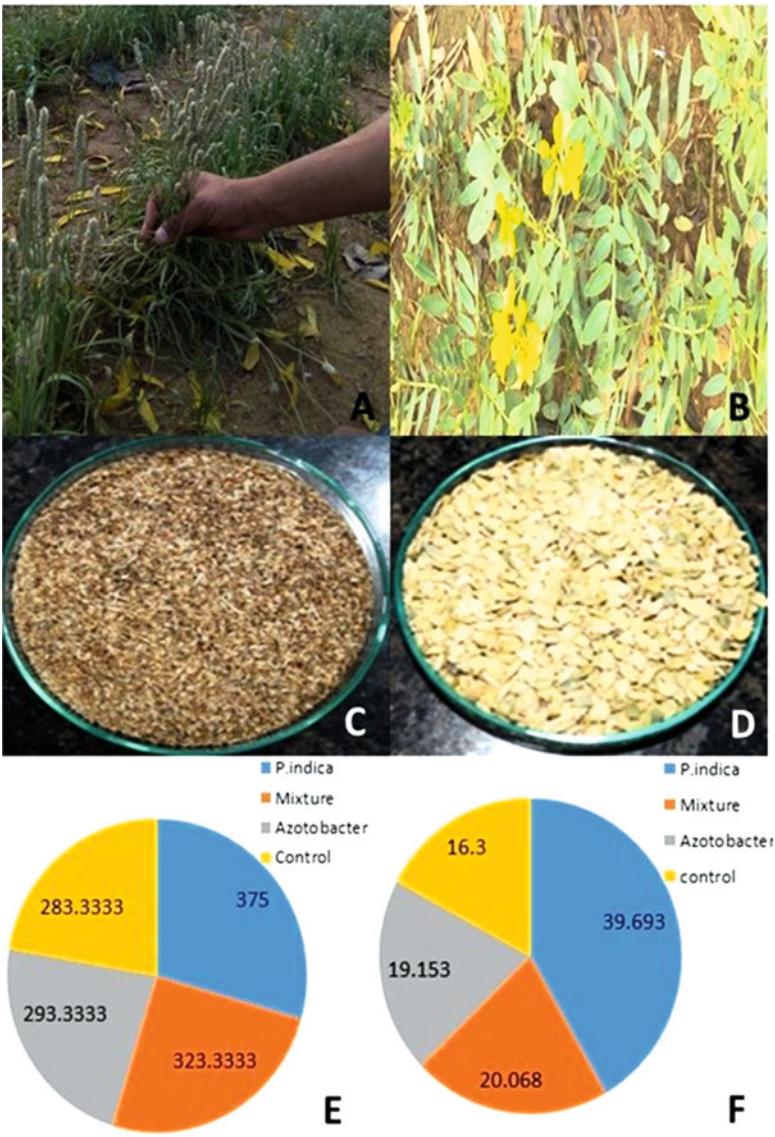

Fig. 9.1 (**a**) Isabgol tillers, (**b**) flowering of Senna, (**c**) seeds of Senna, (**d**) flowering of Senna, (**e**) pie-chart showing yield data (gm/bed) of Senna, (**f**) Isabgol crop after biofertilizer treatment

stress has been suggested by many workers (Stein et al. 2008; Varma et al. 2012). The dual inoculation of AM fungi and *Azotobacter* was found to be the best in the reduction of cadmium stress and promotion of growth parameters (Haneef et al. 2014). Similarly increased overall health of Isabgol plant was observed in light of nitrogen-based fertilizers (Maheshwari et al. 2000).

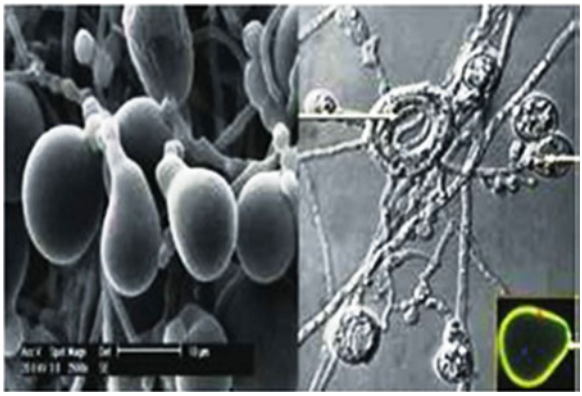

Fig. 9.2 A view of the morphology of the fungus indicating typical autofluorescent pear-shaped spores. A view of the SEM of the spore. (C.F. Varma et al. 2013)

In the present investigation, seeds treated with *S. indica* sowed the maximum yield in both the Isabgol and Senna followed by the treatment of consortium (*S. indica* + *Azotobacter*) and the seeds coated with the formulation of *Azotobacter* strains only. The result suggests that *S. indica* can be alone used as potential biofertilizer for increasing yield and productivity of crops. The fungus has shown strong growth promotion activity with a broad range of plants tested so far (Waller et al. 2005). Its advantage lies in its ease of cultivation on synthetic media. Due to ease of culture as compared to other AM fungi, this fungus could be used as a model organism for the study of beneficial plant-microbe interactions and a new tool for improving plant production systems (Varma et al. 1999). An overall view of Serendipita indica (*S. indica*), commercially called as "ROOTONIC," is given in Fig. 9.2.

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Evaluation of Mixtures of Beneficial Microorganisms on *Brassica chinensis L.*

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Tarzan Sembiring and Lies Sriwuryandari

Contents

Abstract

Brassicaceae (the mustard family, previously Cruciferae or the crucifers) include many foods, forage, ornamental, and weed plants. Organic production of brassica crops, or any commodity, relies on management techniques that replenish and maintain long-term soil fertility by optimizing the soil's biological activity. This is achieved through crop rotation, cover cropping, and composting and by using organically accepted fertilizer products that feed the soil while providing plants with nutrients. Excessive use of chemical fertilizers, pesticides, and herbicides in uncontrolled amounts and methods will damage the land farm, pollute the environment, and contaminate the food due to their residues. The use of aromatic-degrading bacteria *Alcaligenes* sp., *Pseudomonas* sp. in combination with *Azospirillum* sp., *Azotobacter* sp., *Bacillus subtilis*, *Actinomycetes*, and *Lactobacillus* sp. will remediate the soil farm. In this study, we have evaluated the mixtures of these beneficial microorganisms on *Brassica chinensis L.* due to their previous efficacies on various cropping systems. The experiment was a

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randomized complete block design with nine treatments and three replications per treatment. Our results showed that the mixtures of those microorganisms showed beneficial effects and could be implemented as biofertilizers for the production of *Brassica* spp. in the farmlands in Indonesia.

Keywords
Pesticide residue · Biofertilizer · Evaluation · Plant supplement

10.1 Introduction

Aromatics compounds are used as herbicides or pesticides in farmland usually in the form of chlorinated, carboxylic acids of mono-, di-, tri-, or polyaromatic substances (Sembiring 1993). It was reported that some triazine is also primarily used to control broadleaf weeds (US EPA 1999) such as ametrine, atrazine, cyanazine, prometryne, propazine, and simazine. Those substances are harmful and persistence. The use of such substances polluted the farmland and water body. Some of them would also contaminate the farm product such as fruits and vegetables. Some bacteria such as *Pseudomonads* and *Alcaligenes* were able to degrade herbicides or pesticides (Sembiring 1993; Sembiring et al. 1996; Sembiring and Sriwuryandari 2000). Application of synthetic/inorganic fertilizer such as phosphates in the praxis of the farmers is usually introduced in excessive use with the purpose that it will enhance the productivity. This brings to the consequence of hardening the soil and the phosphate rest in the farmland would be binding to the soil and minerals matrix forming undissolved phosphate which latest to be abiogenic. This is one of the problems to be solved in farmland besides the excessive introducing of chemicals pesticide and herbicides.

Meanwhile, some microorganisms are known to be able to carry out fixation of nitrogen from the air and deliver it to the plants in the rhizosphere zones. *Azospirillum* sp. and *Azotobacter* sp. are found in the rhizosphere of plants. These bacteria are nonsymbiotic nitrogen fixers. Therefore, it would be applicable to the wide range of plants. Some other bacteria are able to solubilize phosphates from rock phosphate (Antoun and Kloepper 2001; Suliasih and Sriwidawati 2005) such as *Pseudomonads*, *Bacillus* sp., and *Lactobacillus* sp. *Bacillus* sp. such as *Bacillus subtilis* produces antibiotics as a secondary metabolite, which is useful to suppress the plant pathogens (Sembiring and Fachmiasari 2004; Glick 2012). On the other hand, *Actinomycetes* sp. is able to degrade organic compound and produce substances which promote the growth of the plants.

Combination of some aromatic-degrading bacteria such as pseudomonads and *Alcaligenes* with nitrogen fixers, antibiotic producer, and organic material decomposers would be useful to help in remediation and fertilization of farmland. Before introducing to the farmland, the effectiveness of the mixed culture as biofertilizer should be tested and evaluated.

10.2 Materials and Methods

10.2.1 Biofertilizer Formulation and Preparation

Biofertilizer was formulated by mixed of some microorganisms with biomass extracts obtained from fruits and vegetables. The microorganisms were nitrogen fixer bacteria *Azospirillum* sp. and *Azotobacter* sp., phosphorus diluter *Bacillus subtilis*, and phenolic- and aromatic-degrading *Pseudomonas fluorescens* and *Alcaligenes* sp., lactate producer of *Lactobacillus* sp. and hetero-biopolymer degraders *Actinomycetes* sp. The microorganisms were enriched in the media containing soy flour (1 gL^{-1}), tapioca (1 gL^{-1}), sugar (1 gL^{-1}), and fruits or plant extracts. The broth was prepared by diluting the components in hot and sterile cold water. The enrichment was at ambient temperature with aeration to introduce oxygen for the growth of the cultures. Formation of alcohol odors indicated as the end of enrichment which was around 10–12 days. Microorganism population of each species were determined by selective methods. It was found that the mixture contains *Azotobacter* sp. ($10.^7$ CFU/ml), *Azospirillum* sp. ($10.^7$ $CFUmL^{-1}$), *Lactobacillus* sp. ($10.^7$ CFU mL^{-1}), actinomycetes ($10.^7$CFU/ml), *Pseudomonas fluorescence* ($10.^{11}$ CFU mL^{-1}), *Bacillus subtilis* ($10.^7$ CFU mL^{-1}), and *Alcaligenes* sp. ($10.^7$ $CFUmL^{-1}$).

10.2.2 Experimental Design on the Evaluation of Biofertilizer Efficacy

Efficacy of the biofertilizer evaluated using a randomized block design with nine treatments and three (3) replications (27 blocks of experiments). Each block is 1 m × 2 m or 2 m × 2m. The response of plants to biofertilizer was by measuring the plant's height, amount of leaf, the diameter of the crown, and the yields. Nutrients using the plant's, namely, nitrogen, phosphorus, and potassium absorbed were determined using standard methods. N, P, and K of soil were also determined at the end of the experiment. Analysis of the plant response was determined on the average of the treatments using *F* test at (5 %) the difference of the average followed by Duncan's multiple range test (5 %.)

The experiment was using *Brassica chinensis L.* as a test plant. The experiments were nine treatments in three (3) replications prepared due to the standard methods. The *Brassica chinensis L.* seeds were sowed near the experiment site, and the plant seedlings were planted in the blocks at 12 days old. Planting area was fertilized with 2 kg of compost of cow and goat dungs (1:1) which was finely milled. The compost dungs were mixed with phosphorus and potassium fertilizers according to the treatments and spread on the soil surface in each of block. Plants planted in each block were 50 seedlings with 20 cm × 20 cm of distance. Urea was used at 7 and 14 days after planting and diluted prior to spraying. Biofertilizer was also sprayed on the surface of the plants and the soil around the plants. Biofertilizer was introduced

Table 10.1 Evaluation of the biofertilizer efficacy

Treatments	Biofertilizer (L/ha)	Dungs (ton/ha)	Urea (kg/ha)	SP-36	KCl
A: Control	–	10	–	–	–
B: 0 BF + NPK	–	10	100	100	75
C: 1 BF + 0 NPK	9	10	–	–	–
D: 1 BF + ¼ NPK	9	10	25	25	18.75
E: 1 BF + ½ NPK	9	10	50	50	37.50
F: 1 BF + ¾ NPK	9	10	75	75	75
G: 1/2 BF + NPK	4.5	10	100	100	75
H: 1 BF + NPK	9	10	100	100	75
I: 1½ BF + NPK	13.5	10	100	100	75

*Anonymous (2010)

to the plants at 7 days until 24 days after planting with an interval of 3 days or approximately six times due to the treatments (Table 10.1)

Evaluation of the biofertilizer effectiveness was by observation and determining of the amount and quality of plant's height, the amounts of leaf, the diameters of the crown, the diameter of the stem at the harvesting day, and the yields of harvesting. Soil fertility was evaluated by the determination of soil parameter before planting and after harvesting of the plants.

10.3 Results and Discussion

Brassica chinensis was grown very well. Each block of the experiment was controlled every day to see if there are any trouble due to the animals or insect and pathogen infections. Figure 10.1a. showed the seedling growth at 10th day after planting. Observation on the plant pathogen showed that the vegetables are free from any infections such as blight or others. The greenish plant could be seen at the harvesting day (day 28th) as seen in Fig. 10.1b.

Evaluation of plant growth height could be seen in Table 10.2. It showed the plant's height starting from 14 days after planting to 28 days when the *Brassica chinensis L.* was harvested. It could be seen that the experiments G, H, and I are better than the other treatments in terms of plant's height, but the difference is not a significant one to the other if evaluated by statistic methods followed with Duncan's multiple range test.

Observation of leaf amounts during the experiment showed (Table 10.3) that the development of leaf is similar to the development of plant's height for each treatment. It was found that the leaf amounts of treatments G, H, and I were better than other treatments. In this case, the treatment H where nutrition supplied with biofertilizer (9 L ha^{-1}), compost dung (10 ton/ha), and N (urea), P (SP-36), and K (KCl) in amount of 100 kg ha^{-1}, 100 kg ha^{-1}, and 75 kg ha^{-1}, respectively, was the highest amount on the leaf production. Utilizing the same amount of compost dung,

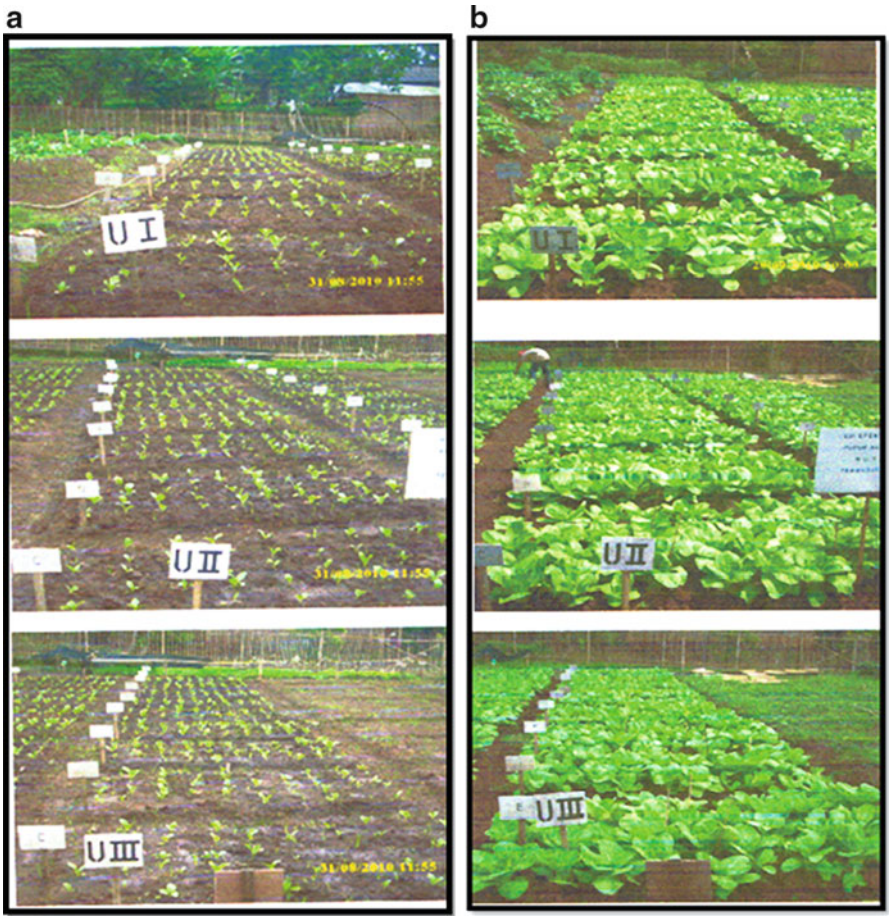

Fig. 10.1 *Brassica chinensis* cultivation due to the experiments (**a**) 10 days after planting, (**b**) 28 days after planting (harvesting day)

Table 10.2 The development of plant's height (*Brassica chinensis L.*) during the experiments

	Measurement (days) after planting (cm)		
Treatments	14 day	21 day	28 (harvesting)
A	15.99	20.61	24.67
B	18.75	24.28	27.67
C	16.98	22.22	24.94
D	16.68	22.61	25.30
E	17.15	23.16	26.00
F	18.07	23.28	26.44
G	18.26	23.61	27.94
H	19.60	25.45	29.04
I	19.19	25.22	28.44

*Anonymous (2010)

Table 10.3 Amount of leaf of *Brassica chinensis L.* during the experiments

Treatments	Measurement (days) after planting		
	14 day	21 day	28 (harvesting)
A	6.00	10.66	12.00
B	7.78	12.33	13.67
C	6.44	11.00	12.33
D	6.67	11.33	13.00
E	7.22	11.78	13.11
F	7.56	11.89	13.22
G	7.78	12.00	13.78
H	8.67	13.44	14.78
I	8.00	13.22	14.11

*Anonymous (2010)

Table 10.4 Development of crown diameter of *Brassica chinensis L.*

Treatments	Measurement (days) after planting (cm)		
	14 day	21 day	28 (harvesting)
A	19.81	27.33	29.87
B	25.58	31.61	34.20
C	20.86	29.11	30.97
D	21.77	29.89	31.77
E	22.54	30.45	32.57
F	23.03	30.89	33.44
G	23.99	31.22	33.87
H	26.01	33.56	36.08
I	25.04	32.55	35.44

*Anonymous (2010)

N (urea), P (SP-36), and K (KCl) in the omitted biofertilizer (Tables 10.1 and 10.3) produced less amount of leaf although the difference is not much.

However, there are difference, and it should be due to the influence of the biofertilizer. In the application of compost dung combined with biofertilizer (treatment *C*) compare to a compost dung (treatment A) for nutrition sources was shown that the introduction of mixed microorganisms as biofertilizer useful in the development of plant's height and leaf produce (Tables 10.2 and 10.3). Variation of chemical fertilizer applied in the experiment (treatments D, E, and F) showed that only small difference could be found (Tables 10.2 and 10.3) on the leaf production, but a contrast result would be detected in the crown diameter of the *Brassica chinensis L.* (Table 10.4.)

Evaluation of the biofertilizer influence on the development of stem diameter was shown in Table 10.5. The stem measurement was on the day of harvesting. It is shown that introducing biofertilizer to the plant has a positive impact on the development of the stem. Control of the treatment found to be the smallest one. The best result was shown by the treatment *H*. The treatment *H* was a treatment

Table 10.5 The diameter of the stem of *Brassica chinensis L.* at the harvesting day

Treatments	Diameter of sample (cm)			Diameter of stem average (cm)
	Replication I	Replication II	Replication III	
A	1.13	1.23	1.33	1.30a
B	1.37	1.50	1.73	1.53bc
C	1.33	1.27	1.37	1.32a
D	1.43	1.40	1.60	1.48b
E	1.47	1.40	1.60	1.49b
F	1.47	1.47	1.60	1.51bc
G	1.47	1.47	1.60	1.51bc
H	1.57	1.50	1.90	1.66c
I	1.50	1.50	1.87	1.62bc

*Anonymous (2010). The same notation is not significant based on the Duncan's multiple range test at 5%

using mixture of beneficial microorganisms or biofertilizer (9 L ha^{-1}), compost dung (10 ton ha^{-1}), N (urea), P (SP-36), and K (KCl) in amount of 100 kg ha^{-1}, 100 kg ha^{-1}, and 75 kg ha^{-1}, respectively.

The growth and quality of the plant are good, and there is a positive influence of biofertilizer observed compared to the control. The plants fertilized with biofertilizer of mixed bacteria showed that the leaf color was green, glossy, and fresh. The texture is crunchy, and the taste is slightly sweet better than the control which was green yellowish, gristly, and fibrous gristly. The leaves were smooth, and there was no leaf blight caused by phytopathogenic *Xanthomonas* (Ignatov et al. 2011). Healthy greenish plant was obtained because of the availability of siderophore excreted by microorganisms contained in the biofertilizer. Fluorescent pseudomonads are characterized by the production of yellow-green pigments termed pyoverdines which fluoresce under UV light and function as siderophores (Demange et al. 1987). Siderophores are an iron-binding extracellular compound with low molecular weight and high affinity for ferric iron that are secreted by microorganisms to take up iron from the environment (Hofte 1993). *Pseudomonas fluorescens* has a gene cluster that produces antibiotics including compounds such as 2, 4-diacetylphloroglucinol (DAPG), phenazine, pyrrolnitrin, pyoluteorin, and biosurfactant antibiotics (Velusamy et al. 2006). All of the growth-promoting substances such as indole acetic acid, gibberellic acid, and cytokinin excreted by *Azospirillum* sp. and *Azotobacter* sp. as well as metabolites from *Lactobacillus sp.*, *Bacillus subtilis* (Adesemoye et al. 2008), *Actinomycetes* sp. (Sneh 1981), and *Alcaligenes* sp. support the growth of the plant (Kumar et al. 2011; Sriwuryandari and Sembiring 2010; Glick 2012) fertilized with the mixed bacteria in the formula (Tables 10.6 and 10.7).

Tables 10.6 and 10.7. Shown that the treatment H with biofertilizer (9 L ha^{-1}), compost dung (10 ton ha^{-1}), and N, P, and K in the amount of 100 kg ha^{-1}, 100 kg ha^{-1}, and 75 kg ha^{-1}, respectively, was the best result compare to the treatment I where the using of more biofertilizer. It was observed that leaf of *Brassica chinensis L.* in treatment I attacked by caterpillar and the taste is better

Table 10.6 Measurement of the sample weight (g plant^{-1})

Treatments	Replication			Average
	I	II	III	
A	126.67	148.33	113.33	129.44a
B	196.67	255.00	203.33	219.33d
C	130.00	173.33	151.67	151.67b
D	166.67	185.00	173.33	175.00c
E	168.33	206.67	180.00	185.00c
F	216.67	223.33	188.33	209.44d
G	220.00	255.00	203.33	226.11de
H	260.00	298.33	240.00	266.11f
I	236.67	271.67	213.33	240.56e

*Anonymous (2010). The same notation is not significant based on the Duncan's multiple range test at 5%

Table 10.7 Overall yield of *Brassica chinensis L.*

Treatments	Replication			Average (kg/block)	Yield conversion (kg/ha)
	I	II	III		
A	3.60	5.20	4.60	4.47a	19,997.50
B	4.60	6.30	6.90	5.93cd	25,202.50
C	4.30	5.50	5.20	5.00ab	21,250.00
D	4.40	6.50	5.40	5.43bc	23,077.50
E	4.60	6.90	5.60	5.70bc	24,225.00
F	4.60	7.00	6.30	5.97cd	25,372.50
G	4.90	6.60	6.60	6.03cd	25,627.50
H	5.30	8.10	8.30	7.23e	30,727.50
I	5.10	7.90	7.20	6.73de	28,602.50

*Anonymous (2010). The same notation is not significant based on the Duncan's multiple range test at 5%

than *Brassica chinensis L.* obtained from treatment H. which is bring to the consequence of less of biomass. It might be the reason that the yield of treatment I with the addition of biofertilizer 13.5 L ha^{-1} is less than the yield of treatment H.

Evaluation of the soil fertility after harvesting the plants showed in Table 10.8. It could be seen that the acidity (pH) of the soil in all the treatments is almost similar as well as the carbon and nitrogen content's of the soil. There is no significant deviation of C-organic content of soil treated either with chemical fertilizer or in addition of biofertilizer. It may be influenced by the addition of compost dung in the same amounts. The content C organic initially (2.65%) has no significant difference with the C organic at the end of the experiment (2.31–2.41%). It is also the same for the nitrogen content, where there is no significant difference in the evaluation by Duncan's multiple range test although the biofertilizer contains nitrogen fixers *Azotobacter* sp. and *Azospirillum* sp. Presumably, the nitrogenfixer fixation is not happen, because of the high content of nitrogen originated from compost dung and the addition of urea during the experiment.

Table 10.8 Nutrition contents of soil at initial and after the harvesting

Treatment	pH	C- org (%)	N-total (%)	C/N	P_2O_5 (mg 100 g^{-1})	K_2O (mg 100 g^{-1})
Initial	5.70	2.65	0.20	13	21.22	13.88
A	5.72a	2.33a	0.25a	9.32	26.60ab	22.81a
B	5.72a	2.32a	0.25a	9.28	28.53bc	25.84b
C	5.71a	2.33a	0.30a	7.77	28.45bc	26.29bc
D	5.71a	2.41a	0.26a	9.27	29.42bc	27.37bc
E	5.74cd	2.35a	0.27a	8.70	29.93bc	27.57bc
F	5.75d	2.35a	0.23a	10.22	23.37a	27.51bc
G	5.73bc	2.32a	0.25a	9.28	31.14c	27.08bc
H	5.76d	2.31a	0.23a	10.04	26.26ab	28.01bc
I	5.74cd	2.33a	0.30a	7.77	29.52bc	28.33c

*Anonymous (2010). The same notation is not significant based on the Duncan's multiple range test at 5%

Although there is no addition of urea (treatment *C*) with 0.30 % N Total, there is no difference between the control (treatment *A*) where the N total was 0.25%. The addition of compost dung as a base organic fertilizer caused the suppressed nitrogen fixer ability of *Azotobacter* and *Azospirillum*. However, the mixed microorganisms as biofertilizer in the experiment supported the cultivation of *Brassica chinensis L.* The introduction of biofertilizer in the experiment showed that the yield was increased significantly (Tables 10.6 and 10.7). It was found that treatment *H* was the best one compared to the treatment *B*; the latest is the standard fertilization method in *Brassica chinensis L.* cultivation. Furthermore, if the seed were soaked in diluted liquid biofertilizer prior to sowing in the seedling preparation, the yields would be better

Alcaligenes sp. and *Pseudomonas fluorescens* are able to degrade aromatic and polycyclic compound which are the precursor of pesticides and herbicides (Sembiring 1993; Sembiring et al. 1996; Sembiring and Sriwuryandari 2000). The phenoxy carboxylic acids such as 2-4 D and 2,4,5 T known as the oldest herbicide were used frequently in the farmland. *Alcaligenes eutrophus* was known as bacteria containing plasmid which could degrade 2-4 D by transformation to the 2,4-DCP and later on mineralized to $CO_2 + H_2O$. In the experiment, those bacteria were used as the consortium with *Azotobacter sp*., *Azospirillum* sp., *Lactobacillus* sp., *Bacillus subtilis*, and *Actinomycetes* in the biofertilizer. It was also reported that fluorescent pseudomonads produce plant growth-promoting hormones and enzymes which suppress the growth of phytopathogenic fungi (Antoun and Kloepper 2001; Albert and Anderson 1987) and ACC (1-aminocyclopropane-1-carboxylic acid) deaminase from those pseudomonads enhances the tolerance of poppy (*Papaver somniferum* L.) plants against biotic stress of downy mildew caused by *Peronospora* sp. (Barnawal et al. 2017) and the saline resistance in groundnut plants (Saravanakumar and Samiyappan 2006). Application of biofertilizer or plant growth-promoting rhizobacteria in onion production found that *Pseudomonas fluorescens* strains were better at producing siderophores and solubilizing

phosphates, whereas *Bacillus subtilis* was the best producer of IAA (Čolo et al. 2014). This means that those beneficiary mixture bacteria could support the plant growth and would degrade persistence compound residue available in the soil farms.

10.4 Conclusion

Application of aromatic degraders *Alcaligenes* sp. and *Pseudomonas fluorescens* combine in consortium with *Azotobacter* sp., *Azospirillum sp.*, *Lactobacillus sp.*, *Bacillus subtilis*, and *Actinomycetes* as biofertilizers, to support plant growth and soil fertility and health facing the aromatic pesticide's is promising and fruitful for the obtaining of healthy vegetables, fruits, and foods. The contaminated landfarming caused by excessive aromatics in the agricultural fields could be remediated simultaneously. As shown in the experiment, the mixture of those beneficial microorganisms as biofertilizer on *Brassica chinensis L.* cultivation was succeeding and supports the growth and enhances the productivity of the plants.

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Characterization of Sugarcane Mosaic Disease and Its Management with PGPR

11

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Contents

Abstract

Sugarcane mosaic caused by sugarcane mosaic virus (SCMV) is a serious problem in India's sugarcane production. SCMV was first reported in India from Pusa during 1921 in sugarcane variety D-99, and now it has been reported in every sugarcane-growing areas across India due to its perpetuation through vegetative cuttings and regarded it as a potential threat to sugarcane industry. Our present study was focused on the characterization of SCMV and use of PGPR strains to manage the disease. A survey was undertaken in sugarcane-grown areas of Andhra Pradesh, India, and found the sugarcane aphid (*Melanaphis sacchari*) and corn leaf aphid (*Rhopalosiphum maidis*) as potential vectors for ScMV. Vector transmission was confirmed using DAC-ELISA. Further, the ScMV was detected in diseased leaves through DAC-ELISA and RT-PCR during our survey. Scanning electron microscopy (SEM) was also used to detect ScMV from

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diseased leaf samples. The results showed that all the leaf samples collected showed a positive reaction to the presence of ScMV in RT-PCR with a band at around 0.98 kbp. Further, asymptomatic leaves also have shown a positive reaction with RT-PCR for the presence of ScMV. Whereas, SEM studies showed the presence of potyvirus filamentous particles related to ScMV. To manage ScMV, several PGPR strains were isolated from the rhizosphere of sugarcane samples collected during our surveys using standard isolation methods. Studies are being undertaken to identify these strains and further to evaluate their efficacy against ScMV in sugarcane under in vitro and in vivo conditions.

Keywords
Sugarcane mosaic virus (SCMV) · Biocontrol · PGPR · *Melanaphis sacchari* · *Rhopalosiphum maidis* · vectors

11.1 Introduction

Sugarcane is a preeminent commercial crop of India and is grown in tropical and subtropical regions. The crop is cultivated to a tune of 4.5 M ha in India with an annual production of 27.2 MMT (India Sugar Annual 2016). Sugarcane productivity is hampered by several biotic stresses of which plant diseases cause a major havoc. Approximately, 55 diseases of sugarcane caused by fungi, bacteria, viruses, phytoplasmas, and nematodes have been reported in India (Rao et al. 2002). Several disease epidemics in sugarcane have also been reported in India like red rot, smut, wilt, rust, leaf scald, and viral diseases (Viswanathan and Rao 2011). The extent of damage of sugarcane disease epidemics would depend on the nature of the disease and its spread on the affected varieties. Despite continuous efforts in the areas of breeding for disease resistance, sugarcane is subjected to economic losses due to the incidence of plant diseases. Sugarcane mosaic disease caused by sugarcane mosaic virus (ScMV) is hitherto a minor disease and is now considered a major one especially in states like Andhra Pradesh (Raja Kumar et al. 2016a).

Sugarcane mosaic virus was first reported in India from Pusa, in 1921, on sugarcane variety D 99 and Sathi 131, an indigenous cane of Bihar (Dastur 1923). Since then, virus isolates causing mosaic disease on sugarcane in India continue to be a potential threat to sugarcane industry (Bhargava 1975) as it is a very common disease because of the virus perpetuation through vegetative cuttings (Rao et al. 1995). In India, sugarcane mosaic disease of late is causing significant yield losses (Raja Kumar et al. 2016b). Earlier, in India, sugarcane mosaic disease was perceived to be caused by different strains of ScMV (Rao et al. 2002). However, researchers have later established new strains of mosaic disease in sugarcane in India. For example, Hema et al. (2003) reported the sugarcane streak mosaic virus (ScSMV) as the new casual virus of the mosaic disease in tropical India. This confirms that the mosaic disease on sugarcane in India is not only caused by the strains of ScMV subgroup but also by the newly described ScSMV. Both ScMV and ScSMV have been studied more extensively in India. Research results have indicated that ScSMV

is the most widely spread and major cause of mosaic disease complex in India on sugarcane (Viswanathan et al. 2008; Raja Kumar et al. 2016a). However, both the SCMV and SCSMV cause significant yield losses in promising cultivars of sugarcane throughout India (Viswanathan and Balamuralikrishnan 2005). Mixed infection of SCMV and SCSMV was also recorded on commercial crops of sugarcane all over India (Viswanathan et al. 2007).

Management of viral diseases in crop plants is an uphill task especially when the diseases assume an epidemic form. For timely control of viral diseases, and to reap a satisfactory harvest, understanding the disease progression over years is mandatory. For this, a comprehensive survey in determining the prevalence of sugarcane mosaic disease, the extent of losses it causes, and farmers' practices in overcoming the disease is essential. Sugarcane, in Coastal Andhra Pradesh, has grown approximately to a tune of 65,000 ha, and viral diseases such as mosaic and yellow leaf disease cause sizeable losses. Further, cane and jaggery quality is also deteriorated with the incidence of these viral diseases (Viswanathan and Rao 2011). Sugarcane mosaic disease incidence in Andhra Pradesh, India, is also assuming a major biotic stress, and several popularly grown cultivars are showing increased susceptibility over the years. It is precisely at this juncture, understanding the exact susceptibility of ruling cultivars in a particular area over time to mosaic disease is essential. This is very important since it facilitates in advocating effective management strategies to sugarcane farmers for timely interventions.

Our present research, therefore, attempted to understand the prevalence of sugarcane mosaic disease, determining the risk and sensitive areas in Coastal Andhra Pradesh since 2010. Further, comprehensive understanding on varietal susceptibility, vector transmission, serological studies, and ultrastructural studies using scanning electron microscopy for confirming viral diseases in Coastal Andhra Pradesh was carried out. Our long-term goal is to manage sugarcane mosaic disease through healthy seed material.

11.2 Material and Methods

11.2.1 Survey for the Incidence of Sugarcane Mosaic Disease and Identification of Hot Spot Areas

A survey was undertaken in Coastal Andhra Pradesh in selected districts such as Visakhapatnam, Vizianagaram, Srikakulam, and East Godavari districts from 2010–2011 to 2016–2017. Surveys were conducted thrice in a crop year in the selected districts. A total of ten mandals were selected in each district and three villages from each mandal. Mosaic incidence was recorded from ten selected plots in each village, and the data were pooled to arrive at a mean mosaic disease incidence. Same villages were visited every year, and proper care was ensured to visit the same farmers' fields every year from 2010–2011 to 2016–2017. The percent mosaic incidence was calculated, and the mandals were categorized as mosaic incidence percent as <10%, 11–16%, 17–23%, 24–37%, and 38–65% and above. Areas with

the mosaic incidence of 38–65% and above were categorized as high-risk and sensitive areas, and these areas were mapped using Global Positioning System duly recording the coordinates.

11.2.2 Cultivar Susceptibility to Sugarcane Mosaic Virus

In screening trials for the incidence of viral diseases at the Regional Agricultural Research Station, Anakapalle, the mean disease incidence (%) of sugarcane mosaic disease was enumerated based on visual observation, annually. The cultivars that were selected for the present study were 87A298, 2003 V46, and Co86032, and these cultivars are the popularly grown cultivars in Coastal Andhra Pradesh. Data on % mosaic incidence on these cultivars were recorded from 2010–2011 to 2016–2017.

11.3 Confirmation of Mosaic Disease Using Serological, Molecular, and Scanning Electron Microscopy

11.3.1 Serological Assays (DAC-ELISA)

Direct antigen coating enzyme-linked immunosorbent assay (DAC-ELISA) was carried out using the kit obtained from ScMV-specific antibodies obtained from Bioreba, Germany, by following the standard protocol as detailed herewith (Lockhart et al. 1992). The plates were first coated with coating antibody supplied with the kit (diluted with coating buffer in 1:10 dilution) at 100 µl per well, covered tightly, and incubated for 4 h at room temperature, i.e., 21–24 °C (RT) in a humid box. In the second step, wells are coated with 100 µl of the diseased leaf extract/aphid vectors, duly preparing the loading diagram along with positive and negative control. The plate was incubated at 4C overnight for the binding of the antigen on the plate walls in a humid box. The enzyme conjugate was prepared just before use by diluting 1000 times in conjugate buffer and coated to the wells at 100 µL each and incubated at RT for 2.5 h. In the final step, the PNP substrate was added to the wells 100 µl each and incubated at RT in dark for 30 to 60 minutes and observed for color development. After each step, the wells are emptied and washed thoroughly with PBST washing buffer for 4–6 times. Observations were taken visually and also photometrically at 405 nm using Thermo Fisher Scientific Multiscan-X, ELISA reader, and the readings are documented.

11.3.2 Molecular Studies (RT-PCR)

For RT-PCR, the protocols adopted by Chatenet et al. (2005) were used with slight modifications. Total RNA from sugarcane leaves showing mosaic symptoms by using standard protocols. Total RNA was eluted in a final volume of 40 µL of diethyl pyrocarbonate-treated (DEPC) water and stored at −20 °C. RT-PCR assays to detect

Table 11.1 Details of SCMV primer pairs used

Primer code	Primer sequence	Location	Expected amplicon size
SCMV-F3 (24 mer)	5′-TTT YCA CCA AGC TGG AA-3′	CP	0.98 kbp
SCMV-R3 (24 mer)	5′-AGC TGT GTG TCT GTC TGT ATT CTC-3′	CP	0.98 kbp

SCMV with primer pairs as detailed below in Table 11.1 were used according to the protocol suggested by Alegria et al. (2003). RT-PCR assays to amplify fragments specific to potyviruses of *Poaceae* with primer pair oligo 1 n-oligo2n were performed according to Marie-Jeanne et al. (2000). The RT-PCR program was 50 °C for 30 min, 95 °C for 15 min, 30 cycles at 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min with a final 72 °C extension for 5 min. A 10 μL aliquot of each amplified product was analyzed by electrophoresis through a 1.2% agarose gel.

11.3.3 Scanning Electron Microscopy (SEM)

Sugarcane mosaic virus-infected leaf material from sugarcane plants was collected from surveyed areas. Later, partially purified leaf extracts were prepared from 1 g of leaves according to the protocol described by Lockhart et al. (1992). Preparations were later used for observation by scanning electron microscopy using standard leaf-dip protocols.

11.4 Results

11.4.1 Survey for the Incidence of Sugarcane Mosaic Disease and Identification of Hotspot Areas

Our survey results indicated that the incidence of mosaic disease steadily increased over years (2010–2011 to 2016–2017) in the surveyed areas. The incidence was least during 2010–2011 (2%) and progressed steadily and reached a peak during 2016–2017 (41%). In general, the higher incidence of mosaic disease was observed since 2013–2014 (>20%) (Fig. 11.1). Higher incidence of sugarcane mosaic disease in Coastal AP is attributed to increased susceptibility of all cultivars.

Our survey results have also indicated that in the surveyed areas, mosaic disease incidence was least (11–16%) in Narsipatnam, Etikoppaka, and Devarapalli mandals (Visakhapatnam); Rajam, Salur, Jami, Ramabhadrapuram, Terlam, Bobbili, Merakamudi, Gajapathinagaram, Parvathipuram, and Nemalam mandals (Vizianagaram); and Sankili, Santhakaviti, and Mandasa mandals (Srikakulam). High-risk and sensitive areas (38–65%) in these districts include Munagapaka, Atchutapuram, Kasimkota, and Anakapalle mandals (Visakhapatnam) (Fig. 11.2).

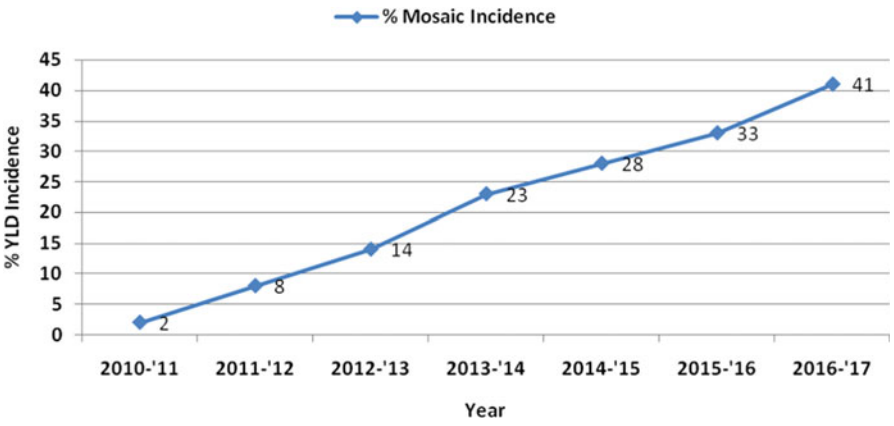

Fig. 11.1 The incidence of Mosaic disease on sugarcane in Coastal Andhra Pradesh, India, during 2010–2017

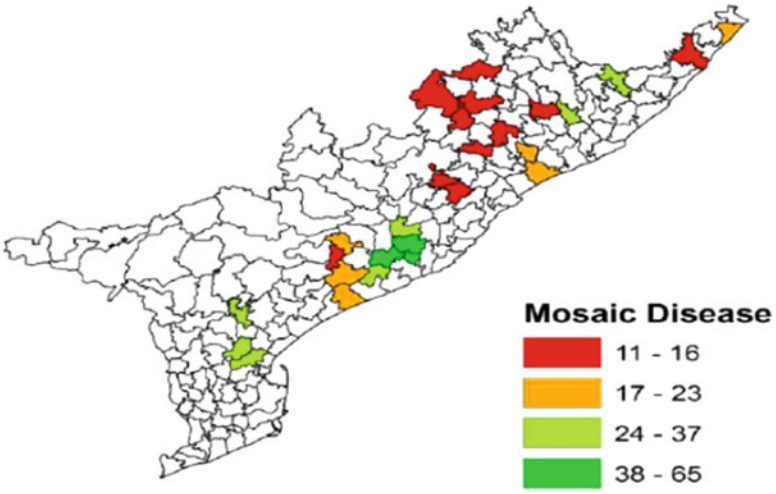

Fig. 11.2 Mean mosaic disease incidence on sugarcane in different mandals of Visakhapatnam, Vizianagaram, Srikakulam, and East Godavari districts of Andhra Pradesh, India, during 2010–2016

11.5 Cultivar Susceptibility to Sugarcane Mosaic Virus

Further, our studies at experimental fields of Regional Agricultural Research Station, Anakapalle, indicated that all the popularly grown sugarcane cultivars such as 87A298, 2003 V46, and Co86032 have shown increased susceptibility in general over the years from 2010–2011 to 2016–2017. As a slight exception to this, a marginal decrease in mosaic incidence was noticed on the cultivar, 87A298 in

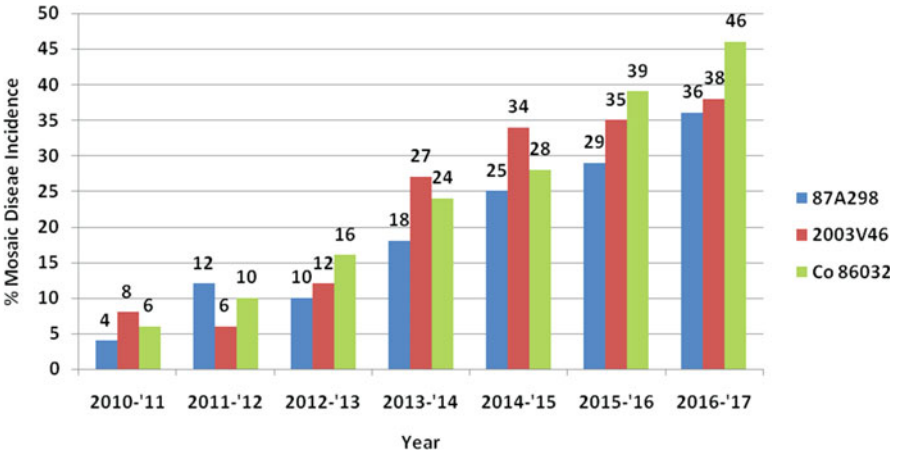

Fig. 11.3 Mean percent mosaic disease incidence in popularly grown sugarcane cultivars of Coastal Andhra Pradesh, India, during 2010 to 2017

2012–2013 (10%) when compared to during 2011–2012 (12%) (Fig. 11.3). The highest incidence of mosaic disease (36% in 87A298; 38% in 2003 V46; and 46% in Co86032) was recorded on all the three cultivars during 2016–2017. Overall, our results suggest that all the three sugarcane cultivars under study were found susceptible to mosaic disease over the due course (Fig. 11.3).

11.6 Confirmation of Mosaic Disease Using Serological, Molecular, and Scanning Electron Microscopy

11.6.1 Serological and Molecular Studies

Further, the ScMV was detected in diseased leaves through DAC-ELISA and RT-PCR.

The samples collected during the survey and the tissue culture seedlings were tested for the presence or absence of the virus using DAC- ELISA, and the absorbance values were recorded at OD 405 nm. In the case of positive reaction, the OD values ranged from 2.66 to 2.515, but in the case of negative reaction, the values are 0.254 to 0.212. Based on these results, the presence or absence of the virus was detected.

Two types of aphid samples were observed from the mosaic-infected sugarcane plants surveyed during the study period. The identification carried out by using aphid species identification keys. The two aphid species collected during the survey were identified as sugarcane aphid, *Melanaphis sacchari* (Zehntner), and corn leaf aphid, *Rhopalosiphum maidis* (Fitch).

11.6.2 RT- PCR

The samples collected during the survey were tested for the presence or absence of the virus using RT-PCR. Even though two of the samples didn't show any symptoms at field level, all the samples showed a positive reaction for the virus in RT-PCR test with the presence of a band at around 0.98kbp length confirming the presence of ScMV in all samples (Fig. 11.4).

11.6.3 Scanning Electron Microscopy

Scanning electron microscopy (SEM) results have indicated the SEM studies showed the presence of non-enveloped, flexuous filament characteristic of the *Potyviridae* family. The amount and size of particles varied among different diseased samples. The mean particle size was measured up to 800 nm length and 15 nm in width in SEM (Fig. 11.5).

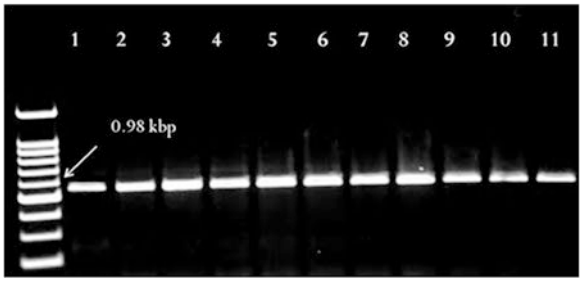

Fig. 11.4 Agarose gel 1.2% showing the RT-PCR amplification product obtained from using the sugarcane mosaic-specific primers (SCMV- F3 and SCMV- R3). Lane showing positive PCR amplification

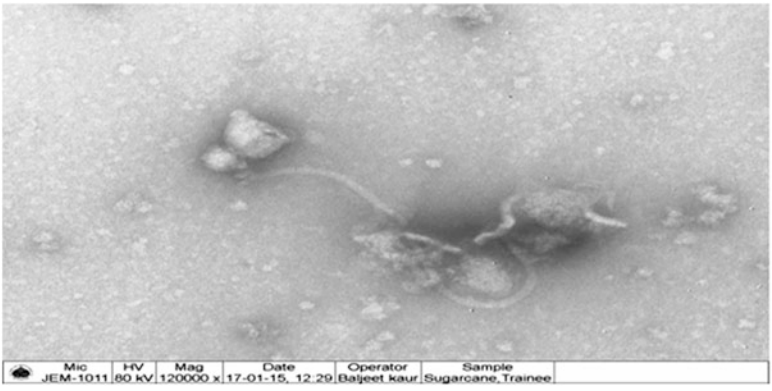

Fig. 11.5 Scanning Electron Microscopy studies of diseased sugarcane leaf samples infected with mosaic disease showing sugarcane mosaic virus (ScMV)

11.7 Discussion

Our studies indicated the prevalence of sugarcane mosaic disease in Coastal Andhra Pradesh. Further, popularly grown cultivars of Coastal Andhra Pradesh are being prone over time to mosaic, thus indicating the need to act swiftly in devising plant protection tactics comprehensively to this disease. In the surveyed districts, there was an increase in mosaic disease over time, and the hot spot areas in each of the surveyed districts are of concern (Fig. 11.2). The steady increase in mosaic disease from 2010–2011 to 2016–2017 over years (Fig. 11.3) is majorly attributed to poor vector management and rationing of the mosaic diseased crop. Earlier reports also established the relationship between high mosaic disease with the use of diseased seed material, mono-cropping, increased number of rationing, and poor vector management (Raja Kumar et al. 2016a). In particular, aphids play a significant role in the spread of virus diseases of sugarcane (Viswanathan and Balamuralikrishnan 2005) thereby causing huge economic losses (Singh et al. 2005). Proper care hence must be taken to educate the farmers on disease progression through various factors and on the ambient climatic conditions that prevail for taking up the prophylactic measures to overcome the same.

In our studies, all the popularly grown cultivars have shown susceptibility over time from 2010–2011 to 2016–2017 to mosaic incidence (Fig. 11.3). Increased susceptibility of CVs: 87A298, 2003 V46, and Co86032 over time to mosaic disease is also majorly attributed to an increased number of rationings, use of diseased seed material and poor vector management. Our vector transmission studies have established the presence of virus particles in aphids collected from diseased plants/fields. Previously, researchers have established that proper vector management in conjunction with other virus management strategies can significantly bring down mosaic and other viral disease incidences in sugarcane (Singh et al. 2005).

11.8 Conclusions

It is precisely at this juncture, the role of integrated disease management of viral diseases assumes significance.

Plant growth-promoting rhizobacteria (PGPR) have been used for sustainable management of several soil-borne diseases in different crops (Vijay Krishna Kumar et al. 2012). In sugarcane, PGPR such as *Pseudomonas fluorescens* has been used earlier to manage diseases and in promoting seedling vigor (Guru Prasad et al. 2012). However, no comprehensive information is available on the efficacy of PGPR in reducing disease levels induced by viruses in sugarcane. Our future studies are therefore directed to devise comprehensive integrated management strategies for viral diseases of sugarcane in general and mosaic disease in particular, through the application of antiviral compounds such as IAA and GA; PGPR proper and timely vector management, along with bringing up awareness to farmers on the precise use of healthy seed; avoiding mono-cropping; and more rationing.

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Effect of Different Doses of Pendimethalin on Microbial Activities and Nodulation in Chickpea

12

Ashok S. Jadhav, M. G. Patil, and R. K. Sonwane

Contents

Abstract

Field trial was conducted at research farm of weed science research center, Parbhani, in split plot design with an objective to study the effect of herbicides on biochemical activities and nodulation in chickpea (maize-chickpea cropping system) during 2012–2013.The soil of experimental plot was black cotton soil with medium fertility and pH 7.9, EC 0.32 dsm^{-1}, and OC 0.57%. The treatments comprised of four weed control measures (weedy check, mechanical weeding, weed free, and pendimethalin 1.0 kg ha^{-1}). The soil samples were collected from rhizospheric soil from each plot at 30, 50 DAS and at harvest of crop and were analyzed for soil microbial biomass carbon and basal soil respiration and number of nodules and nodule dry matter. The results revealed that there were nonsignificant variations observed due to various treatments in affecting soil physicochemical properties of soil at various crop growth stages. Highest microbial population, microbial biomass carbon, and basal soil respiration were recorded in mechanical weeding, whereas lower activities were recorded in pendimethalin applied plots at 1.0 kg ha^{-1} at 30 DAS. These values were enhanced at harvest.

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Application of pendimethalin1.0 kg ha^{-1} did not influence nodal count and nodal dry matter significantly.

Keywords
Pendimethalin · Effect · Physicochemical properties of soil · Soil microflora · Chickpea

12.1 Introduction

Soil microbial populations are immersed in a framework of interactions known to affect plant fitness and soil quality. They are involved in fundamental activities that ensure the stability and productivity of both agricultural systems and natural ecosystems. The soil samples were collected from the rhizospheric layer of plant from herbicide-treated plots; it is to be used for all the microbial and biochemical analysis. It is to be collected at three stages of crop growth, viz., maximum vegetative growth stage (30 DAS), flowering stage (60 DAS), and at harvest. The soil samples were analyzed for following observations: pH, EC and organic carbon, microbial biomass carbon, basal soil respiration, total number of "N" fixers, total number of "P" solubilizers, nodule numbers, nodule dry matter, and the percent root colonization by VAM fungi.

12.2 Materials and Methods

The field experiment was conducted on permanent herbicide trial in maize-chickpea cropping system during *kharif* season of 2011–2012 in split plot design replicated three times. This investigation was an attempt to check the effect of different pre and postemergence herbicide on soil physicochemical and biological properties in relation to chickpea rhizosphere (Table 12.1).

12.3 Method for Microbial Count

The soil samples were collected up to 15 cm depth and processed for microbial analysis following the dilution plate technique. The population of bacteria and fungi were determined by the standard pour plate technique using soil extract agar media for bacteria and Rose Bengal Agar media for fungi. Triplicate plates for each sample

Table 12.1 Treatments details

Treatments in 1st crop (maize)	Treatments in 2nd crop (chickpea)
T_1 = weedy check	T_1 = weedy check
T_2 = mechanical weeding	T_2 = mechanical weeding
T_3 = atrazine at 0.75 ha^{-1}	T_3 = pendimethalin at 1.0 kg ha^{-1} PE
T_4 = atrazine at 1.50 kg ha^{-1}	T_4 = pendimethalin at 0.75 kg ha^{-1} PE fb-mechanical weeding
T_5 = atrazine at 0.75 kg ha^{-1} fb-2,4-D at 0.5 kg ha^{-1}	

and microbial group were used for microbial population before sowing and after harvest.

12.4 Results and Discussion

12.4.1 Effect of Weed Control Treatments in Chickpea (*Rabi* Season)

12.4.1.1 Microbial Biomass Carbon and Basal Soil Respiration

The basal soil respiration was not influenced due to weed control treatment. Significantly higher basal soil respiration were observed in T_2 (mechanical weeding) followed by T_1, i.e., (weedy check), whereas significantly lowest biochemical activities were observed in treatment C4, i.e., pendimethalin at 0.75 kg ha^{-1} fb 2,4-D at 0.5 kg ha^{-1} followed by treatment C3 pendimethalin at 1.5 kg ha^{-1} (Table 12.2).

12.5 Total "N" Fixers and "P" Solubilizers

In chickpea a total number of "N" fixer were observed more than total number of "P" solubilizer. Both the beneficial microorganisms were increased in their numbers from initial to harvest. At initial days, beneficial microflora were significantly lowest in treatment C_4 - pendimethalin 0.75 kg ha^{-1} fb mechanical weeding and C_3 - pendimethalin 1.00 kg/ha, whereas highest count was observed in T_2 mechanical weeding, but at the time of harvest, the number of beneficial microflora was increased, and differences were nonsignificant (Table 12.3). Similar results were also reported by Bhutada et al. (2014), Singh et al. (2013), Yadav et al. (1983), Narendra Kumar et al. (2014), and Sunil Kumar et al. (2010).

12.6 Nodule Number and Nodule Dry Matter

Effect of different herbicide on nodule number and nodule dry matter is shown above. Nodule number and nodule dry matter were not influenced by different herbicide in chickpea. Numerically highest nodule numbers were observed in T_2

Table 12.2 Effect of different doses of herbicide on soil microbial biomass and basal soil respiration in maize-chickpea cropping system

Treatments	Microbial biomass carbon (ug/g soil)[a]			Basal soil respiration: (ug Co_2/100 g of soil/h)[a]		
	30 DAS	60 DAS	At harvest	30 DAS	60 DAS	At harvest
Main treatments – 1st crop (maize)						
M_1 weedy check	349.2	343.7	357.7	403.25	403.34	391.3
M_2 mechanical weeding (2)	346.5	359.8	352.4	398.33	410.0	393.0
M_3 atrazine at 0.75 kg ha^{-1} PE	342.6	371.6	358.3	400.17	405.0	397.6
M_4 atrazine at 1.0–1.50 kg ha^{-1} PE	349.0	353.8	369.5	397.67	406.9	394.7
M_5 atrazine at 0.75 kg/ha fb 2,4-D at 0.5 kg ha^{-1}	349.7	371.5	354.0	394.50	401.1	395.4
SE+	2.71	2.23	1.23	3.51	1.24	0.83
CD at 5%	7.84	6.44	3.55	11.01	3.58	2.41
Sub-treatments – 2nd crop (chickpea)						
C_1 weedy check	345.8	359.2	351.2	404.27	405.0	395.0
C_2 mechanical weeding (2)	341.4	351.0	347.2	405.33	416.1	401.8
C_3 pendimethalin at 1.0 kg ha^{-1} PE	351.3	365.3	354.9	393.20	401.8	390.8
C_4 pendimethalin at 0.75 kg ha^{-1} fb mechanical weeding	351.2	365.9	370.2	392.33	398.2	390.1
SE+	1.79	1.87	1.23	3.63	1.44	0.90
CD at 5%	5.16	5.39	3.55	11.04	4.17	2.59
Interaction						
SE+	4.00	4.18	4.01	8.11	3.23	2.01
CD at 5%	11.5	12.0	11.1	22.3	9.33	5.80

[a]Mean of three replications

mechanical weeding, whereas there were no significant differences in nodule numbers and nodule dry matter in herbicide treatment. Lowest nodule number and nodule dry matter were recorded in C_4 - pendimethalin 0.75 kg ha^{-1} fb mechanical weeding (Table 12.4).

Herbicide in the present investigation had only temporary effect on soil health parameter. The herbicide used, i.e., atrazine and pendimethalin, 2,4-D in maize-chickpea cropping system, did not lower the basal soil respiration and microbial

Table 12.3 Effect of different doses of herbicide on soil total "N" fixers and total "P" solubilizers in maize-chickpea cropping system

Treatments	Total "N" fixers[a] (c.f.u. × 10^{-3})			Total "P" solubilizers[a] (c.f.u. × 10^{-3})		
	30 DAS	60 DAS	At harvest	30 DAS	60 DAS	At harvest
Main treatments – 1st crop (maize)						
M_1 weedy check	16.2	16.6	12.8	12.4	13.1	13.0
M_2 mechanical weeding (2)	13.2	14.6	12.1	11.4	10.7	10.7
M_3 atrazine at 0.75 kg ha^{-1} PE	15.2	15.8	12.9	12.9	12.0	12.2
M_4 atrazine at 1.0–1.50 kg ha^{-1} PE	14.6	16.1	11.4	12.5	11.5	10.7
M_5 atrazine at 0.75 kg ha^{-1} fb. 2,4-D at 0.5 ha^{-1} ha	15.1	15.7	11.7	12.5	11.0	9.7
SE+	0.63	0.45	0.22	0.188	O.16	0.25
CD at 5 %	1.83	1.32	0.66	0.54	0.47	0.74
Sub-treatments – 2nd crop (chickpea)						
C_1 weedy check	13.3	14.6	11.1	10.8	10.9	11.2
C_2 mechanical weeding (2)	18.1	18.1	14.3	15.2	14.0	12.6
C_3 pendimethalin at 1.0/1.25 kg ha^{-1} PE	15.0	15.8	11.6	12.0	11.6	11.2
C_4 pendimethalin at 0.75 kg ha^{-1} fb mechanical weeding	13.1	14.0	11.2	11.0	10.2	10.2
SE+	0.46	0.08	0.24	0.20	0.21	0.17
CD at 5%	1.34	0.88	0.74	0.58	0.61	0.50
Inter action						
SE+	1.04	0.68	0.54	0.45	0.47	0.39
CD at 5%	3.0	1.98	1.57	0.13	1.37	1.13

[a]Mean of three replications

biomass production of microbes at the harvest time of the crop. Similar results regarding beneficial microflora were found. Increasing trend in number of colonies was found from initial to harvest. Application of herbicide did not restrict the microbial growth in both the crops. There is nonsignificant effect of herbicide on number of root nodules and nodule dry matter. Total number of "N" fixers was observed more than that of "P" solubilizers.

Table 12.4 Effect of different doses of herbicide on soil total nodule No and total dry matter (mg/plant) in maize-chickpea cropping system

Treatments	Total nodules (Number/plant) 60 DAS	Total dry matter[a] (mg/plant) 60 DAS
Main treatments – 1st crop (maize)		
M_1 weedy check	20.6	0.37
M_2 mechanical weeding (2)	20.8	0.38
M_3 atrazine at 0.75 kg ha^{-1} PE	19.4	0.37
M_4 atrazine at 1.0–1.50 kg ha^{-1} PE	21.0	0.35
M_5 atrazine at 0.75 kg ha^{-1} fb. 2,4-D 0.5 ha^{-1} ha	22.7	0.42
SE+	0.20	0.004
CD at 5%	0.59	0.011
Sub-treatments – 2nd crop (chickpea)		
C_1 weedy check	20.8	0.37
C_2 mechanical weeding (2)	22.2	0.40
C_3 pendimethalin 1.0/1.25 kg ha^{-1} PE	20.8	0.37
C_4 pendimethalin 0.75 kg ha^{-1} fb mechanical weeding	19.9	0.37
SE+	0.14	0.003
CD at 5%	0.40	0.01
Interaction		
SE+	0.31	0.007
CD at 5%	NS	NS

[a]Mean of three replications

Acknowledgments The permission from Vasantrao Naik Marathwada Agricultural University, Parbhani to conduct the field experiments is gratefully acknowledged and a field study is appreciated.

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Effect of Herbicide Application on Soil Microflora and Nutrient Status of Soil

13

Ashok S. Jadhav, M. G. Patil, and R. K. Sonwane

Contents

Abstract

A field experiment was conducted during Kharif season of 2011–2012 at Weed Science Research Center, Parbhani, to study the effect of atrazine as a pre-emergence application on soil microflora and nutrient status of soil when used in sorghum for weed control. The soil samples of 15 cm depth were collected and processed for microbial analysis following the dilution plate technique. The population of bacteria and fungi were determined by the standard pour plate technique using soil extract agar media for bacteria and rose Bengal agar media for fungi. Triplicate plates for each sample and the microbial group were used for microbial population before sowing and after harvest of the sorghum crop. The result showed that there is no detrimental effect of herbicidal (atrazine) application at the recommended dose on soil microflora. The fungal and bacterial population was decreased at harvest with the application of atrazine at 0.50 kg ha^{-1} as compared to its population with recommended cultural practices.

Keywords

Herbicide · Atrazine · Soil microflora · Soil properties

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13.1 Introduction

The complexity of the soil system is determined by the numerous and diverse interactions among its physical, chemical and biological components as modulated by the prevalent environmental conditions. Beneficial effects of rhizosphere microorganisms were observed on plant health and productivity of crops. The principal goal of agriculture is the production of high quality, safe and affordable food for an ever-increasing worldwide population (Avis et al. 2008). Physicochemical soil properties are fundamental for soil quality, with soil structure being one of the most influential factors. Indiscriminate use of chemical pesticides and fungicides leads to environmental pollution and causes serious effects on human health and nontarget organisms (Khokhar et al. 2012). Hence the field experiment was conducted to study Effect of herbicide application on soil microflora and nutrient status of soil. The soil sample were collected from the rhizospheric layer of plant from herbicide treated plots; it is to be used for all the microbial and biochemical analysis at three stages of crop growth, viz., vegetative growth stage (30DAS), flowering stage (50 DAS) and at harvest.

13.2 Materials and Methods

The field experiment was conducted at Weed Science Research Station Farm, Parbhani, during Kharif seasons 2010 and 2011 to study the "Effect of herbicide application on soil microflora and nutrient status of the soil." The experiment was laid out in a randomized block design with six replications: T_1 - atrazine at 0.50 kg ha^{-1} (low dose); T_2 - atrazine at 0.75 kg ha^{-1} (high dose); and T_3 - RCP (recommended cultural practices control). Soil microbial count was analyzed following the dilution plate technique. The population of bacteria and fungi were determined by the standard pour plate technique using soil extract agar media for bacteria and rose Bengal agar media for fungi. Triplicate plates for each sample and the microbial group were used for microbial population before sowing and after harvest of the sorghum crop.

13.3 Results and Discussion

13.3.1 Soil pH

Many soil properties were influenced with the application of herbicides. Soil pH, EC, and organic carbon were significantly affected. Soil pH in sorghum was influenced by herbicide application; highest pH was recorded in control (7.8) and significantly was at par with the other treatments.

13.3.2 EC

Electrical conductivity (EC) was also influenced at initial stage; atrazine spraying at higher dose of atrazine at 0.75 kg/ha was recorded lowest count as compared to other treatments, but at the time of harvest values of EC were nonsignificant.

13.3.3 Organic Carbon

Organic carbon was influenced by herbicide application. At the time of harvest, OC was increased in all treatment plots as compared to initial. Similar results were also reported by Das and Nag (2009).

The best N fixes were found in treatment T_3, (control) at all the stages of crop growth. Whereas significantly lowest population of N fixers was observed in treatment T_2 (atrazine at 0.75 kg ha^{-1}) as compared to T_3 – RCP (recommended cultural practices control). The population of P solubilizes was also significantly lower with application of atrazine as compared to control. However, at harvest population of both N fixer and P solubilizes was found to increase as compared to initial stage in all the treatments. Son et al. (2006) have reported that phosphate-solubilizing *Pseudomonas* spp. enhanced the number of nodules, dry weight of nodules, yield components, and grain yield in soybean. The microbial biomass carbon and basal soil respiration are in the range of value 330.1 to 400.5 mg biomass C/100 gm dry soil and 270.1 to 286.8 CO_2 g $soil^{-1}$ h^{-1}, respectively. These two activities accurately indicate the biological condition of soil. The result from the present study revealing the changes induced by the herbicide treatment on the microbial biomass carbon and basal soil respiration recorded increasing trend in both of the microbial activity from initial to harvest. At harvest the application of herbicide treatment, i.e., atrazine resulted in lower microbial biomass as compared to the recommended cultural practices, i.e., control treatment. Higher microbial biomass and basal soil respiration were observed in treatment T_3 and which was at par with treatment T_1 – lower dose of atrazine whereas, significantly less biochemical activities were observed in treatment T_2, higher dose of atrazine (Table 13.1).

The microbial biomass carbon and basal soil respiration are in the range of value 330.1 to 400.5 mg biomass C per 100 gm dry soil and 270.1 to 286.8 CO_2 g^{-1} soil h^{-1}, respectively. These two activities accurately indicate the biological condition of the soil. The result from the present study revealing the changes induced by the herbicide treatment on the microbial biomass carbon and basal soil respiration recorded an increasing trend in both of the microbial activity from initial to harvest. At harvest the application of herbicide treatment, i.e., atrazine, recorded lower microbial biomass as compared to its recommended dose, i.e., control treatment (Table 13.2.).

Higher microbial biomass and basal soil respiration were observed in treatment T_3 and which was at par with treatment T_1 – a lower dose of atrazine – whereas significantly less biochemical activities were observed in treatment T_2, i.e., higher dose of atrazine (Table 13.3).

Table 13.1 Effect of herbicides on pH, EC, and organic carbon of soil in sorghum

Treatments	Soil physical properties								
	pH[a]			EC (ds m^{-1})			OC%[a]		
	30 DAS	50 DAS	At harvest	30 DAS	50 DAS	At harvest	30 DAS	50 DAS	At harvest
T_1 – atrazine at 0.50 kg ha^{-1} (low dose)	7.7	7.6	7.6	0.39	0.32	0.34	0.71	0.65	0.81
T_2 – atrazine at 0.75 kg ha^{-1} (high dose)	7.5	7.5	7.4	0.28	0.32	0.30	0.58	0.60	0.71
T_3 – RCP (recommended cultural practices control)	7.8	7.7	7.3	0.38	0.36	0.30	0.78	0.70	0.69
SE±	0.019	0.06	0.06	0.01	0.01	0.01	0.02	0.02	0.01
CD at 5%	NS	NS	NS	0.05	NS	NS	0.08	0.07	0.03

[a]Mean of six replications

Table 13.2 Effect of herbicides on total N fixers and P solubilizers of soil in sorghum

	Soil microflora					
	Total N fixers (cfu × 103/gm of soil)[a]			Total P solubilizers (cfu × 103/gm of soil)[a]		
Treatments	30 DAS	50 DAS	Harvest	30 DAS	50 DAS	Harvest
T_1 – atrazine at 0.50 kg ha^{-1} (low dose)	9.7	12.6	13.6	9.3	10.3	12.1
T_2 – atrazine at 0.75 kg ha^{-1} (high dose)	9.6	10.6	11.6	8.5	9.4	12.1
T_3 – RCP (recommended cultural practices control)	19.6	13.6	14.6	12.0	12.0	14.03
SE±	0.6	0.6	0.33	0.3	0.69	0.45
CD at 5%	2.5	2.6	1.3	1.1	2.7	1.77

[a]Mean of six replications

Table 13.3 Effect of herbicides on microbial biomass carbon and basal soil respiration of soil in sorghum

	Soil microflora					
	Microbial biomass carbon (mg biomass C/100 gm dry soil)[a]			Basal soil respiration (CO_2/g soil/hr)[a]		
Treatments	30 DAS	50 DAS	Harvest	30 DAS	50 DAS	Harvest
T_1 – atrazine at 0.50 kg ha^{-1} (low dose)	338.6	351.1	369.8	280.1	260.1	270.3
T_2 – atrazine at 0.75 kg ha^{-1} (high dose)	330.1	332.4	388.5	270.1	255.1	246.8
T_3 – RCP (recommended cultural practices control)	353.1	359.7	400.5	280.2	281.8	286.8
SE±	2.61	1.06	1.01	3.3	2.53	3.4
CD at 5%	10.26	4.1	3.9	13.1	9.45	13.4

[a]Mean of six replications

13.4 Conclusion

Herbicide had an only temporary effect on soil health parameter. Effect of atrazine on physical properties of soil, i.e., pH, EC, and OC, was negligible. It indicates that the applications of atrazine restrict the microbial growth initially, and no sustained ill effect on the growth of microbes up to the harvest of the crop in sorghum. A number of P solubilizers were recorded comparatively less than that of N fixer (Soumen and Ghosh 2013; Ramesh and Nadanassababady 2005).

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Yield Maximization in Pigeon Pea (*Cajanus cajan* L. Millsp.) Through the Application of Plant Growth-Promoting Bacteria

14

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Contents

Abstract

The field experiment was conducted to study the "Influence of NPK, biofertilizers, foliar application of Micronutrients on growth, yield and yield attributes of Pigeonpea (*Cajanus cajan* L. Millsp.)" at Agronomy Department Farm, College of Agriculture, Dapoli, Dist. Ratnagiri, Maharashtra, India, during *Kharif* seasons 2012 and 2013. The response in terms of yield and yield attributes was tested against the application of *Rhizobium* + phosphate-solubilizing bacteria (PSB) in addition to a recommended dose of fertilizer (RDF) and micronutrients' application. The result showed that there is 21.07% increase in pigeon pea with the application of *Rhizobium* + PSB over RDF alone and 11.53% over the application of RDF + micronutrient. The application of *Rhizobium* + PSB helped to increase the various yield attributes, viz., plant height, number of branches per plant, root nodules per plant, dry matter accumulation per plant, number of pods per plant, number of grains per plant, pod length, 100-seed weight, the weight of grains per plant, and the weight of stalk per plant. The application of *Rhizobium* + PSB is helpful for sustaining soil fertility and higher net returns and B:C ratio over RDF application. Thus, for obtaining higher yields and economic returns to sustain soil fertility, pigeon pea variety BSMR-736 should be grown

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during *Kharif* season with the application of seed inoculation of *Rhizobium* + PSB biofertilizers at 25 g each kg^{-1} seeds in addition to RDF alone.

Keywords
Rhizobium sp. · Pigeon pea · Plant growth promotion · Yield improvement

14.1 Introduction

As Pigeon pea being a legume crop, it fixes atmospheric nitrogen symbiotically as well as it adds organic matter to the soil at maturity. Also, having an outstanding deep root system which not only breaks the hardpan of soil but also maintains optimum moisture and nutrients' utilization which enables it to tolerate drought. The yield of any crop depends on regional biotic and abiotic factors, viz., production potential of the cultivar and climatic, edaphic, and management practices to which the cultivar is exposed. There is a worldwide consensus that sole dependence on chemical input-based agriculture is not suitable long-term cultivation. For that, the only option is the use of Integrated Plant Nutrient Systems (IPNS) which involve combining the use of fertilizers, organic manures, and biofertilizers for sustaining crop production. Adaptation of improved varieties and suitable crop management practices are important factors for improving crop productivity (Sharma et al. 2009). Intensive crop cultivation requires the use of chemical fertilizers, which are expensive in developing country like India. The nutrients applied as basal and top dressing may not be effective because nutrients could not reach in the active root zone of the crop. Foliar spray of nutrients can help in nutrition in plants and also grain yield. Foliar fertilization has received considerable attention to increasing grain production.

14.2 Materials and Methods

The present experiment was set in a split-plot design having three replications, main plot with four varieties of pigeon pea, namely, Konkan Tur-1, ICPL-87, Vipula, and BSMR-736 and subplot treatments consist of six nutrient management practices, viz., Control (F_1-No application of nutrients), RDF (F_2 – 25:50:00 NPK kg ha^{-1}), RDF + Biofertilizers – *Rhizobium* + PSB (F_3), 75% RDF + Two foliar sprays of nutrients (Potassium nitrate 1% + DAP 1% + Boron 500 ppm at flowering and 20 days after first spray) + Biofertilizers – *Rhizobium* + PSB (F_4), 100 % RDF + Two foliar sprays of nutrients (Potassium nitrate 1% + DAP 1% + Boron 500 ppm at flowering and 20 days after first spray) + Biofertilizers – *Rhizobium* + PSB (F_5), and application of major and micronutrients based on soil test (NPK through soil + micronutrients through foliar spray) + Biofertilizers – *Rhizobium*+ PSB (F_6). The experimental site was sandy clay loam in texture with slightly acidic in reaction (pH 5.80), medium in available nitrogen (376.80 kg ha^{-1}), potassium (309.74 kg ha^{-1}), and low in phosphorus (9.20 kg ha^{-1}). Soils are low in available

zinc (0.44 mg kg^{-1}) and boron (0.25 mg kg^{-1}), high copper (1.89 mg kg^{-1}), and medium in manganese (56.04 mg kg^{-1}).

14.3 Results and Discussion

Application of NPK through the soil in combination with micronutrients as foliar spray along with biofertilizers (F_6) produced a significantly higher number of pods per plant, grain pod^{-1} and longer pod when sown with variety BSMR-736 over the rest of the varieties and nutrient management practices during both the years (Table 14.1).

Among the varieties tested, BSMR-736 recorded significantly higher 100-seed weight, the weight of grains $plant^{-1}$ and weight of stalk $plant^{-1}$ as compared to the rest of the varieties when the application of NPK through the soil in combination with micronutrients through foliar spray along with biofertilizers (F_6) over the nutrient management practices (Table 14.2).

Variety BSMR-736 produced significantly higher grain yield (16.99 q ha^{-1}), stalk yield q per ha, and maintain higher harvest index as compared to the rest of the varieties during both the years as well as in pooled data. Also among the nutrient

Table 14.1 Number of pods $plant^{-1}$, number of grains pod^{-1}, and pod length (cm) of pigeon pea as influenced by different treatments

	No. of pods per plant		No. of grains per pod		Pod length (cm)	
Treatments	2012	2013	2012	2013	2012	2013
Variety						
V_1 – Konkan Tur-1	107.33	105.95	3.41	3.33	5.24	5.12
V_2 – ICPL-87	104.52	103.19	3.30	3.22	5.09	4.94
V_3 – Vipula	110.02	108.70	3.47	3.41	5.38	5.24
V_4 – BSMR-736	113.86	112.53	3.60	3.58	5.46	5.95
S.Em. ±	1.10	1.09	0.04	0.04	0.06	0.05
CD at 5%	3.81	3.77	0.12	0.12	0.22	0.19
Nutrient management						
F_1 – Control	96.85	95.41	3.13	3.00	4.63	4.72
F_2 – RDF	105.32	104.00	3.27	3.23	4.89	4.95
F_3 – RDF + Biof.	108.81	107.49	3.41	3.37	5.15	5.12
F_4 – 75 % RDF + 2 FS + Biof.	110.07	108.75	3.48	3.40	5.25	5.23
F_5 – 100 % RDF + 2 FS + Biof.	114.27	112.95	3.62	3.58	5.53	5.62
F_6 – STCR approach + Biof.	118.28	116.94	3.77	3.73	6.31	6.26
S.Em. ±	1.21	1.21	0.04	0.02	0.09	0.06
CD at 5%	3.47	3.45	0.13	0.05	0.25	0.18
Interaction						
S.Em. ±	2.43	2.42	0.09	0.038	0.18	0.13
C.D. at 5%	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
General mean	108.93	107.59	3.45	3.38	5.29	5.31

Table 14.2 100-seed weight (g), the weight of grains plant^{-1}, and weight of stalk plant^{-1} of pigeon pea as influenced by different treatments

Treatments	100-seed weight (g)		Wt. of grains plant^{-1} (g)		Wt. of the stalk plant^{-1} (g)	
	2012	2013	2012	2013	2012	2013
Variety						
V_1 – Konkan Tur-1	10.53	10.36	38.91	36.97	109.07	106.99
V_2 – ICPL-87	10.44	10.25	36.27	34.31	80.57	78.08
V_3 – Vipula	10.66	10.49	41.03	39.19	110.59	108.52
V_4 – BSMR-736	10.95	10.74	45.09	43.52	120.61	118.79
S.Em. ±	0.05	0.05	0.84	0.85	0.66	0.78
CD at 5%	0.17	0.16	2.92	2.96	2.29	2.68
Nutrient management						
F_1 – Control	10.12	9.96	30.73	28.68	96.58	94.32
F_2 – RDF	10.40	10.23	35.92	34.36	100.91	98.71
F_3 – RDF + Biof.	10.59	10.42	39.33	37.82	104.23	102.08
F_4 – 75 % RDF + 2 FS + Biof.	10.71	10.53	41.09	39.00	106.41	104.29
F_5 – 100 % RDF + 2 FS + Biof.	10.90	10.72	45.18	43.40	109.87	107.80
F_6 – STCR approach + Biof.	11.14	10.91	49.71	47.71	113.25	111.39
S.Em. ±	0.06	0.06	0.94	0.90	1.11	1.06
CD at 5%	0.18	0.18	2.70	2.58	3.18	3.04
Interaction						
S.Em. ±	0.12	0.13	1.89	1.81	2.22	2.13
C.D. at 5%	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.
General mean	10.64	10.46	40.33	38.50	105.21	103.10

management practices application, NPK through soil and micronutrients through foliar spray along with biofertilizers (F_6) produced significantly higher yield (Table 14.3).

The marked improvement in yield attributes was due to the application of NPK through the soil in combination with micronutrients through foliar spray along with biofertilizers (F_6) which significantly improved in growth parameters which favorably reflect on yield and yield-attributing characters of the pigeon pea. Further, the effect observed with the dual inoculation of both *Rhizobium* and phosphate-solubilizing bacteria (PSB) along with inorganic sources of nutrients which might be due to the fixation of nitrogen by *Rhizobium* and possible solubilization of fixed P (as alumina and iron phosphates) as well as applied P, besides synthesis of growth-promoting substances like auxins, gibberellins, and produced vitamins which augmented plant growth by phosphorus-solubilizing species, improved vigor and resulted in recording higher values of morphological parameters which increased photosynthetic capacity of plants and thereby increasing the biological yield in terms of dry matter production per plant. Foliar application of micronutrients at flowering and 20 days after the first spray would have helped in reducing flower drop which in turn increased in a number of pods per plant. It also may be accounted for significant improvement in yield attributes like a number of pods per plant, a number of grains

Table 14.3 Mean grain, stalk yield, and harvest index of pigeon pea as influenced by different treatments

Treatments	Grain yield (qha^{-1})			Stalk yield (qha^{-1})			Harvest index (%)	
	2012	2013	Pooled	2012	2013	Pooled	2012	2013
Variety								
V_1 – Konkan Tur-1	12.27	11.22	11.75	56.16	54.32	55.24	17.54	16.63
V_2 – ICPL-87	11.14	10.13	10.63	41.48	39.64	40.56	20.89	20.02
V_3 – Vipula	13.33	12.47	12.90	56.94	55.10	56.02	18.72	18.17
V_4 – BSMR-736	16.53	15.64	16.08	62.10	60.31	61.21	20.92	20.47
S.Em. ±	0.34	0.37	0.50	0.34	0.39	0.52	–	–
CD at 5%	1.16	1.28	1.73	1.18	1.36	1.80	–	–
Nutrient management								
F_1 – Control	9.27	8.17	8.72	49.73	47.88	48.81	15.61	14.44
F_2 – RDF	10.73	9.73	10.23	51.96	50.11	51.04	17.01	16.09
F_3 – RDF + Biof.	13.14	12.18	12.66	53.67	51.83	52.75	19.70	19.04
F_4 – 75 % RDF + 2 FS + Biof.	13.89	12.94	13.41	54.79	52.95	53.87	20.22	19.61
F_5 – 100 % RDF + 2FS + Biof.	15.51	14.55	15.03	56.57	54.73	55.65	21.58	21.04
F_6 – STCR approach + Biof.	17.35	16.62	16.99	58.31	56.55	57.43	22.96	22.73
S.Em. ±	0.49	0.52	0.14	0.57	0.54	0.15	–	–
CD at 5%	1.40	1.48	0.39	1.64	1.54	0.43	–	–
Interaction								
S.Em. ±	0.98	1.04	0.56	1.14	1.08	0.61	–	–
C.D. at 5%	N.S.	N.S.	N.S.	N.S.	N.S.	N.S.	–	–
General mean	13.32	12.36	12.84	54.17	52.34	53.26	–	–

per pod, pod length, the weight of grains per plant, 100-seed weight, and harvest index which finally resulted in increased grain yield. The results are in agreement with those of Dixit and Elamathi (2007), Singh and Singh (2011), Malik et al. (2013), Raman and Venkataramana (2006), Ganapathi et al. (2008), and Singh and Yadav (2008).

14.4 Conclusion

Therefore for obtaining higher yields and economic returns by sustaining soil fertility, pigeon pea variety BSMR-736 should be grown during Kharif season with the application of seed inoculation of *Rhizobium* + PSB biofertilizers at 25 g each kg^{-1} seeds in addition to RDF alone.

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Financial Analysis of Biofertilizer Application: Case Study of Gliocompost Utilization on Red Chili Farming

15

Irawan and L. Pramudyani

Contents

Abstract

In Indonesia, chili is one of the strategic commodities and defined as staple food other than rice, corn, and soybeans. Chili is also a horticultural commodity cultivated by many farmers, and it has a significant effect on inflation. Based on the results of the Indonesian National Survey (SUSENAS), chili consumption is estimated to increase by 0.81% every year. Considering such a condition, the increase of pepper production nationally needs to be continuously cultivated, either through the expansion of planting area or the increase in land productivity. At the level of chili farming fertilization with synthetic chemicals are very attractive to farmers, often excessive fertilization occurs that damages soil fertility

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and biological balance in the soil. As a result, farming efficiency is low, and the price of chili pepper becomes high. Therefore, it needs to find another alternative to chili farming at farmer's level; one of this is using biological fertilizer. The Indonesian Agency of Agricultural Research and Development (IAARD) has assembled an agriculture input product of biofertilizer called Gliocompost, and it was tested in an on-farm research in Tapin District, South Kalimantan, in 2016. This paper presents part of the results of the research, in particular with regard to the objective of knowing the technical and economic efficiency of Gliocompost utilization in chili farming. Gliocompost was applied to two varieties of red pepper (Gandewa and Pilar varieties), three doses of chemical fertilizer (100% NPK, 75% NPK, and 50% NPK), and control (100% NPK without Gliocompost). Each treatment was replicated twice with a plot size of 300 m^2 each. The research was managed by a farmer with the guidance of field assistant and researcher. The results show that the use of Gliocompost can increase the productivity of red chili and financially feasible to the farmer.

Keywords
Biofertilizer application · Gliocompost · Red chili farming

15.1 Introduction

Nationally, chili is one of the strategic vegetable commodities. Chili prices are very volatile and at certain times increased sharply enough to affect inflation. In 2016, the increase in the price of red pepper had contributed inflation up to 0.7% (BPS 2016). Internationally, chili peppers attract scientists' attention because chili contains antimicrobial and antifungal (Omolo et al. 2014) and as an important component in the diet to reduce the risk of sudden death (Chopan and Littenberg 2017).

Some of the government's efforts to overcome the price fluctuation of chili are through the increase of planting area of chili in the rainy season, regulation of planting area and an increase of chili production in the dry season, institutional development of the partnership, and application of cultivation technology to increase chili productivity at farmer level. Chili needs for large cities with a population of one million or more about 800,000 tons per year or 66,000 tons per month. In certain seasons, such as festivals or religious holidays, chili needs increase by about 10–20% of normal needs (Pusdatin 2016).

The national chili productivity for the last 5 years is about 5.6 ton ha^{-1}; therefore, to meet them on the needs of urban communities, it is necessary to harvest chili area about 12,000 ha/month under normal conditions and around 12,500–-13,500 ha $month^{-1}$ in the special months. The need for chili is not yet included for people in small towns and rural areas. The increase of chili production, either through the expansion of the planting area and the increase in productivity should continue to be cultivated to meet the national consumption needs as well as export requirements. In 2014, the increase of chili production reaches 6.1% from the expansion of planting area and productivity increment (BPS 2014).

Application of horticulture cultivation technology of vegetables is generally very intensive. The use of chemical fertilizers and pesticides in the cultivation of chili is very high, even excessive, so that the negative impact on chili farming itself, which reduces profits, reduces competitiveness, and produces products that harm human health due to chemical residues contained in the chili. The results of the Novel study (2012) indicate that the type and dosage of fertilizer used by farmers in chili farming include manure of 12–15 t ha^{-1}, urea 200–250 kg ha^{-1}, ZA 500–600 kg ha^{-1}, SP36 400–450 kg ha^{-1}, and KCl 300–350 kg ha^{-1}. On the other hand, the farmers also use pesticides, insecticides, and fungicides intensively, as the type and number of pests and diseases of chili plants are quite diverse.

Accordingly, the Researcher of the Agency for Agricultural Research and Development through the National Innovative Bio-fertilizer Team (called as PHUN) has conducted research on the use of Gliocompost on food crops and vegetables, including chili (Supriyo et al. 2014). Gliocompost is a fungicide that can act as a biological fertilizer (Djatnika et al. 2003). The active ingredient in this product is *Gliocladium* sp. which is very effective in controlling various plant pathogens such as *Fusarium*. The Gliocompost carrier material is an organic material rich in micro- and macronutrients as well as various soil microbes that play a role in the supply and absorption of nutrients for plants. In addition, Gliocompost can also improves the structure and soil airborne so that the movement of roots and nutrient absorption by plants would be optimal. This paper presents part of the study results, in particular, related to the financial analysis of Gliocompost application on chili farming.

15.2 Research Approach

The research was conducted in Harapan Masa Village, Tapin Selatan District, Tapin District, South Kalimantan Province. Two varieties of red chili were used as indicator plants, namely, Gandewa and Pilar. Both varieties of chili were favored by the farmers because it was more resistant to the pest attack and also favored by consumers. The seedlings of chili were done on June 23, 2016, and planted on July 23, 2016. Chili was planted with a spacing of 100 cm times 50 cm in beds measuring 50 m times 1.2 m.

The research approach was with and without Gliocompost treatment combined with 50%, 75%, and 100% doses of recommended inorganic (NPK) fertilizers, whereas treatment without Gliocompost only used inorganic fertilizers at 100% recommended dosage. Each treatment consists of ten plant beds. The executor of the field research was a cooperator farmer, namely, Mr. Sukarlisasa, head of the local cooperative farmer group. Researchers and field technicians explained SOP of Gliocompost applications and guided farmers in the implementation of such research activities. The stages of research implementation consisted of (a) soil processing, (b) preparation of seedling media and planting, (c) fertilization, and (d) maintenance and monitoring of plants.

15.3 Soil Processing

The land was formerly used for tomato plant. The remaining tomato plants and weeds were cleaned as of farmers' existing techniques. The bed of the former plant was hoisted again and the soils were smoothed. Further, dolomite application of 3 t ha^{-1} was done 2 weeks before planting by distributing it evenly on the entire surface of the land. The use of dolomite was based on soil acidity of soil test result using upland soil test kits (PUTK). The next activity was the installation of plastic mulch and making planting holes in accordance with the plant spacing. Then the application of manure was applied in accordance with the treatments. Treatment of Gliocompost used manure that has been fermented with Gliocompost for10 days, while other treatments used manure without Gliocompost fermentation. Manure was applied to the planting hole at a dose of 10,000 kg ha^{-1}. The next stage was the application of TSP fertilizer with a dose of 200 kg ha^{-1}about 7 days after the application of manure.

15.4 Preparation of Seedling Media and Planting

The seeding medium for Gliocompost treatment was made by mixing 2 grams of Gliocompost into 1 kg of soil prepared in a plastic bag. Then the planting medium was inserted into banana leaves. Planting media for other treatments contains only soil. After 30 days the seedlings were planted in the planting hole in the bed.

15.5 Fertilization

Fertilization NPK Mutiara (16:16:16) as much as 600 kg ha^{-1} (100% NPK treatment) was applied three times, i.e., at age 3, 7, and 10 weeks after planting with 1/3 dose for each fertilizer application. The amount of 75% NPK and 50% NPK treatments were adjusted accordingly.

15.6 Monitoring and Maintenance of Plants

Plant monitoring began when the plants were 7 days after planting, especially against pests and plant diseases, and then the installation of plant springs and straps at the age of chili plants 15 days after planting. Furthermore, the maintenance of vegetative and generative phase cultivation to chili harvesting was referred from the book *Technical Guide PTT Cabai Merah* written by Sumarni and Muharam (2005).

Parameter observed in this research includes yield, revenue, and cost of chili farming in order to calculate the incremental net benefit of Gliocompost application. The effectiveness of Gliocompost in increasing productivity was measured based on

the difference in chili yield between Gliocompost and control treatments. If the difference was higher than 7% than it declared effective since nationally the increased production of chili in 2014 was about 6.1% (BPS 2014).

Incremental net benefit (INB) was calculated as follows:

$$\text{INB} = \text{IB} - \text{IC} \tag{15.1}$$

$$\text{IB} = \text{GL\_B} - \text{CB} \tag{15.2}$$

$$\text{IC} = \text{GL\_C} - \text{CC} \tag{15.3}$$

where

IB = incremental benefit, IC = incremental cost, GL_B = Gliocompost benefit, GL_C = Gliocompost Cost, CB = control benefit, CC = control cost.

The decision was made if INB > 0 that Gliocompost was more efficient compared to control treatment in chili farming. Chili farming benefit was calculated by multiplying the yield and farmers' gate price of chili. Meanwhile, the farming cost was measured by calculating the variable cost, especially due to Gliocompost application. Data processing and data analysis were conducted by tabulation and description.

15.7 Research Site Description

Tap in Regency in South Kalimantan Province is geographically located at 2° 32′43″-3° 00′43″ SL and 114°46′13″-115°30′33″ EL. The Regency is about 70 km from the city of Banjarbaru and adjacent to Hulu Sungai Selatan Regency in the north, Banjar regency in the south, Barito Kuala regency in the west, and Hulu Sungai Selatan Regency in the east.

The research location belonged to Harapan Masa Village, Tapin Selatan District, South Kalimantan Province. It is about 19 km from the capital city of Tapin Regency. The altitude of the research site was 100 m above sea level. The average rainfall over the last 20 years was 2.280 mm $year^{-1}$ with wet months (rainfall>200 mm) occurred in November–March and dry months (rainfall < 100 mm) occurred in July–September (Anonymous 2015). The dry land agricultural ecosystems in this village were generally planted with corn and vegetable horticulture, including chili. The soil texture at the study site was sandy clay with soil pH of 4.5–5.5. Organic matter content was low-moderate, and nutrient content of N, P, and K was low-moderate. The slope of the study land was quite flat. The source of irrigation farming on dry land was mainly rainwater (BPS Tapin 2013).

15.8 Effect of Application Gliocompost on Chili Yield

The productivity of chili was quite diverse, and the use of Gliocompost significantly increased the productivity of chili (Fig. 15.1). Chili productivity among varieties was also different where the yield of Pilar variety was relatively higher than Gandewa variety. Interestingly, Gliocompost application with high doses of inorganic fertilizer (G + NPK 100%) did not always result in higher yield compared with lower fertilizer doses (G + NPK 75% and G + NPK 50%). It showed that the use of Gliocompost could suppress the use of inorganic fertilizer with high chili productivity. The highest chili productivity was obtained by G + NPK 50% treatment. This means that the use of Gliocompost on Gandewa and Pilar chili varieties could save 50% NPK inorganic fertilizer.

In this study, the highest chili yield was achieved by G + NPK 50% treatment, i.e., 9.8 t ha^{-1} for Gandewa variety and 8.7 t ha^{-1} for Pilar variety, respectively. The lowest yield of chili besides the control was obtained by G + NPK 75% treatment for Gandewa variety (5.9 t ha^{-1}) and G + NPK 100% treatment for Gandewa variety (6.8 t ha^{-1}). The productivity of chili in this study was relatively similar to that reported by Hendrawanto (2008) in Cianjur but lower than the findings of Nofita and Hadi (2015) in Jember. Many factors may cause differences in the yield of chilies, including soil fertility, cultivation techniques, and its varieties.

The difference of chili yield between Gliocompost treatment and control was quite high, as much as 88.5% for Gandewa variety, i.e., at G + NPK 50% treatment (Fig. 15.2). Considering the treatment of chili cultivation on the control was the same, except for varieties and application of Gliocompost, then simply that the difference of the yield obtained in the same chili variety could be concluded as the impact of Gliocompost application. For example, at the treatment of G + NPK 100%, the additional chili yield obtained by Gliocompost application reached 30.8% (1.6 t ha^{-1}) for Gandewa variety and 31.6% (1.8 t ha^{-1}) for Pilar variety. The higher

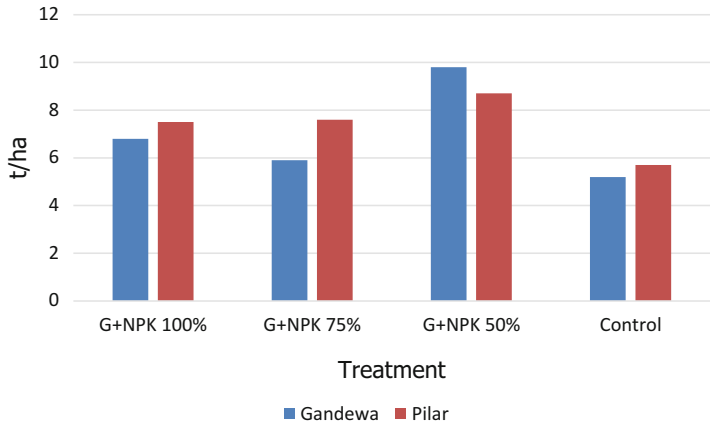

Fig. 15.1 The productivity of chili varieties Gandewa and Pilar due to Gliocompost treatments (t/ha), Tapin, South Kalimantan, 2016

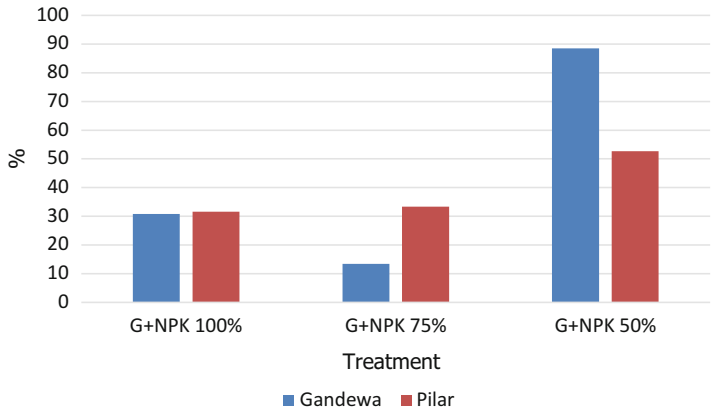

Fig. 15.2 Differences in chili yields of varieties Gandewa and Pilar due to Gliocompost treatment and control (%), Tapin, South Kalimantan, 2016

difference in chili yield was at G + NPK 50% treatment, i.e., 88.5% (4.6 t ha^{-1}) for Gandewa variety and 52.6% (3.0 t ha^{-1}) for Pilar variety. It showed that Gliocompost was very effective in increasing chili productivity.

The higher chili yield difference in lower inorganic fertilizer doses indicated a high technical efficiency in Gliocompost treatment with low NPK fertilizer dosage or 50% NPK dose. If only paying attention to the cost of purchasing and application of inorganic NPK fertilizer that was IDR 6,480,000 ha^{-1} for NPK 100%; IDR 4,860,000 ha^{-1} for NPK 75%; and IDR 3,240,000 ha^{-1} for NPK 50% treatments, then financially the use of Gliocompost as biological fertilizer with an inorganic fertilizer dose of 50% NPK was more profitable than the dosage of 75% NPK and 100% NPK fertilizer, respectively. The amount of 50% NPK fertilizer savings was 300 kg ha^{-1} NPK Mutiara, and it was equivalent to IDR 3,240,000 ha^{-1}.

15.9 Financial Analysis of Application Gliocompost on Chili Farming

The feasibility of farming is influenced by productivity, product selling price, and farming cost. The selling price of the product is classified as an external variable where the farmer can only accept it. The selling price of chili at the time of research at the location was relatively fluctuating with the range IDR 10,000–20,000 kg^{-1}. At the average of chili price (IDR 15,000 kg^{-1}), the additional revenue of chili farming as a result of Gliocompost application was as follows: (a) Gandewa variety at G + NPK 100% treatment = IDR 22,600,000 ha^{-1}; at G + NPK 75% treatment = IDR 9,555,000/ha; and at G + NPK 50% treatment = IDR 69,810,000 ha^{-1} and (b) Pilar variety at G + NPK 100% treatment = IDR 38,220,000 ha^{-1}; at G + NPK 75% treatment = IDR 40,560,000 ha^{-1}; and at G + NPK 50% treatment = IDR 60,810,000 ha^{-1}.

Table 15.1 Labor and production materials for chili farming used in the analysis, Tapin, South Kalimantan Province 2016

Description	Unit	Quantity	Remark
Land preparation/soil tillage	IDR/ha	800,000	Bulk wage/tractor
Beds preparation and basic fertilizer application	IDR/ha	14,000,000	Bulk wage
Seedling and planting activities	Day/ha	40	Daily wage
Installation of plant springs and straps	Day/ha	30	Daily wage
Pruning and weeding	Day/ha	98	Daily wage
Fertilizer application by plowing	Day/ha	24	Daily wage
Fertilizer application by spraying	Day/ha	64	Daily wage
Insecticide/pesticide spraying	Day/ha	96	Daily wage
Harvesting and postharvest activities	Day/ha	320	Daily wage
Man wage	IDR/day	80,000	Without in kind
Woman wage	IDR/day	60,000	Without in kind
Chili seed price	IDR/ha	1,875,000	
Plastic mulch price	IDR/ha	3,750,000	50% of the actual value[a]
Springs and straps price	IDR/ha	1,720,000	60% of the actual value[b]
NPK Mutiara (16-16-16) price	IDR/kg	10,800	
Manure price	IDR/kg	550	
Insect/pesticides cost	IDR/ha	1,420,500	
Manure dose	Kg/ha	10,000	
Gliocompost price	IDR/kg	20,000	Farmer's WTP[c]
Gliocompost dose	kg/ha	50	[d]

Chili selling price (minimum) = IDR 10,000/kg (farmer's gate price)
Chili selling price (maximum) = IDR 20,000/kg (farmer's gate price)
Source: Cooperator farmer's record keeping and interview
Note: [a]The normal size was folded into two sides; [b]40% of it can be reused; [c]Willingness to pay; [d]It was mixed with manure

Based on the record keeping and the results of discussions with cooperator farmer, the financial parameters and the cost of chili farming are presented in Table 15.1. In this study, the average cost of chili farming was IDR 88,280,500 ha^{-1}. The proportion of labor cost was 62.2%, and material cost was 37.8%. The cost of chili farming was relatively higher when compared with the results of the BPS survey (2014) and Hendrawanto research (2008). This was due to an increase in the price of wages and agricultural materials in farming. Furthermore, as a sensitivity analysis, the profit of farming based on treatment and selling price of chili IDR 15,000 kg^{-1} and IDR 20,000 kg^{-1} is presented in Table 15.2. Considering the productivity of Pilar variety, the selling price of chili IDR15,000 kg^{-1} had benefited farmer in applying Gliocompost. The highest profit of chili farming was obtained at G + NPK 50% treatment, both for Gandewa and Pilar varieties, respectively.

Table 15.2 Sensitive analysis of chili farming profit under Gliocompost treatment at difference price of chili, Tapin, South Kalimantan Provinces, 2016 (IDR)

Gliocompost	Profit at chili price of IDR 15,000/kg		Profit at chili price of IDR 20,000/kg	
Treatment	Gandewa var.	Pilar var.	Gandewa var.	Pilar var
G+NPK100%	(1,100,500)	10,655,000	27,954,500	43,155,000
G+NPK75%	(10,785,500)	15,790,000	13,914,500	48,875,000
G+NPK50%	49,849,500	32,135,000	97,634,500	75,875,000
Control	(22,320,500)	(16,610,000)	(805,500)	6,335,000

Notes: number in the (...) is negative value

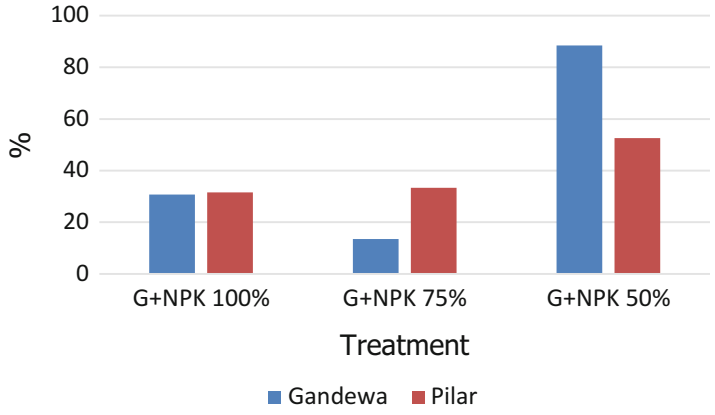

Fig. 15.3 Incremental net benefit (INB) of chili farming under Gliocompost treatments, Tapin, South Kalimantan Province, 2016

The additional cost of farming as a result of Gliocompost application was IDR 1,400,000 ha^{-1} for G + NPK 100% treatment; -IDR 1,980,000 ha^{-1} for G + NPK 75% treatment; and -IDR 1,760,000 ha^{-1} for G + NPK 50% treatment, respectively. Negative additional cost of G + NPK 75% and G + NPK 50% treatments meant that the value of NPK fertilizer reduction in those treatments was higher than the cost of Gliocompost application. Furthermore, the incremental net benefit of chili farming can be calculated, and the results in unit of percent are presented in Fig. 15.3. In line with the high yield difference and the efficiency of farming cost due to Gliocompost treatments, the incremental net benefit of chili farming as a result of Gliocompost application was IDR11.5–71.6 million ha^{-1} for Gandewa variety and IDR 27.3–-49.1 million ha for Pilar variety. It showed that the use of Gliocompost in chili farming could increase farmers' income.

15.10 Conclusions

Gliocompost, as biofertilizer, was effective in increasing yield of chili. The application of Gliocompost at a 50% NPK fertilizer dose provided higher yield and more effective than Gliocompost at 75% NPK and 100% NPK fertilizer dosages. The difference of chili yield between the treatment of Gliocompost at 50% NPK dose and control was 88.5% for Gandewa variety and 52.6% for Pilar variety.

Financially, the application of Gliocompost benefited farmer because the incremental net benefits of chili farming with Gliocompost treatment was higher than zero or positive. The use of Gliocompost at a dose of 50% NPK fertilizer dose was most advantageous to the farmer.

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Viability of *Pseudomonas plecoglossicida* and *Rhizobium* sp. LM-5 as Liquid Bacterial Fertilizers in Various Formulated Carriers

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Contents

Abstract

Microbial fertilizers, known as "biofertilizers," have the ability to convert the nonusable to usable form into nutritionally important elements through biological processes. The effectiveness and viability of biofertilizer inoculants are correlated with carrier formulations. The purpose of this study was to evaluate the efficiency of various carrier materials for the shelf life of N-fixing *P. plecoglossicida* strain NBFPALD_RAS144 and *Rhizobium* sp. LM-5 to produce as liquid formulations. The experiments to investigate the effect of carrier formulation were conducted under laboratory conditions from January to July during 2015. The experiment

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was set up as a completely randomized design, consisting of nine different formulations each in three replications. The population of *P. plecoglossicida* strain NBFPALD_RAS144 and *Rhizobium* sp. LM-5 were enumerated at 2, 4, 8, 12, 16, 20, and 24 weeks of incubation at room temperature. The population of *P. plecoglossicida* strain NBFPALD_RAS144 after 24 weeks of incubation varied in different carrier formulations. Maximum cell density (12.8×10^9 CFU mL^{-1}) was recorded in 5% molasses + 0.05% polyvinyl alcohol, while the lowest population (2.65×10^9 CFU mL^{-1}) was recorded in 2.5% Triton. The population of *Rhizobium* sp. LM-5 remained relatively high even after 6 months of incubation in all the formulation of carriers under tested.

Keywords
Carrier formulation · *P. plecoglossicida* strain NBFPALD_RAS144 · *Rhizobium* sp. LM-5 · Liquid biofertilizer · Cell density

16.1 Introduction

Biofertilizer is the preparations that contain living microorganisms and are used to increase the growth and yield of crops through nitrogen fixation, phosphate solubilization, and induction of plant resistance (Singh and Purjit 2011; Sharma et al. 2004; Purwanto et al. 2016). Since biofertilizer are low cost, eco-friendly, and sustainable, they are being used as an alternative to chemical fertilizers (Simarmata 2013). Their demand is increasing due to public awareness, environmental concerns, and ever-increasing prices of chemical fertilizer.

Among the various biofertilizers, nitrogen (N_2)-fixing bacteria have been widely used biofertilizers. They have been used as an alternative source of N_2 for rice crops and in remediation of wetland soils (Shrestha and Maskey 2005; Widowati et al. 2011). N_2-fixing bacteria colonize plant roots, provide N to the crop plant and stimulate plant growth through the production of phytohomones, and induction of systemic resistance in plants (Singh et al. 2011), and It has been reported that utilization of N_2 fixing bacteria in intensive farming systems greatly contributes to the availability of N for plants (Vakadattu and Peterson 2006). The contribution of N_2-fixing bacteria ranges from 50 to150 kg ha^{-1} N (Chowdhury and Mukherjee 2006). N_2-fixing bacteria have the ability to synthesize various phytohormones like auxin in the form of indoleacetic acid and produce antibiotics under optimum environmental conditions (Reddy et al. 2000; Chowdhury and Mukherjee 2006; Razie and Iswandi 2005).

The effectiveness of biofertilizers in agriculture field is often correlated with the carrier material and the soil organic content or organic ameliorant supply as a source of energy (Sharma et al. 2004; Simarmata et al. 2016; Sudjana et al. 2017). Various formulations such as liquid, solids, granules, peat, freeze-dried powders have been developed; however, their success rates are highly dependent on crop type, cost, market availability, environmental constraints, and ease of use (Herrmann and Lesueur 2013). To be the best carrier, it should be cheap and available in low cost in ample amount, soluble in various materials/solvents, should permit gas exchange

(oxygen), should possess high organic matter, and bear high water-holding capacity. In order to increase the inoculant quality and efficiency and to reduce costs and environmental impacts, alternative carrier materials are needed. A variety of carriers materials were evaluated to improve the survival, biological effectiveness, and protection offered against biotic and abiotic stresses.

Liquid biofertilizer formulation offers improved viability of the cells, contains adequate nutrients, and protects the cells so that microbial cells or spores can remain alive in a long time. The viability of microbes in liquid formulations can be up to 2 years and resistant to high temperatures, while solid formulas are only about 6 months (Brar et al. 2012). Dormant cell technology is a technique for maintaining the viability of microbial cells with the addition of various additives such as acetone, sodium benzoate, sodium azide, glycerol, polyethylene glycol, gum arabic (Kumaresan and Reetha 2011; Pindi and Satyanarayana 2012). The purpose of this study was to evaluate the efficiency of various carrier materials for the shelf life of N-fixing *P. plecoglossicida* strain NBFPALD_RAS144 and *Rhizobium* sp. LM-5 to produce as liquid formulations.

16.2 Materials and Methods

The research to study the effectiveness of different formulated liquid carriers for nitrogen biofertilizers was conducted from January to July 2015 in the production unit of biofertilizers at CV. Bintang Asri Arthauly Bandung, West Java, Indonesia. The experiment was arranged as completely randomized design and was carried out in triplicate. The following nine formulations were used:

A. Molasses 5% + yeast extract 1%
B. Molasses 5% + glycerol 2.5%
C. Molasses 5% + Tween 20 2,5%
D. Molasses 5% + PEG 400, 0.5%
E. Molasses 5% + sucrose 5%
F. Molasses 5% + gum arabic 0.3%
G. Molasses 5% + polyvinyl alcohol 0.5%
H. Molasses 5% + Triton 2.5%
I. Molasses 5%

A pure culture of *P. plecoglossicida* strain NBFPALD_RAS144 was cultivated in Ashby's broth. And *Rhizobium* sp. LM-5 was cultivated in Okon broth. Each pure culture of bacteria was separately grown at room temperature and shaking at 120 rpm for 3 days. Following the incubation, the liquid biofertilizer was separately packed in plastic bottles and stored at room temperature (28 °C) and periodically observed for cell density. Observations for cell count were made periodically, i.e., 2, 4, 6, 8, 12, 16, 20, and 24 weeks after production by plate count, and the count was expressed as CFU mL^{-1}. The data obtained were statistically analyzed using analysis of variance (ANOVA) by using SAS software version 9.1, and any significant difference in cell density was further processed for analysis by DMRT at 95% confidence level.

16.3 Results

The addition of addictive ingredients in the liquid formulation significantly enhanced the availability of *P. plecoglossicida* strain NBFPALD_RAS144; however, there was no significant difference on the viability of *Rhizobium* sp. LM-5 due to the addition of these ingredients. Addition of 0.05% polyvinyl alcohol strengthened the viability of *P. plecoglossicida* strain NBFPALD_RAS144, and cell density remained at maximum count (12.8×10^9 CFU mL^{-1}) up to 6 months of storage, while the lowest population density ($2.65\ 10^9$ CFU mL^{-1}) was obtained with Triton 2.5% (Table 16.1). The viability of *Rhizobium* sp. LM-5 in various liquid formulations of biofertilizer with the addition of addictive ingredients did not show any significant difference until 24 weeks of storage (Table 16.2.). The result of the present study showed that the addition of sucrose (5%), Tween 20, yeast extract (1%), and polyvinyl alcohol (0.5%) could maintain the viability of *Rhizobium* sp. LM-5.

The pH of various liquid formulations of N_2-fixing microbial fertilizer remained unchanged and stable in the pH range of 3.84 to 4.44. Total nitrogen in various liquid fertilizer formulations varied from 0.3% to 0.5%. The total N_2 content in the liquid formula can be correlated to the ability of diazotrophic bacteria to fix the atmospheric nitrogen (Table 16.3).

16.4 Discussion

Carrier formulation as a media for microbial N_2 fertilizer-based molasses medium is relatively cheap and readily available and contains sufficient sucrose (50%) (Peighami-Ashnaei et al. 2009). The efforts to maintain the viability of bacteria over a long period and provide suitable conditions for rapid bacterial growth in the media requires the addition of addictive substance as osmoprotectant (sucrose), cell p, and cell protectants (glycerol, gum arabic, PEG 400, polyvinyl alcohol). A liquid formulation of biofertilizer contains a consortium of indigenous N_2-fixing bacteria, i.e., *P. plecoglossicida* strain NBFPALD_RAS144 and *Rhizobium* sp. LM-5.

Addition of addictive ingredients in liquid formulation significantly enhanced the cell population of *P. plecoglossicida* strain NBFPALD_. Addition of addictive ingredient osmoprotectant offers greater viability. The addition of the cell protectants such as polyvinyl alcohol (PVA) maintained the viability of *P. plecoglossicida* strain NBFPALD_RAS144 of 12,8 × 109 CFU mL^{-1} for 6 months of storage. Whereas in *Rhizobium* sp. LM-5 cell population maintained up to 9.07×10^9 CFU mL^{-1}. Addition of polyvinyl alcohol proved to be significant vis-à-vis other cell protectants. Use of PVA offers many advantages; it is water-soluble, biodegradable, and nontoxic to microorganisms. Its effect on bacterial viability is also negligible as compared to PEG 4000 or PEG 6000 (Rivera et al. 2014). Deaker et al. (2007) have reported the increased viability cells in presence of PVA. PVA hydrolysis in media provides suitable conditions to protect the cell protection against high temperatures and dehydration due to its hydrophilic nature

Table 16.1 The viability of *P. plecoglossicida* strain NBFPALD_RAS144 in a liquid formulation

	The population of *P. plecoglossicida* strain NBFPALD_RAS144 (10^9 CFU mL^{-1})						
Treatments	2 weeks	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks
A	21.90a	15.30a	6.82a	9.70ab	5.20a	5.47a	4.48bc
B	16.30a	15.70a	9.70a	5.97bc	4.26a	6.10a	7.92ab
C	14.10a	12.50a	7.93a	14.10a	4.72a	3.07a	6.45ab
D	14.40a	10.80a	8.03a	14.00a	5.42a	4.57a	7.42ab
E	16.70a	16.20a	17.60a	14.80a	5.90a	5.67a	8.48ab
F	16.60a	11.20a	11.70a	10.60ab	4.72a	4.00a	6.65ab
G	12.80a	14.30a	11.00a	10.80ab	5.23a	5.18a	12.80a
H	20.90a	14.70a	4.88a	14.80a	6.47a	3.77a	2.65c
I	15.90a	9.03a	5.65a	4.67c	4.25a	5.73a	6.08b

Remark: A, molasses 5% + yeast extract 1%; B, molasses 5% + glycerol 2.5%; C, molasses 5% + Tween 20 2.5%; D, molasses 5% + PEG 400 0.5%; E, molasses 5% + sucrose 5%; F, molasses 5% + gum arabic 0.3%, G, molasses 5% + polyvinyl alcohol 0.5%, H, molasses 5%. + Triton 2.5%; dan I, molasses 5%. The value within the column followed by the same letters are not significantly different according to DMRT 5%

Table 16.2 The viability of *Rhizobium* sp. LM-5 in a liquid formulation of biofertilizers

Treatments	The population of *Rhizobium* sp. LM-5 (10^9 CFU mL^{-1})						
	2 weeks	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks	24 weeks
A	25.10a	8.70a	8.42a	5.40a	4.15bc	3.85a	9.32a
B	24.50a	10.50a	12.90a	6.88a	6.40a	2.77a	8.83a
C	26.30a	10.80a	13.70a	5.17a	4.62abc	1.27a	9.35a
D	12.60a	10.40a	12.80a	6.65a	5.03ab	2.77a	7.53a
E	19.20a	10.40a	13.90a	6.42a	6.27a	5.25a	11.3a
F	23.20a	9.02a	12.50a	5.42a	4.00bc	2.38a	5.80a
G	12.60a	5.65a	11.80a	5.10a	5.05ab	2.67a	9.07a
H	23.90a	4.88a	11.10a	4.40a	3.40c	2.80a	6.58a
I	22.10a	9.07a	9.17a	4.38a	3.72bc	3.05a	8.10a

Remark: A, molasses 5% + yeast extract 1%; B, molasses 5% + glycerol 2.5%; C, molasses 5% + Tween 20 2.5%; D, molasses 5% + PEG 400 0.5%; E, molasses 5% + sucrose 5%; F, molasses 5% + gum arabic 0.3%; G, molasses 5% + polyvinyl alcohol 0.5%; H, molasses 5% + Triton 2.5%; dan I, molasses 5%. The numbers followed by the same letters are not significantly different according to DMRT 5%

Table 16.3 Chemistry characteristic of liquid formulated biofertilizer

Treatments	C-organic	N total	C/N ratio	pH
A	1.47d	0.05a	29.00c	4.11a
B	2.54b	0.05a	58.00b	4.14a
C	3.12a	0.03a	91.00a	4.18a
D	1.48d	0.04a	35.67c	4.41a
E	2.11c	0.04a	55.00b	3.84a
F	1.16e	0.04a	33.67c	4.25a
G	1.52d	0.04a	36.67c	4.30a
H	3.03a	0.03a	91.33a	4.20a
I	1.26de	0.04a	32.67c	4.44a

Remark: A, molasses 5% + yeast extract 1%; B, molasses 5% + glycerol 2.5%; C, molasses 5% + Tween 20 2.5%; D, molasses 5% + PEG 400 0.5%; E, molasses 5% + sucrose 5%; F, molasses 5% + gum arabic 0.3%; G, molasses 5% + polyvinyl alcohol 0.5%; H, molasses 5% + Triton 2.5%; dan I, molasses 5%. The numbers followed by the same letters are not significantly different according to DMRT 5%

(Hubalek 2003). Ruiz-Valdiviezo et al. (2015) reported that pH and the viscosity of the medium are physical and chemical characters which affect the shelf life of the strains of rhizobia in carrier media.

The addition of 2.5% of Tween and 2.5% Triton showed the highest C-organic content, while the addition of 0.3% gum arabic yielded the lowest C-organic content. The content of C-organic liquid formulation directly correlates ($r = 0.929$) with the C/N ratio, whereas the C/N ratio of other formulations showed the differences between the formulations. This reflects that the magnitude of the C/N ratio of 92.9% is influenced by the amount of C-organic content in the liquid biofertilizer. The availability of carbon source in medium sufficient to maintain bacterial viability remained high during the storage period up to 24 weeks in both the isolates *P. plecoglossicida* strain NBFPALD_RAS144 and *Rhizobium* sp. strain LM-5.

16.5 Conclusion

Biofertilizer formulation of molasses (5%) and polyvinyl alcohol (0.05%) proved as effective carrier for as it maintained the viability and high cell population density (12.8×10^9 CFU mL^{-1}) of P. *plecoglossicida* strain NBFPALD_RAS144 for longer period up to six months of in a mixture of enrichment with additive compound in liquid formulation of biofertilizer resulted in the same population of *Rhizobium* sp. LM-5 in all treatments. The viability of biofertilizers inoculant remained high (more than 10^8 CFU mL^{-1}) even after prolonged storage of six months as this cell population density was higher than the cell density prescribed by the government regulation, such formulations can be used as a best material for biofertilizer.

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Studies on the Performance of Coropulse on Black Gram (*Vigna mungo* L.) in Rice Fallows

17

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Contents

Abstract

Studies on the performance of *Coropulse* (a unique emulsifiable concentrate formulation) were taken up on black gram (*Vigna mungo*). The experiment was laid out in a randomized block design with seven treatments and three replications. The objective was to study the effect of *Coropulse* toward improving the *Rhizobium* nodulation and a concomitant increase in the available nitrogen for crop growth and yield. *Coropulse* at 2.0 mLL^{-1} of water was sprayed on PU 31 black gram variety on 10, 15, and 20-day-old seedlings for one time and at 10 days followed by 15/20/25 DAS for two times. *Coropulse* was shown to significantly increase the nodule number as against untreated control at 25 and

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49 DAS. Fresh weight of 50 *Rhizobium* nodules at 49-day age of crop recorded more in *Coropulse* sprayed plot compared to untreated control at 10 and 25 days of crop growth. Further *Coropulse* increased leaf area, root length, number of pods plant^{-1}, number of seeds pod^{-1}, 1000-grain weight, and seed yields (18.97 q ha^{-1}) as against untreated control (10.77 q ha^{-1}) that resulted in higher benefit-cost ratio.

Keywords
Coropulse · Black gram · *Vigna mungo* · *Rhizobium nodulation* · Rice fallows

17.1 Introduction

Urdbean or black gram (*Vigna mungo* L), an important pulse crop, has been cultivated both as pure and mixed crop along with maize, cotton, sorghum, and other millets and used as seed and vegetable. In India, it is cultivated both in Kharif and Rabi seasons in an area of 31.00 lakh hectares with a production of 14.00 lakh tons and productivity of 451.61 kg ha^{-1}. In both Telangana and Andhra Pradesh states put together, the crop covers an area of 4.22 lakh hectares with a production of 2.50 lakh tons and productivity of 572 kg ha^{-}1. An important feature of this plant has been its ability to establish a symbiotic partnership with specific bacteria, setting up the biological N_2 fixation process in root nodules by *rhizobia* that supply the plant's nitrogen needs when the plant is young and cannot meet its own requirement.

The essential mineral nutrients for symbiotic legume nitrogen fixation are those required for the normal establishment and functioning of symbiosis. The following chemical elements are known to be essential for the legume-*Rhizobium* symbiosis, viz., C, H, O, N, P, S, K, Ca, Mg, Fe, Mn, Cu, Zn, Mo, B, C1, Ni, and Co. Each of these nutrients has specific physiological and biochemical roles, and there are minimal nutrient concentrations required within both legumes and *Rhizobium* to sustain metabolic function at rates which do not limit growth (Graham et al. 1988).

Robson (1978) concluded that Co and Mo are required in high amounts for symbiotic nitrogen fixation for host plant growth. In some species of legumes for symbiotic development, only four of these elements (Ca, P, Fe, and Mo) appear to cause significant limitations on the productivity (O'Hara 2001). There are reports that foliar applications of Mo to grain legumes in field conditions increase the levels of N_2 fixation and nodule mass, resulting in higher overall N content and seed yield (Yanni 1992; Vieira et al. 1998). Weisany et al. (2014) indicated the essentiality of other elements like iron, manganese, zinc, boron, copper, and molybdenum for legumes and multiplication of *Rhizobium*. Similarly, Ahmad et al. (2013) indicated that *Rhizobium* inoculation along with Mo at 2 kg ha^{-1} increased plant height, root length, 100-grain weight, and nodule number. *Coropulse* a unique formulation developed by M/s Dhana Crop Sciences Limited, Secunderabad, Telangana, India, in emulsifiable concentrate form is rich in amino acids, different unique micronutrients toned with wintergreen oil, plant activators, and several other ingredients which have the capacity to induce systemic acquired resistance in treated plants. Our objective was to know whether Coropulse in black gram would increase *Rhizobium* nodulation and thereby increase the available nitrogen to enhance the

crop physiological efficiency and yield. *Coropulse* an eco-friendly product does not contain any synthetic organic material and is nonhazardous to nontarget organisms. To facilitate the farmers to quickly apply and take advantage, the *Coropulse* effect on black gram (*Vigna mungo* L.) was found, and the results obtained in the present study have been presented.

17.2 Material and Methods

Coropulse a unique new product and formulation developed by M/s Dhana Crop Sciences (Pvt.) Limited, Secunderabad, Telangana, India, was utilized for testing the "Bioefficacy of this product to induce growth and increase nitrogen fixation by nodules in leguminous crops like black gram" in rice fallows on PU 31 black gram (*Vigna mungo* L.). The experiment was laid out in randomized block design with seven treatment and three replications. Plot size adopted was 8x5 m. Treatment details included T1-first spray at 10 DAS, T2-first spray at 15 DAS, T3-first spray at 20 DAS, T4-spray at 10 and at 15 DAS, T5-spray at 10 and 20 DAS, T6-spray at 10 and 25 DAS, and T7-control (no spray).

17.3 Seeds and Sowing

A 18 kg PU 31 black gram seed was used following the package of practices prescribed by Professor Jayashankar Telangana State Agricultural University, Hyderabad, Telangana State, India.

17.4 Counting of Nodules

Data on a number of nodules was collected at 15, 25, and 49 DAS from 10 plants which were carefully uprooted by digging 15 cm around the plant. Data was collected to flowering stage as most of the nodules lose their activity afterward. Nodules collected from treatments 6 and 7 were analyzed for nitrogen. At harvest data was collected on (1) average pod length (cm), (2) number of seeds per pod, (3) number of pods per plant, (4) thousand seed weight, (g) and (5) grain yield ha^{-1}. Data collected were subjected to statistical analysis for testing the differences between treatments and untreated control.

17.5 Results and Discussion

A perusal of the results from indicated that 5 days after spray of Coropulse, i.e., at 15 days of crop growth, nodule number per plant did not differ significantly. Significant differences were recorded between treatments and untreated control.

Table 17.1 Nodules, leaf area, root length, pods per plant, pod length, seeds per pod, the weight of 1000 seeds (g), grain yields, and net benefit upon a spray of *Coropulse* in black gram

Treatments	Rhizobial nodules at 15 DAS	Rhizobial nodules at 49 DAS	Nodule diameter (mm)	Leaf area (cm^2)	Root length (cm)	Pods/ plant	Pod length (cm)	Seeds/ pod	Weight of 1000 seeds (g)	Grain yield (Q ha^{-1})	Net benefit over control (Rs)	Benefit (US$)
T1	6.47	32.46	2.16	11.60	23.98	37.78	4.78	6.27	37.11	13.31	16,594	255.29
T2	6.32	38.92	2.47	14.08	17.67	36.52	4.72	7.40	38.75	15.57	32,414	498.67
T3	6.24	50.32	2.49	16.17	28.17	45.67	5.11	7.47	39.82	16.15	36,474	561.14
T4	6.08	57.46	2.21	17.99	21.20	34.3	4.64	7.60	38.17	16.24	36,368	559.51
T5	6.17	68.27	26.86	18.09	26.86	41.50	4.95	7.40	37.37	16.73	39,798	612.27
T6	6.25	77.11	29.72	27.12	29.72	51.28	5.80	8.77	44.06	18.97	55,478	853.51
T7 (C)	6.27	18.13	14.34	9.20	14.34	28.12	4.20	5.40	33.51	10.77	–	
C.D (P = 0.5)	NS	8.34	8.67	0.58	8.67	2.56	0.34	0.31	0.86	2.2		
SE(d)		8.17	3.93	0.26	3.93	1.16	0.15	0.14	0.39	1.0		
SE(m)		0.12	2.78	0.19	2.78	0.82	0.11	0.1	0.28	0.71		
CV		1.16	21.13	1.98	21.13	3.61	3.87	2.43	1.24	7.98		

Coropulse sprayed at 10 (T1), 15 (T2), 20 (T3), 10 + 15 (T4), 10 + 20 (T5), 10 + 25 (T6)day-old seedlings and untreated control (T7)

Coropulse applied at 10 and 25 DAS (T6) was superior (Table 17.1). The effect has been observed in the following parameters.

17.6 Nodule Number

At 25 and 49 days crop growth, Coropulse (T6) applied at 10 and 25 DAS recorded an increased number of nodules over control (52 and 12). Further at 49 days of crop growth, T6 recorded more nodules (77) as against control T7 (18) an increase of 325% (Table 17.1).

17.7 Root Length

At 60 days of crop growth, T6 compared to untreated control recorded more root length (29.72 and 14.34 cm) (Table 17.1).

17.8 Physiological, Biometric, and Yield Attributes

17.8.1 Pods Per Plant

T6 – Coropulse sprayed at 10 and 25 DAS compared to untreated control recorded maximum pods per plant (51, 28) (Table 17.1).

17.8.2 Average Pod Length (cm)

T6 – Coropulse applied at 10 and 25 DAS recorded more pod length over control (5.8 and 4.2 cm) (Table 17.1).

17.9 1000-Seed Weight (g)

T6 – Coropulse spray at 15 and 25 DAS recorded superior seed weight compared to control (44.66 and 33.64 g) (Table 17.1).

17.10 A Number of Seeds per Pod

T6 recorded 8.77 seeds per pod, while control recorded 5.40 seeds per pod. v) Grain yield (q ha^{-1}): significant differences were recorded in grain yields between treatments and untreated control. Grain yields were maximum in T6- 18.97 q ha^{-1} when Coropulse was applied at 10 and 25 DAS) compared to T7 - Untreated control

which recorded10.77 q ha^{-1} followed by T5 Coropulse applied at 10 and 20 DAS (16.73 q ha^{-1}). Further, in T4 when Coropulse was applied at 10 and 15 days of crop growth, yield recorded was 16.73 q ha^{-1} indicating that T4 and T5 are statistically at par. T1 recorded minimum yield (13.31 q ha^{-1}) among the Coropulse treatments but superior over untreated control (Table 17.1).

17.11 Net Benefit Over Untreated Control

Based on the yield data, cost of cultivation has been worked out. Results indicated maximum net benefit in T6 – i.e., Rs.55,478 ha^{-1} – over T7-untreated control, followed by T5 Coropulse applied at 10 and 20 DAS which resulted in a benefit of Rs.39,798 ha^{-1}. T3 and T4 treatments with Rs.36,474 and 36,368, respectively, were found to be at par. T1 recorded the minimum net benefit of Rs.16,594. The present findings indicate that all Coropulse treatments were found to be superior over control. Among all the treatments (T6), Coropulse applied at 10 and 25 DAS (total two rounds) was found to be superior over all other treatments. The results strongly suggest the superiority of this treatment and can be recommended to farmers who cultivate black gram in rice fallows during Rabi season.

The present findings are in full agreement with that of Graham et al. (1988) who indicated that each of the essential nutrients, viz., O, N, P, S, K, Ca, Mg, Fe, Mn, Cu, Zn, Mo, B, C1, Ni, and Co, has specific physiological and biochemical roles and is required within both legumes and *Rhizobium* to sustain metabolic function at rates which do not limit further growth. Weisany et al. (2014) indicated the essentiality of other elements like iron, manganese, zinc, boron, copper, and molybdenum for legumes and multiplication of *Rhizobium*. Similarly, Ahmad et al. (2013) indicated that *Rhizobium* inoculation along with Mo at 2 kg ha^{-1} increased plant height, root length, 100-grain weight, and nodule number in mung bean. In the present studies also with *Coropulse* applied on Blackgram crop (*Vigna mungo* L.) in Rice fallows at 10 and 25 DAS increased plant height, root length, 1000 grain weight, nodules number and subsequently recorded maximum grain yields compared to other treatments and untreated control.

17.12 Conclusion

Coropulse a unique emulsifiable concentrate formulation is a rich source of amino acids, micronutrients toned with wintergreen oil, plant activators, and several other ingredients which have the capacity to induce systemic acquired resistance in treated plants. *Coropulse* at 2 ml l^{-1} of water applied on black gram crop cultivated in rice fallows at 10 followed by 25 DAS (2 times) was found to be superior over other treatments including control. Results clearly showed an increased number of *Rhizobium* nodules, fresh weight, nitrogen in nodules and plants, leaf area, root length at 60 DAS which increased the number of pods $plant^{-1}$, number of seeds pod^{-1}, 1000-grain weight, grain yields, and maximum benefit-cost ratio.

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Part II

PGPR for Sustainable Management of Pathogens and Stress

Microbial Surfactants and Their Significance in Agriculture

18

Hameeda Bee, Mohamed Yahya Khan, and R. Z. Sayyed

Contents

Abstract

Plant health and crop productivity need to be monitored to maintain the quality and availability of food to meet the growing demands of the population worldwide. Continuous efforts by researchers and scientists have explored novel bacteria and their metabolites (green compounds) to achieve sustainability in agriculture. Green surfactants produced by bacteria, actinomycetes, yeast, and fungi are less toxic and eco-friendly and have significant application in agriculture. Both epiphytic and endophytic plant-associated microbes are known to produce biosurfactants and have a vital role in motility, signaling, and biofilm formation. Biosurfactants aid in seed germination, inhibit phytopathogens and act as biocontrol agents used for bioremediation of contaminated soils, mobilize nutrients and enhance plant-microbe interactions. Based on their physicochemical properties, biosurfactants are glycolipids, lipopeptides, phospholipids, and polymeric. These biosurfactants are produced from renewable resources and exhibit

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excellent surface activity, possess high specificity, and are effective under extreme conditions. Here we report the isolation of bacteria from different rhizosphere samples and their screening for plant growth-promoting (PGP) traits and antifungal activity. Ten bacterial isolates exhibited PGP traits, antifungal activity, and biosurfactant activity. Two potential isolates (*Pseudomonas aeruginosa* DR 1 and *Bacillus amyloliquefaciens* RHNK 22) were selected for production of biosurfactant using synthetic media (nutrient broth, Bushnell has broth and mineral salt). Characterization of biosurfactant revealed it as rhamnolipid by DR 1 and surfactin and iturin by RHNK 22. Extracted biosurfactants (rhamnolipid and iturin) were evaluated for antifungal studies against *Fusarium oxysporum f.* sp. *ricini*, and fungistatic activity was around 40 μgmL^{-1} (with iturin) and 600 μgmL^{-1} (with rhamnolipid) and hence can be recommended toward their application as biocontrol agents.

Keywords
Biosurfactant · Plant growth promotion · Antifungal activity

18.1 Introduction

In this era of awareness for pesticide-free food and the increase in pesticide resurgent pathogens, rhizosphere microorganisms and/or their metabolites play a significant role in the management of plant diseases. Rhizobacteria belonging to genera *Bacillus* and *Pseudomonas* are known for the production of versatile metabolites and their use in agriculture as plant growth promoters and fungicidal agents (Petatán et al. 2011). Antifungal metabolites produced by the plant growth-promoting rhizobacteria (PGPR) include siderophores, phenazines, pyrrolnitrin, cyclic peptides, biosurfactants, and hydrolytic enzymes such as chitinase and glucanase (Raaijmakers et al. 2010; Rahmoune et al. 2017). Biosurfactants (BS) are surface-active molecules produced by microorganisms and attracted interest as alternatives to current petroleum-based synthetic surfactants. These green compounds have low critical micelle concentration and reduce the air/water surface tension to very low levels. Based on structural variability, they are classified into four classes, i.e., glycolipids, lipopeptides, phospholipids, and polymeric surfactants (Otzen 2017). Recent studies reveal that biosurfactants (rhamnolipids, lipopeptides) are molecules of the twenty-first century with wide prospective and potential applications in the food, cosmetics, pharmaceutical, agricultural and petrochemical industries (Santos et al. 2016). Biosurfactants have drawn the interest of different industries due to their diverse structures, low toxicity, biodegradable nature, and production using renewable/agro-industrial substrates (Radzuan et al. 2017; Nurfarahin et al. 2018). These green surfactants, due to amphipathic structure, promote seed germination, exhibit biocontrol activity by inhibition of phytopathogens and inducing systemic resistance in plants, and also play a significant role in bioremediation of pollutants (Reddy et al. 2016; da Silva et al. 2015). With this focus, we have screened rhizobacteria for plant growth-promoting traits and biosurfactant activity and

evaluated fungistatic activity against *Fusarium oxysporum f.* sp. *ricini.* Plant growth-promoting studies were carried out using maize seeds to evaluate the role of bacteria, rhamnolipid, and iturin on seed germination and plant growth of maize using paper towel method.

18.2 Materials and Methods

18.2.1 Source of Bacterial Isolates

Bacteria were isolated from the rhizosphere of different cereals (rice, sorghum, millets), legumes (pigeon pea, chickpea, groundnut, soybean), oil crops (sunflower, castor), grass, etc. from different districts of Telangana and Andhra Pradesh, India, and maintained as germplasm collection in the laboratory. All the bacterial isolates were purified onto nutrient agar (NA) medium and characterized by morphological and cultural characteristics and screened for plant growth-promoting (PGP) traits.

18.2.2 Screening for Plant Growth-Promoting (PGP) Traits and Antifungal Activity

Based on morphological and cultural characteristics, a total of 107 *Pseudomonas* sp. and 100 *Bacillus* sp. were selected for further studies. All the 207 bacterial isolates were screened for PGP traits (as mentioned below) and antagonistic activity against *F. oxysporum f.* sp. *ricini* obtained from Indian Institute of Oilseeds Research (IIOR), Rajendranagar, Hyderabad.

18.2.2.1 Ammonia Production

For detection of ammonia production by bacterial isolates, 10 mL of peptone water was inoculated with 100 µl of bacterial culture and incubated at 28 ± 2 °C for 48 h. After incubation, 1 mL broth culture was taken in a separate test tube, and 1 mL Nessler's reagent was added and observed for color change (Dey et al. 2004). Development of yellow to orange red color indicate ammonia production.

18.2.2.2 Phosphate Solubilization

Phosphate-solubilizing activity of isolates was checked on Pikovskaya's agar containing tricalcium phosphate, all the bacteria were individually spot inoculated in each Pikovskaya's agar plates, and plates were incubated at 28 ± °C for 4–7 days and observed for P clearing or P solubilization zones around the colonies (Mehta and Nautiyal 2001).

18.2.2.3 Phytase Activity

Phytase activity of isolates was screened on phytic acid medium prepared as mentioned by Richardson and Hadobas (1997). Bacterial isolates were separately spot inoculated on each phytic acid medium, and these plates were incubated at 28 ± 2 °C for 2–4 days and observed for the hydrolysis zone around the colonies.

18.2.2.4 Production of Indole Acetic Acid (IAA)

Bacteria were grown in nutrient agar medium amended with 5 mM of L-tryptophan at 28 ± 2 °C for 2 days followed by overlaying with 10 mm-diameter nitrocellulose membrane (NCM) disk as saturated with Salkowski reagent (Gordon and Weber 1951) and observation for color change of paper to pink.

18.2.2.5 Screening for Siderophore Production

Screening for siderophore production was carried out on CAS agar plate (Schwynn and Neiland 1987; Patel et al. 2018).

18.2.2.6 Antifungal Activity

Antifungal activity of isolates was detected by the dual culture method using plant pathogenic fungus *F. oxysporum f.* sp. *ricini* on potato dextrose agar (PDA) media. An agar block (5 mm diameter) was cut from an actively growing (96-hour-old) fungal culture and placed on the surface of fresh agar medium at the center of Petri plate. A loopful of 24-hour-old culture of each bacterium was streaked in a straight line on one edge of a 90 mm diameter Petri plate; plates were incubated at 28 ± 2 °C, and the inhibition zone was measured 5 days after inoculation. Plates inoculated with the same fungus without bacteria were used as a control. Three replications were maintained for each, and reduction in radial growth was measured, and percent inhibition over control was calculated using the formula (Anandaraj and Thivakaran 2010):

$$I\% = \frac{(C - T) \times 100}{C}$$

where:

I = inhibition % of mycelial growth (growth reduction over control)
C = radial growth of fungus in the control plate (mm)
T = radial growth of fungus on the plate inoculated with bacteria (mm)

18.2.2.7 Screening for Biosurfactant Activity

Bacterial isolates were selected based on plant growth-promoting traits and screened for biosurfactant activity by microplate and penetration methods, oil spread method, blue agar plate method, blood hemolysis test, lipase assay, emulsification activity (EI %), and reduction in surface tension (Plaza et al. 2006).

18.2.2.8 Production of Biosurfactant Using Selective Bacterial Isolates (DR1 and RHNK 22)

Based on biosurfactant activity and reduction in surface tension, two bacterial isolates, DR1 and RHNK 22, were selected as best biosurfactant producers for further studies, and bacterial biosurfactant production was studied using the mineral salt medium (MSM) containing 2% glucose as carbon source, Bushnell-Hass broth medium (BH medium), and nutrient broth (NB) medium. A 24-h log culture of each bacterial strain (1 x10^5 cells mL^{-1}) was inoculated into each of the MSM, BH, and NB media and incubated at 35 ± 2 °C, 180 rpm for 48 to 72 h, followed by extraction of biosurfactant.

18.2.2.9 Extraction of Biosurfactant

Following the incubation, each broth was centrifuged at 10000 rpm for 15 min, and the supernatant was collected. The supernatant was acid precipitated by adding 6 N HCl to bring down the pH to 2 (Kim et al. 2004; Heyd et al. 2008) and kept overnight at 4 °C followed by centrifugation at 10000 rpm for 15 min, and the pellet (precipitate) was collected. The pellet was lyophilized and used for antifungal studies.

18.2.2.10 Antifungal Activity of Biosurfactant

For testing antifungal activity, an agar plug of actively growing fungal culture was placed in the center of the plate. Then, wells were made (using sterile agar borer), 2 cm away from the center where the fungus was placed, and different concentrations of biosurfactant were added in separate wells and incubated at 30 ± 2 °C for 24 to 96 h. Sterile distilled water was used as a control. The inhibition percentage (I %) was calculated using the formula as mentioned above (Anandaraj and Thivakaran 2010).

18.2.2.11 Evaluation of Bacteria, Biosurfactant on Plant Growth of Maize Using the Paper Towel Method

Maize seeds (Variety: Aparanji −1) were surface sterilized using sodium hypochlorite (3%) treated and washed with sterilized distilled water for 3–5 times and dried in laminar. Both the bacterial isolates (DR 1 and RHNK 22) were inoculated into 3 mL NB medium separately, and the cell pellet was prepared and used for seed coating (10^8 CFU per seed) using carboxymethyl cellulose as adhesive. The crude extract of biosurfactant (rhamnolipid and iturin, prepared as mentioned above) was applied at 40 µgmL^{-1} to see their effect on germination and plant growth of maize. Fifty seeds were placed in each paper towel and three replications were maintained. The percentage of germination was assessed by the procedure recommended by International Seed Testing Association (ISTA 1993), and the experiment was incubated for 10 days under greenhouse conditions and assessed for a percentage of germination and seed vigor index and compared with control.

18.2.2.12 Statistical Analysis

All experiments were performed in triplicates, and the results obtained are mean of three independent experiments showing consistent results. ANOVA, means, the

coefficient of variance (CV) %, ranking, and standard errors were calculated using the Microsoft Office Excel (version 7).

18.3 Results

18.3.1 Source of Bacterial Isolates

A total of 500 bacterial isolates were obtained based on morphological variation, cultural characteristics, purified and preserved as glycerol stocks, NA slopes and maintained as germplasm collection at microbiology laboratory of Osmania University, Hyderabad, Telangana, India. Based on cultural characteristics and Gram staining, 107 bacterial isolates were of *Pseudomonas* sp., and 100 isolates belonged to *Bacillus* sp. All the 207 were screened for PGP traits and antagonistic activity.

18.3.1.1 Bacteria with Plant Growth-Promoting Traits, Antifungal, and Biosurfactant Activity

Following the addition of Nessler's reagent in inoculated peptone water, instant color change from yellow to orange-red indicated the production of ammonia by isolates. A clear zone of solubilization due to organic acid production on Pikovskaya's medium and zone of hydrolysis on phytate medium were recorded as inorganic and organic phosphate solubilizers, respectively. Production of IAA production was detected by characteristic pink to purple color on nitrocellulose membrane disk. Bacterial isolates, when inoculated on CAS plate, developed a halo of orange/yellow color, that indicated siderophore production (Fig. 18.1).

Antifungal activity of the bacterial isolates against *F. oxysporum f.* sp. *ricini* ranged from 72 to 80% (data not shown). Out of the total 207 bacterial isolates, 67% of *Pseudomonas* sp. and 58% of *Bacillus* sp. showed one or more than one PGP traits. Five of *Pseudomonas* spp. (DR1, SW7, SHC, SHH, MYKH 12) and five *Bacillus* spp. (RHNK 10, RHNK 22, RHNK 33, RHNK 75, RHNK 85) showed PGP traits and significant inhibition of *F. oxysporum f.* sp. *ricini*, and all the ten bacterial isolates showed biosurfactant activity. Maximum antifungal activity was recorded with *P. aeruginosa* DR 1 and *B. amyloliquefaciens* RHNK 22 (Fig. 18.2).

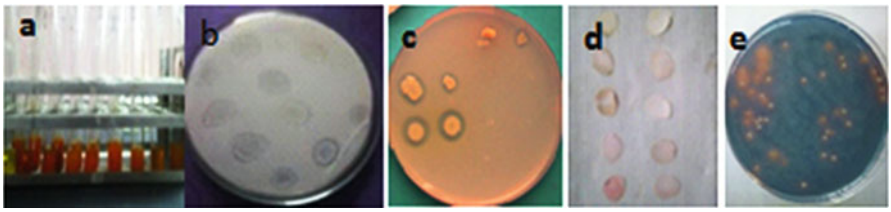

Fig. 18.1 Plant growth-promoting traits of bacterial isolates on different media
a = ammonia production, b = phosphate solubilization, c = phytase activity, d = indole production, e = siderophore production

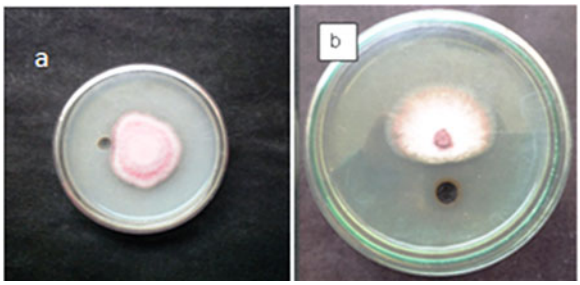

Fig. 18.2 Antifungal activity of biosurfactant against *F. oxysporum f. sp. ricini* a = lipopeptide inhibiting *F. oxysporum* f. sp. ricini; b = rhamnolipid inhibiting *F. oxysporum* f. sp. ricini

18.3.1.2 Production and Detection of Biosurfactant Using Selective Bacterial Isolates (DR1, RHNK 22)

Both the isolates, *P. aeruginosa* DR1 and *B. amyloliquefaciens* RHNK 22, produced varying amounts of biosurfactants; isolates DR1 produced 4.95 $gL^{-1}$and 3.74 gL^{-1} biosurfactant in MSM and BH medium respectively and 2 gL^{-1} in NB medium. RHNK22 showed biosurfactant production in NB medium (270 mgL^{-1}) and in BH medium (250mgL^{-1}).

18.3.1.3 Antifungal Activity of Extracted Biosurfactant

Antifungal activity of crude biosurfactant (rhamnolipid and iturin) against *F. oxysporum f.* sp. *ricini* exhibited fungistatic activity. Both iturin (40 µg mL^{-1}) and rhamnolipid (600 µgmL^{-1}) showed antifungal activity against *F. oxysporum f.* sp. *ricini.*

18.3.1.4 Evaluation of Bacteria on Plant Growth of Maize Using the Paper Towel Method

Seed treatment with bacterial isolates (DR1, SW7, SHC, SHH, MYKH 12, RHNK 10, RHNK 22, RHNK 33, RHNK 75, RHNK 85) promoted seedling emergence, shoot length, root length, and wet weight of maize. The maximum increase (17%) in plant biomass and seed vigor was observed in RHNK 22 inoculated seeds. *Pseudomonas* sp. SHC and *Bacillus* sp. RHNK 22 and RHNK 33 inoculation resulted in an increase in germination (Table 18.1). Rhamnolipid and iturin application also promoted seed germination (11%) and did not show any inhibitory effect on germination of maize and showed a slight increase in plant biomass.

Microorganisms with potential PGP traits and known to promote plant growth has attracted the researchers global wise due to their positive effect on environment and reduction of pollution (Shaikh et al. 2016). In this study, bacteria were isolated from the rhizosphere of different crop samples, during different crop growth periods. Morphological and cultural identification revealed them as 107 *Pseudomonas* spp. and 100 *Bacillus* spp. Preliminary screening revealed 67% of *Pseudomonas* and 58% of Bacillus group showed PGP traits. Bacteria of diverse genera, especially *Pseudomonas* and *Bacillus*, were predominant among the plant growth-promoting and antagonistic bacteria (Podile and Kishore 2006; Hameeda et al. 2006; Narendra Kumar et al. 2017).

Table 18.1 Effect of bacterial isolates, biosurfactant on maize growth by paper (roll) towel method in greenhouse conditions

Isolates	Shoot length (cm)	Root length (cm)	Plant wet weight (mg)	Germination (%)	Seed vigor index
Pseudomonas aeruginosa DR1	11	30	184 (12)	94	2831
Pseudomonas sp. SW7	11	28	170 (4)	92	2587
Pseudomonas sp. SHC	11.5	29	184 (12)	96	2796
Pseudomonas sp. SHH	10	28	162	92	2586
Pseudomonas sp. MYKH 12	10	29	170 (4)	92	2587
Bacillus sp. RHNK 10	12	29	185 (13)	94	2738
Bacillus amyloliquefaciens RHNK 22	11	32	192 (17)	96	4128
Bacillus sp. RHNK 33	12.5	30	180 (10)	96	2893
Bacillus sp. RHNK 75 RHNK 75	12	28	180 (10)	88	2476
Bacillus sp. RHNK 85	11	29	168 (2)	94	2737
Rhamnolipid	11	28	168 (2)	94	2643
Iturin	11	29	170 (4)	95	2766
Control (uninoculated)	9	27	164	90	2439
LSD ($P = 0.05$)	0.9	12	10	6	174
CV (%)	5	3	5	4	5

Data is the mean of 50 seeds (in each roll) with three replications

Two isolates, namely, DR1 and RHNK 22, exhibited potent antifungal activity against *F. oxysporum f.* sp. *ricini*. This antagonism can be attributed to the secretion of an array of antifungal metabolites by these PGPR, and the biosurfactants produced by *Pseudomonas* and *Bacillus* sp. have been implied in biocontrol due to their potential positive effect on competitive interactions with organisms including bacteria, fungi, oomycetes, protozoa, nematodes, and plants (Raaijmakers et al. 2010). Our isolates, RHNK 22 and DR1, also showed highest lipase activity and a significant reduction in surface tension. Detection of biosurfactant activity is usually performed with multiple screening methods such as penetration assay, hemolytic assay, drop collapse assay, oil-spreading capacity, and surface tension reduction (Satpute et al. 2008). Hence we incorporated all the above mentioned screening methods to select potential bacterial isolates with significant biosurfactant activity.

Isolate DR1 produced rhamnolipid while isolate RHNK 22 produced surfactin and iturin; results of the present study are in accordance with previous reports (Romero et al. 2007). Both rhamnolipid and iturin inhibited the fungal pathogen tested in this study. Surfactants from *Pseudomonas* sp. and *Bacillus* sp. are known for antagonism and biocontrol (de Bruijn et al. 2007). Enhanced seed germination and growth promotion of maize without any inhibitory effect by biosurfactant indicated their usefulness for treatment of seeds. Phytotoxicity assay using a chemical surfactant in *Brassica nigra* and *Triticum aestivum* showed inhibitory effect on seed germination, while biological surfactants used were nontoxic (Sharma et al. 2014). A similar study of the use of rhamnolipid for seed development was done at a varied concentration of 250 mgL^{-1} to 1000 mgL^{-1}, and germination of different cultivars (lettuce, corn, soybean, and sunflower) was evaluated (da Silva et al. 2015). After treatment of seeds with rhamnolipid, lettuce showed 75% increase in germination, favored germination of sunflower seeds; which might be due to tension-active nature of rhamnolipid however, soybean did not show any effect. Rhamnolipids, due to their amphipathic structure, may act on external wrapping tissue and enhance the permeability of seeds and hence facilitate germination. This study indicates the importance of biosurfactants both for seed germination and for efficient anti-fungal activity.

18.4 Conclusion

Rhizobacteria are known for their plant growth-promoting and antifungal properties and their use as bioinoculants for sustainable agriculture. However, crop yield and sustainable agriculture are still set back due to the continuous application of chemicals leading to environmental pollution. In this scenario, the metabolites of bacteria (biosurfactants) which are nontoxic and aid in seed germination while inhibiting the growth of fungal pathogens seem to be promising alternatives for sustainable agriculture. However, further studies of evaluating the efficacy of these biosurfactants under greenhouse and field studies will determine their role as potential plant growth promoting and biocontrol agents.

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Compatibility Potential of *Brassica* Species and Mustard Seed Meal with *Pseudomonas fluorescens* for Biological Control of Soilborne Plant Diseases

19

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Contents

Abstract

The biofumigant potential of different *Brassica* sp. and onion for compatibility with PGPR strain of *Pseudomonas fluorescens* under in vitro conditions was studied. The local varieties of biofumigant crops, viz., cabbage, cauliflower, mustard, and onion, were grown in pots under greenhouse conditions. Treatments included were macerated tissue containing shoots, roots, and leaves alone and in combination with one another. Actively growing *P. fluorescens* cultures were streaked onto the inverted bottom of the Petri plate filled with nutrient agar and

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kept above the Petri plate containing macerated plant tissue in different treatments at room temperature for 48–72 h. Results showed that there was no reduction of CFU per plate compared with untreated control. No statistically significant effect was recorded for any of the amended plant material tested against *P. fluorescens* growth. In another study, the growth of *P. fluorescens* was observed for 72 h after continuous exposure to volatiles produced by hydrated mustard seed meal (different quantities) under in vitro conditions. Fungistatic effect was not observed for *P. fluorescens* growth against different concentrations of seed meal, and this attributed tolerance of *P. fluorescent* toxic volatiles produced by seed meal. This work could be important in the future for the integrated use of biofumigants/mustard seed meal along with *P. fluorescens* for the management of plant diseases.

Keywords
Compatibility · Biofumigation · *Brassica* species · Mustard seed meal · *Pseudomonas fluorescens*

19.1 Introduction

A major concern of sustainable agriculture is higher crop yields with reduced chemical fertilizers and pesticides. Integrated use of eco-friendly strategies along with bioproducts often gives synergistic effect for pest management. The combined use of biofumigant (incorporation of the mustard crop) and biocontrol agents like *Trichoderma* and *Fluorescens* paves the way for sustainable management of soilborne plant pathogens. There are several approaches to improve the soil microbial population to promote plant growth by reducing the inoculum of soilborne pathogens. Agronomic practices like crop rotation, the addition of organic matter, tillage operations, application of fertilizers, and chemical fungicides directly affect the population of beneficial microorganisms which are playing a major role in biological control. Recently it was found that the use of Brassicaceae plant species as a cover crop or incorporation into the soil results in good disease control in all cropping systems because of the production of isothiocyanates (ITCs) released by the reaction of glucosinolates (GSLs) with myrosinase enzyme. Incorporation of plants containing GSLs which results in suppression of soilborne plant pathogens and insect pests by their biocidal activity is referred to as biofumigation (Kirkegaard et al. 1993, 1998). During the seventeenth century, unique properties of GSLs and ITCs were first observed when the research was conducted to identify the different taste of mustard seeds (Challenger 1959). GSLs are sulfur-containing compounds produced by some crops and release isothiocyanates when the tissue is macerated and hydrolyzed by myrosinase enzyme by a process called GL-MYR system (Wathelet et al. 2004). It was found that ITCs have toxic effect against several soilborne plant pathogens (Sarwar et al. 1998).

19.2 Concept of Biofumigation

The concept of biofumigation was first reported by Theophrastrus in 300 BC when he observed the harmful effect on vines by the odors of cabbage (Willis 1985). The term biofumigation was coined by Kirkegaard et al. (1993) to describe the suppression of soilborne pathogens by isothiocyanates released by different *Brassica* species. Biofumigation is the process of growing the plants containing GSLs as green manures and incorporating it into the soil resulting in the reduction of soilborne pests (Angus et al. 1994). It is the agricultural practice of using volatile chemicals (allelochemicals) released from the incorporated plant tissues to reduce pest population (Brown and Morra 1997; Rosa 1997). Use of different compounds as soil fumigants was studied as alternatives to methyl bromide for the management of soilborne pests after the ban of chemical fumigants (Duniway 2002; Porter and Mattner 2002). The range of pests suppressed includes germinating weed seeds, nematodes, bacteria, fungi, viruses, and insects. The principle involved in the biofumigation is the release of isothiocyanates, thiocyanates, nitriles, oxazolidine, dimethyl sulfide, and methanethiol from the GSLs containing plant tissues by the action of myrosinase enzyme (Matthiessen and Kirkegaard 2006; Gimsing and Kirkegaard 2008). *Brassica* sp. containing glucosinolates releases toxic volatile isothiocyanates (ITCs) which are effective against different plant pathogens (Kirkegaard et al. 1993; Matthiessen and Kirkegaard 2006; Madhavi et al. 2015).

19.3 The Response of Plant Pathogenic Fungi to Isothiocyanates Released by Biofumigation

The inhibition of fungal pathogens using ITCs is majorly in two ways, fungistatic and fungitoxic. The growth of the fungal organism is slow in the presence of fungistatic compound, whereas the organism was killed in the presence of the fungitoxic compound. Isothiocyanates have a toxic effect on an array of microbial communities (Walker et al. 1937). Mayton et al. (1996) reported that allyl isothiocyanate released from the macerated tissue of *Brassica juncea* cv. Cutlass completely suppressed the growth of five common plant pathogens, *Pythium ultimum*, *Rhizoctonia solani*, *Verticillium dahlia*, *Verticillium albo-atrum*, and *Colletotrichum coccodes*, under in vitro conditions. Among the ten brassica species studied, *Brassica napus* (winter rapeseed) had the highest aboveground biomass (7.7 ± 3.4 kg m^{-2}). They react with sulfur-containing compounds by a simple and unreversible reaction (Brown and Morra 1997). Fungitoxic and fungistatic effects of GSL were reported by Smolinska et al. (2003). Exposure of *Alternaria* fungus to allyl and benzyl isothiocyanates recorded fungitoxic effect on the mycelial growth (Sellam et al. 2006). Several scientists reported that the bioactive compounds released during biofumigation suppressed plant pathogens and weeds and have an impact on soil microorganisms (Matthiessen and Kirkegaard 2006; Hoagland et al. 2008). Effect of biofumigation against different soilborne fungal pathogens like

Rhizoctonia, *Verticillium*, *Colletotrichum*, *Fusarium*, *Pythium*, and *Phytophthora* sp., was studied (Steffek et al. 2006; Mattner et al. 2008; Friberg et al. 2009). Among different *Brassica* spp. tested, maceration of plant tissue of mustard is highly effective in inhibiting the radial growth and sclerotial formation of *R. solani* (Madhavi et al. 2015).

19.4 *Brassica* sp. as Biofumigant

Brassicaceae family consists of several cruciferous vegetables, cabbages, and mustards which are reused as food crops. Among different *Brassica* sp., six species are particularly important (*Brassica carinata*, *B. juncea*, *B. oleracea*, *B. napus*, *B. nigra*, and *B. rapa*) because of high concentration of vitamin C and soluble fibers and oils with antimicrobial properties (Walker et al. 1937). Within 24 h of incorporation of *Brassica* residues, the ITCs concentration had been shown to decrease by 90% (Brown et al. 1991). Inhibition of microbial growth occurs when isothiocyanates react with sulfhydryl groups, amine groups, and the disulfide bonds of proteins causing the degradation of enzymes (Brown and Morra 1997). Based on the side organic chain, approximately 20 different GSL were found. The concentrations of glucosinolates differ with the age of the plant and conditions in which they are grown (Kirkegaard and Sarwar 1998). It was found that aliphatic GSLs degrade more easily when compared to aromatic GSLs. Shoots contain aliphatic ITCs whereas the aromatic ITCs are more prevalent in roots (Kirkegaard and Sarwar 1998).

Different cultivars of *Brassica* species have varying levels of efficacy due to the difference in concentration and types of the glucosinolates present in their plant tissue (Kirkegaard et al. 1996; Kirkegaard and Sarwar 1998). The effective fungal suppression by *Brassica* plant materials mainly depends upon the concentration and type of toxic volatile gases released (Kirkegaard et al. 1996). Blok et al. (2000) reported increased yields in the biofumigant treatments, although it was not significantly related to disease suppression induced by the release of ITCs into the soil. This result might be related to the increased anaerobic soil conditions created following the incorporation of green manure into the soil compared to the fallow (+) VIF treatment.

Smolinska et al. (1997) studied the efficacy of *Brassica napus seed* meal against *Aphanomyces* causing root rot of pea. Seed inoculated with zoospore suspensions and incubated for 24 h in the presence of toxic volatiles released from rapeseed meal had 50% decreased root rot disease severity than in the control treatment. The results indicated that *B. napus* volatile compounds released by hydrolysis were responsible for toxic effects against *Aphanomyces euteiches* f. sp. *is*.

In greenhouse trials the impact of *Brassica napus* seed meal on the microbial complex that causes apple to replant disease was studied by Mazzola et al. (2001) who found that seed meal amendment at a rate of 0.1% (vol/vol) significantly increased the growth of apple and decreased apple root infection by *Rhizoctonia* and *Pratylenchus penetrans* regardless of glucosinolate content. The impact of *Brassica napus* seed meal on the microbial complex that causes apple to replant

disease was studied in greenhouse trials by Mazzola et al. (2001) who found that seed meal amendment at a rate of 0.1% (vol/vol) significantly increased the growth of apple and decreased apple root infection by *Rhizoctonia* and *Pratylenchus penetrans* regardless of glucosinolate content. Seed meal amendments improved the soil microbial communities of total bacteria and actinomycetes which were phytotoxic to apple when applied at a rate of 2% (vol/vol).

Cohen et al. (2005) found that suppression of *R. solani* by *Brassica napus* seed meal was related to significant changes in soil microbial population but not related to levels of glucosinolate. Larkin and Griffin (2007) documented the significant reduction of *R. solani* population in greenhouse assays and in field tests followed by incorporation of *B. juncea* plant tissue, resulting in disease suppression.

The enzyme myrosinase hydrolyzes the glucosinolates (thioglucoside glucohydrolase) (Brown and Morra 1997; Kirkegaard et al. 2000; Matthiessen and Kirkegaard 2006) into ITCs, thiocyanates, nitriles, epithionitriles, and oxazolidinediones (Fahey et al. 2001). Dhingra et al. (2004) also found that mustard oil concentration (*Brassica rapa*) significantly reduced the saprophytic growth of *R. solani* both in vitro and in field soils, but for field conditions, high concentrations of oil were required. These products, especially ITCs, are highly toxic to several microorganisms and can be used as biofumigants (Fahey et al. 2001; Yulianti et al. 2007). It has been reported that after 1–2 weeks of incorporation, no ITC could be detected (Gimsing and Kirkegaard 2006). The degradation rate of ITCs can be influenced by soil type also (Gimsing et al. 2006). In clay soils degradation of ITCs is faster than in sandy soil because clay soil contains more nutrients and microbial populations and stabilizes cellular enzymes through the adsorption to clay particles. Larkin and Griffin (2007) reported that plants of the family Brassicaceae contain GSLs that are hydrolyzed into various toxic volatiles upon tissue incorporation. *Brassica* species include cabbage (*Brassica oleracea*), broccoli, radish (*Raphanus sativus*), canola (*Brassica napus*), cauliflower, mustard (*Brassica juncea*), and white mustard (*Sinapsis alba*), and the volatiles released from chopped leaf material of *Brassica* crops inhibited the growth of a range of soilborne plant pathogens of potato, including *R. solani*, *Phytophthoraerythroseptica*, *Pythium ultimum*, *Sclerotinia sclerotiorum*, *and Fusarium sambucinam*, with Indian mustard resulting in absolute inhibition (80–100%) in vitro. When compared with plant pathogens, potato, *Trichoderma virens*, and *Penicillium chrysogenum* were much less sensitive to inhibition effects from Indian mustard. Incorporation of *Brassica* plants improves organic matter and soil structure and increases beneficial microbial populations as well as their biocidal effects on plant pathogens. The persistence ITCs up to 45 days of incorporation was demonstrated (Gimsing and Kirkegaard 2008; Poulsen et al. 2008).

Galletti et al. (2008) studied the tolerance of the 40 isolates of *Trichoderma* sp. to the *Brassica carinata* seed meal in vitro for the integrated use of both in field conditions and found that *Trichoderma* spp. are more tolerant than the plant pathogens (*P. ultimum*, *R. solani*, *F. oxysporum*), and at the highest dose (10 μmole of sinigrin), fungistatic effect was observed. Rahmanpour et al. (2009) studied the response of toxic volatiles produced by the glucosinolate-myrosinase system against *Sclerotinia sclerotiorum* by exposing mycelial plugs to inoculated

leaf discs of oilseed rape cultivars and two related species, black mustard (*Brassica nigra*) and white mustard (*Sinapis alba*). The growth rate of fungal colonies was inhibited by higher than 87% compared with control. Repeated exposure of fungus to hydrated mustard seed powder resulted in growth inhibition decreasing from initial levels up to 80% after 2–3 days of exposure, indicating that *S. sclerotiorum* has the ability to adapt to volatiles released during biofumigation.

Fayzalla et al. (2009) observed the efficacy of mustard seed meal at different levels against *R. solani* in vitro and found that the linear growth of the *R. solani* was inhibited (3.8, 1.2, and 0.7 cm) at 5, 10, and 25 mg of seed meal, respectively. Results of field experiment demonstrated that mustard seed meal reduced the disease incidence over the control by 69.7% after planting for 4 months.

Motisi et al. (2009) studied the effect of incorporation of *Brassica juncea* aboveground parts (AP), belowground parts (BP), or both (AP + BP) into the soil in controlled conditions on the persistence of infection caused by *R. solani* and *G. graminis* var. tritici. Long-term persistence of all types of the residue was recorded and retained at least 13 days, while the glucosinolates contained in AP (20.9 μmol sinigrin g^{-1} dry matter) and BP (2–3 μmol gluconasturtiin g^{-1} dry matter) were hydrolyzed in less than 3 days. The toxic effect of residues may be due to the release of isothiocyanates during the first few days following incorporation, but the other mechanisms contributing to long-lasting persistence may not be determined. *B. juncea* plant species contain high levels of glucosinolates in their plant tissue resulting in effective inhibition of fungal growth. As the dose of *B. juncea* plant tissue amended into soil increases, AITC emission increases, and consequently antimicrobial activity becomes stronger. Even at an application rate, up to 1.0% (wt/wt) of *B. juncea* amendment completes the AITC emission within 72 h of post-amendment. Disease suppression is attributed to the efficiency of glucosinolate conversion into isothiocyanates (Mazzola and Zhao 2010).

The influence of Brassica sp. as plant material or its seed meal has been tested by several researchers against different microbial populations (Vera et al. 1987; Williams-Woodward et al. 1997; Mazzola et al. 2001; Cohen and Mazzola 2006; Hoagland et al. 2008; Friberg et al. 2009; Omirou et al. 2013).

Yulianti et al. (2007) reported that incorporation of *Brassica* green manure into the soil reduced the population of *R. solani* pathogen. Incorporation takes place at a depth of 10–20 cm (Bellostas et al. 2007). After crushing the plant material, the GSL products are released (Motisi et al. 2010). In a study conducted by Friberg et al. (2009), the incorporation of mustard (*Brassica juncea*) as a green manure decreased the propagules of *R. solani* in the soil. *R. solani* produce pseudosclerotia or thick-walled hyphae as survival bodies; hence high concentrations of GSL hydrolysis are needed to decrease the pathogen growth. The survival propagules are less susceptible to hydrolysis products of GSL; this character limits the use of mustard green manure to suppress fungal pathogens (Yulianti et al. 2006).

It was demonstrated that the compounds released from certain *Brassica* cultivars inhibited potato pathogens, viz., *P. ultimum*, *R. solani*, *F. sambucinam*, and *V. dahlia* (Charron and Sams 1999; Mayton et al. 1996).

19.5 Response of Biocontrol Agents Against Biofumigation

When compared to fungi and oomycetes, bacteria and actinomycetes are more resistant to volatile toxics (Kreutzer 1963). When Canola (*Brassica napus*) is plowed into the soil as a green manure, crop suppression of soilborne pathogens was recorded. Roots of *B. napus* green manure crops contribute as the major source of biocidal ITCs when the crop is plowed into the soil (Gardiner et al. 1999). Smith and Kirkegaard (2002) reported that the sensitivity of fungi and oomycetes is higher for aromatic ITCs than for aliphatic ITCs as the 2-phenylethyl ITC was the product of the root GSL of canola. It was found that the persistent use of ITCs may directly suppress soilborne pathogens or indirectly cause changes in the diversity of soil microbial communities. Among the eukaryotes, *Aphanomyces*, *Gaeumannomyces*, *Phytophthora*, and *Thielaviopsis* were very sensitive, whereas the *Trichoderma* spp. were the most tolerant to 2-PE ITC. Different anastomosis groups of *Rhizoctonia solani* and *Pythium* sp. exhibited varied responses to 2-PE ITC. Generally, bacteria were found to be more tolerant to 2-phenylethyl ITC than the eukaryotic organisms tested, and *Trichoderma* spp. were found to be more tolerant than the other eukaryotes tested, including *Aphanomyces*, *Gaeumannomyces*, *Phytophthora* sp., *Thielaviopsis*, *Rhizoctonia solani*, and *Pythium* sp.; 2-phenylethyl ITC was found to be suppressive to all microorganisms tested, even though the lethal dose varies attributing its potential in biofumigation. The incorporation of brassica plant material for biofumigation has been shown to increase or decrease the population of the microorganisms such as *Trichoderma* sp., *Pythium* sp., fluorescent pseudomonads, *Streptomyces* sp., actinomycetes, and other antagonists present in the rhizosphere depending on the plant species and soil type (Cohen and Mazzola 2006; Perez et al. 2007; Mazzola and Zhao 2010). The addition of plant materials can improve the native microbial populations with competition, parasitism, antagonism, and predation against the soilborne pests (Raaijmakers et al. 2009). The growth of *R. solani* f. sp. *sasakii*, *T. harzianum*, and *P. fluorescens* was monitored for 72 h after exposure to volatiles released from different quantities of hydrated mustard powder in vitro, and it was found that with the increase in concentration of mustard seed powder, there was a gradual decline in the growth of *R. solani*, whereas the inhibition of growth of *T. harzianum* was less compared to *R. solani*. Fungistatic effect was not observed for *P. fluorescens* growth, and this indicates the tolerance of *P. fluorescens* to mustard seed meal (Madhavi et al. 2016).

19.6 Biofumigant Incorporation Methods

The common method is the *Brassica* crops are grown on the field before sowing of the main crop as green manure, and at the time of flowering, the plant material was chopped, mulched, and pulverized and plowed into the soil (Matthiessen and Kirkegaard 2002). The green manuring process will disrupt the *Brassica* tissues, and hydrolysis of glucosinolates takes place, releasing isothiocyanates into the soil. In addition to this, the incorporation of *Brassica* or canola seed meal in the soil can

be done because the seeds may contain high concentrations of the glucosinolates release of isothiocyanates (Borek and Morra 2005). Added dried green manure plant material can be plowed into the soil by the addition of water resulting in the release of the toxic allelochemicals (Lazzeri et al. 2004).

19.7 Materials and Methods

19.7.1 Compatibility of the Biofumigant Potential of Brassica Species with *Pseudomonas fluorescens*

Brassica sp. contains glucosinolate compounds which release volatile isothiocyanates (ITCs) by hydrolysis with myrosinase enzyme and are toxic to different plant pathogens; hence an experiment was conducted to evaluate the potential of different *Brassica* sp. to release isothiocyanates by the maceration of different plant parts against *P. fluorescence* in vitro. Local varieties of different *Brassica* species such as cabbage (*Brassica oleracea* var. capital) cauliflower (*Brassica oleracea* var. botrytis), mustard (*Brassica nigra*) and onion (*Allium cepa*) were grown in pots in the greenhouse for the bioassay studies. Crushed plant material method (Kirkegaard et al. (1996)) was used for the bioassay of volatiles released from different *Brassica* sp. against *P. fluorescens.* Whole plants were harvested at the time of flowering, roots were washed to remove soil, and later shoots and roots were separated. Separated plant material was dried on absorbent paper. Later, leaves, shoots, and roots of each species were taken separately, then **roughly** chopped, and then macerated with a mortar and pestle. Additional water was not required for maceration other than that contained in the tissues themselves. Macerated tissue was immediately placed on the lids of inverted Petri plates. Three-day-old culture of *P. fluorescens*, grown on KB medium, was streaked on Petri dishes containing nutrient agar medium. For control plates, no plant material was added, but they were also inverted.

19.8 Results and Discussion

The results of the effect of volatiles released from the macerated tissue of different *Brassica* sp. against *P. fluorescens* were presented in Table. 19.1. The growth of *P. fluorescens* was categorized as good (+++), moderate (++), and poor (+) based on visual observation of colony-forming units.

Macerated leaves – Good growth of *P. fluorescens* was observed in treatments of macerated leaves of cabbage, cauliflower, mustard, and onion. *P. fluorescens* recorded good growth in all the treatments at all three concentrations. Fungistatic effect was not observed for *P. fluorescens* growth in all the treatments.

Macerated shoots – In all the treatments at all the concentrations, good growth of *P. fluorescens* was recorded.

Table 19.1 Effect of volatiles released from different plant parts of different *Brassica* species against the growth of *Pseudomonas fluorescens* under in vitro conditions

	The growth of *Pseudomonas fluorescens*											
	Cabbage			Cauliflower			Mustard			Onion		
Macerated Mustard plant part	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
Leaf	++	+++	+++	++	+++	+++	+++	+++	+++	++	+++	+++
Shoot	++	+++	+++	++	+++	+++	+++	+++	+++	++	+++	+++
Root	++	+++	+++	++	+++	+++	+++	+++	+++	++	+++	+++
Whole plant	++	+++	+++	++	+++	+++	+++	+++	+++	++	+++	+++
Control	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

[1]Rate of growth of *P. fluorescens* strain: +++ = good; ++ = moderate; + = poor; and − = no growth

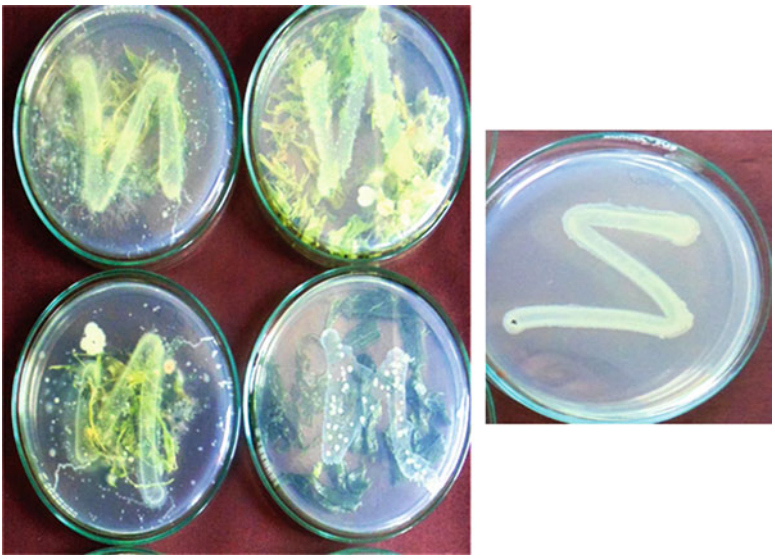

Fig. 19.1 Effect of volatiles released from whole plant maceration of different *Brassica* species against the growth of *P. fluorescens* under in vitro conditions after 72 h

Macerated roots – *P. fluorescens* growth was moderate after 24 h in all cabbage, cauliflower, mustard, and onion root macerated treatments. Fungistatic effect was observed for 24 h; later the growth was normal in all the treatments and at all the concentrations.

Whole plant tissue – Good growth of *P. fluorescens* was observed in treatments of macerated whole plant tissue of cabbage, cauliflower, mustard, and onion. Fungistatic effect was not observed for *P. fluorescens* growth in all the treatments at all three concentrations (Fig. 19.1).

These results indicated the tolerance of *P. fluorescens* to macerated tissue of all *Brassica* species; hence *P. fluorescens* can be integrated with the biofumigant crop for the management of soilborne pathogens. This finding is in accordance with the report of Smith and Kirkegaard (2002) as bacteria were found to be generally more tolerant of 2-phenylethyl ITC than the eukaryotic organisms tested. Depending on the plant species and soil type, the incorporation of *Brassica* plant material increases or decreases the population of the beneficial microorganisms such as *Trichoderma* sp., fluorescent pseudomonads, *Streptomyces* sp., actinomycetes, and other antagonists (Mazzola et al. 2001). Total bacteria, *Trichoderma* sp., and *Pseudomonas* sp. population was high in all biofumigant treatments 50 days after incorporation of mustard plant material in both greenhouse and field studies (Madhavi and Umadevi 2018).

Table 19.2 Effect of volatiles released from mustard seed powder at different concentrations against the growth of *Pseudomonas fluorescens* in vitro

Treatment	The growth of *P. fluorescens*[a]		
	24 h	48 h	72 h
1 g	++	+++	+++
2 g	++	+++	+++
5 g	++	+++	+++
Control	++	+++	+++

[a]Rate of growth of *Pseudomonas fluorescens* strain 8: +++ = good; ++ = moderate; + = poor; and − = no growth

19.9 Compatibility of *P. fluorescens* with Different Concentrations of Mustard Seed Meal

The present study was conducted under in vitro conditions as per the procedure of Rahmanpour et al. (2009) to test the compatibility of *P. fluorescens* with mustard seed meal. Commercially available mustard seeds were taken, and the mustard seed meal was prepared using a pestle and mortar just before the experiment. Mustard powder weighing up to 1 g, 2 g, and 5 g was added in the aluminum foil which was kept in the lid of the Petri plate. Sterile distilled water (10 μL mg^{-1}) was added to the mustard powder to hydrolyze the glucosinolates (GSLs). Twenty ml of NA medium was poured into 90 mm sterilized Petri plates and allowed to solidify. The freshly grown culture of *P. fluorescens* was streaked on the Petri plate. The Petri plate was turned upside down so that the bottom plate contained the mustard powder. Control plates without mustard powder were maintained. The plates were incubated at 26 ± 2 °C for 3 days. The *P. fluorescens* cultures were exposed to volatiles for 3 days with the replacement of fresh mustard powder every 24 h. Six replications were maintained for each treatment. The data for the growth of *P. fluorescens* was measured at 24, 48, and 72 h, and the growth of *P. fluorescens* was categorized as good (+++), moderate (++), and poor (+) based on visual observation of colony-forming units.

19.10 Tolerance of *P. fluorescens* to Mustard Seed Powder

The results pertaining to tolerance of *P. fluorescens* against different concentrations of mustard seed powder are presented in Table 19.2. The growth of *P. fluorescens* was categorized as good (+++), moderate (++), and poor (+) based on visual observation of colony-forming units. *P. fluorescens* recorded good growth in all the treatments at all time intervals. Fungistatic effect was not observed for *P. fluorescens* growth in all the treatments.

This indicates the tolerance of *P. fluorescens* to mustard seed meal. This finding is in accordance with the report of Smith and Kirkegaard (2002) as bacteria were found to be generally more tolerant of 2-phenylethyl ITC than the eukaryotic organisms

tested. Mazzola et al. (2001) reported that *B. napus* cv. Dwarf Essex seed meal applied at lower rates enhanced the populations of fluorescent *Pseudomonas* sp., but these bacteria were not recovered from soils amended with seed meal at a rate of 2% (vol/vol).

19.11 Conclusions

*P. fluorescen*s were compatible with the macerated tissues of different biofumigant crops, cabbage, cauliflower, mustard, and onion, in different concentrations in vitro. Fungistatic effect was not observed for *P. fluorescens* growth when exposed to volatiles of mustard seed meal, and this indicates the tolerance of *P. fluorescens* to mustard seed meal. This work could be important in the future to integrate the use of biofumigants/mustard seed meal in combination with *P. fluorescens* for biological control of plant pathogens.

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Diversity Assessment of Antagonistic *Trichoderma* Species by Comparative Analysis of Microsatellites

20

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Contents

Abstract

Recent genome sequencing of *Trichoderma* species has opened the way for their diversity in the assessment and comparative analysis of microsatellites. Here, we compared microsatellites of three taxonomically different *Trichoderma* species such as *T. asperellum*, *T. citrinoviride*, and *T. longibrachiatum.* Our results highlight abundance and diversity of microsatellite patterns in all the test genomes. The highest relative abundance (283.6) with a relative density (3424.7) of SSRs was identified in all three sequence sets of *T. citrinoviride*. Among all three sequence sets, selection of these isolates were based on maximum frequency of SSRs which was of trinucleotide repeats (79.8%), whereas the dinucleotide repeat represented <8%. With the help of bioinformatics tools,

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species-specific diagnostic primers were developed and validated by *silica* PCR. Out of 31 primer sets, only 18 pairs of primer indicated successful amplification among all the species tested. A total of 34 alleles were detected and 7 loci have polymorphism information content (PIC) values greater than 0.40. In cross-species examination, of 31 markers, amplification of 5 of them was in the corresponding microsatellite regions of 18 different isolates of *Trichoderma* and showed monomorphic banding pattern. Microsatellite locus ThSSR3 was highly specific for *Trichoderma*, as amplification was not detected in closely related other 29 taxa. Primer set, ThSSR3F/ThSSR3R, amplified a specific amplicon of 600 bp from all *Trichoderma* species. According to intensive literature survey, the motif-based comparison is first reported here and was performed in recently sequenced three different species of *Trichoderma*.

Keywords
Trichoderma sp. · Biocontrol · Diversity · Microsatellite · Allele

20.1 Introduction

Trichoderma is a hyper-diverse genus and gained importance due to their antagonistic capability in contradiction of plant pathogen in crop plants. They have different mechanisms of parasitism, stimulation of plant growth, competition for nutrients, and induction of systemic resistance against abiotic and biotic stresses (Kubicek et al. 2011; Solanki et al. 2011; Woo et al. 2006). *Trichoderma* species such as *T. asperellum* (TAs), *T. citrinoviride* (TC), and *T. longibrachiatum* (TL) are a well-known antagonist and are being used in agriculture and allied sectors (Carpenter et al. 2005; Hermosa et al. 2001; Rai et al. 2016). Among *Trichoderma* species, high levels of morphological variability from different geographical regions and hosts have been observed, and therefore their differentiation is very difficult (Bissett et al. 2003; Błaszczyk et al. 2011; Chaverri et al. 2015; Jaklitsch and Voglmayr 2015). To circumvent these problems, it is utmost important to develop the capability to discriminate between closely related species and isolates. To characterize genetic variation and diversity within and among *Trichoderma* populations, molecular markers such as restriction fragment length polymorphism (RFLP) analysis (Dodd, Hill, and Stewart 2004), random amplified polymorphic DNA (RAPD) analysis (Chakraborty et al. 2011), amplified fragment length polymorphism (AFLP) analysis (Al-Sadi et al. 2015; Buhariwalla et al. 2005), sequence-characterized amplified region (SCAR) analysis (Cordier et al. 2007), cleaved amplified polymorphic sequence (CAPS) (Skoneczny et al. 2015), inter-simple sequence repeats (ISSRs) analysis (Shahid et al. 2014), and sequence analysis (Contreras et al. 2013) have been developed. Besides these, the loci containing microsatellites themselves could also be ideal markers to study genetic variation and gene flow because of their high polymorphism, containing a wide range of repeats and usually undergo Mendelian inheritance. Microsatellite simple sequence repeats (SSRs) are random repeated DNA sequences of 1–6 bp, and they have abundant and random distribution

throughout the genome (Dutech et al. 2007). Due to reproducibility, codominant inheritance, multi-allelic nature, wide abundance and genome coverage potential of SSR markers in comparative genomics, genetic fingerprinting, and evolutionary studies are considered (Kumar et al. 2013; Singh et al. 2014). This has prompted us to utilize whole genome and EST resources available for several species of *Trichoderma* including *T. harzianum* (Scherm et al. 2009), *T. reesei* (Martinez et al. 2008), *T. atroviride* (Seidl et al. 2009), *T. asperellum* (Liu et al. 2010), *T. virens* (Kubicek et al. 2011), *T. longibrachiatum* (Xie et al. 2015), and *T. citrinoviride* (http://genome.jgi.doe.gov/Trici1/Trici1.home.html) for the development of reliable microsatellite markers. In the light of this, the present study is undertaken to (i) determine the frequency and distribution of microsatellites in the transcripts' consensus of *Trichoderma* spp.; (ii) find correlation between fungal SSR content and genome size; (iii) access sharing of all SSR motifs and also investigate the uniqueness of SSRs in available transcripts of three *Trichoderma* spp.; and (iv) develop and validate the monomorphic and polymorphic markers for easier identification of members of the genus *Trichoderma* and to study the overall fungal diversity, respectively.

20.2 Materials and Methods

20.2.1 Genome Survey

Three *Trichoderma* species were selected on the basis of their completed and easily accessible genomic sequences in public databases. Annotated transcripts of *Trichoderma asperellum* (TAs), *T. citrinoviride* (TC), and *T. longibrachiatum* (TL) sequences were downloaded from "Joint Genome Institute" (http://genome.jgi.doe.gov).

20.2.2 SSR Analysis

The sequence data screened for SSR motifs were obtained from the whole genome sequences available in the public domain. Using WebSat online software, the retrieved sequences were analyzed (Martins et al. 2009). Parameters were set for detection of perfect di-, tri-, tetra-, penta-, and hexanucleotide motifs with a minimum of three repeats. Microsoft Excel 2013 was used to process and count within the data. SSRs' analysis was performed based on their types (mono- to hexanucleotides), a number of repeats, the frequency of occurrences of each SSR motif, and their distribution in the sequence. Forward and reverse primers were designed for the identified SSR using primer3 software with the default parameters. The relative abundance and density of SSRs were calculated as described by Kumar et al. (2013). For PCR amplification, 31 SSR primers representing 6 from each species of *T. harzianum* and *T. virens* and the rest 19 from *T. asperellum* were randomly selected.

The allocation of repeats was inspected within transcribed sequences in order to comprehend improved outcomes of evolutionary relationship among the species of *Trichoderma*. Microsoft Excel workbook, 2013, was used to analyze the sharing of repeats within the transcripts. Identified motifs were placed in transcript sequences of one species and checked for its corresponding item in the transcripts of remaining species in Excel spreadsheets. Common transcripts were chosen if motif was present in all the transcript sequences. The motif with no matching transcripts was deliberated as unique.

20.2.3 Amino Acid Prediction and Estimation

The amino acid prediction was made with an assumption that trinucleotide SSR sequences had an equal chance to be translated in all the possible reading frames of the trinucleotide repeats. For instance, SSR sequences GCTGCTGCTGCTGCTGCT can be translated in three different frames: (i) GCT GCTGCTGCTGCTGCT, which will be translated into Ala-Ala-Ala-Ala-Ala-Ala; (ii) CTG CTGCTGCTGCTGCTG, which will be translated into Leu-Leu-Leu-Leu-Leu-Leu; and (iii) TGC TGCTGCTGCTGCTGC, which will be translated into Cys-Cys-Cys-Cys-Cys-Cys. Only one of the three possible reading frames would be used to generate the "observed" amino acid repeats. Only trinucleotide repeats falling in coding regions were taken into consideration and examined for the possible encoded amino acid motif.

20.2.4 *Trichoderma* Isolates and Culture Conditions

A set of five isolates of antagonistic *Trichoderma* include fourteen of *T. harzianum* (4-NBAIM and 9-Uttaranchal), eight of *T. virens* (4-NBAIM and 4-Uttaranchal), seven of *T. viride* (4-NBAIM and 3-Uttaranchal), five of *T. asperellum* (3-NBAIM and 2-Uttaranchal), five of *T. koningii* (4-NBAIM and 1-Uttaranchal), and one of *T. atroviride* (1-Uttaranchal), obtained from National Agriculturally Important Microbial Culture Collection (NAIMCC, NBAIM), Mau, Uttar Pradesh, India (Table 20.1). Selection of these isolates was done on the basis of diverse host sources and geographic origin (Table 20.1). The culture of fungal isolates was done on potato dextrose agar (PDA) (Hi-Media, India) at 28 ± 2 °C for 2–5 days.

20.2.5 Amplification of Markers

Extraction of total genomic DNA and amplification of microsatellite markers were performed as described by Kumar et al. (2013). Polymorphism score of individual isolates for each of the selected SSR primers was generated by Jaccard's similarity coefficient (Niwattanakul et al. 2013) using NTSYS-PC, software version 2.1

Table 20.1 Details of *Trichoderma* isolates used in the diversity analysis of microsatellites

Accession no/culture code	Species name	Code	Biological origin	Geographical region
NAIMCC-F-01738	*Trichoderma harzianum*	Th1	Sugarcane rhizospheric soil	Coimbatore, Andhra Pradesh
NAIMCC-F-01741	*Trichoderma harzianum* MTCC7105	Th2	Soybean rhizospheric soil	Indore, Madhya Pradesh
NAIMCC-F-02033	*Trichoderma harzianum* S1–2	Th3	Crop rhizospheric soil	Bharatpur, Rajasthan
NAIMCC-F-02039	*Trichoderma harzianum* TH-4	Th4	Crop rhizospheric soil	Andhra Pradesh
NAIMCC-F-02564	*Trichoderma harzianum* PBAT-42	Th5	Rice rhizospheric soil	Uttarakhand, Uttaranchal
NAIMCC-F-01797	*Trichoderma virens*	Tvr1	Crop rhizospheric soil	Varanasi, Uttar Pradesh
NAIMCC-F-02045	*Trichoderma virens* MML-3152	Tvr2	Crop rhizospheric soil	Chennai, Tamil Nadu
NAIMCC-F-02231	*Trichoderma virens* tri-15	Tvr3	Cotton rhizospheric soil	Shakthinagar, Karnataka
NAIMCC-F-02529	*Trichoderma virens* PBAT-07	Tvr4	Crop rhizospheric soil	Uttarakhand, Uttaranchal
NAIMCC-F-01824	*Trichoderma viride*	Tvd1	Wheat rhizospheric soil	New Delhi
NAIMCC-F-01825	*Trichoderma viride*	Tvd2	Garden soil	Varanasi, Uttar Pradesh
NAIMCC-F-01953	*Trichoderma viride* RHS/T-460	Tvd3	Tea rhizospheric soil	Darjeeling, West Bengal
NAIMCC-F-02060	*Trichoderma viride* TV-12	Tvd4	Wheat rhizospheric soil	Andhra Pradesh
NAIMCC-F-01713	*Trichoderma asperellum*	Ta1	Crop rhizospheric soil	Mau, Uttar Pradesh
NBAIM-F-01937	*Trichoderma asperellum* UTP-16	Ta2	Crop rhizospheric soil	Uttaranchal
NBAIM-F-02170	*Trichoderma asperellum* TWD1	Ta3	Crop rhizospheric soil	Andaman and Nicobar
NBAIM-F-01758	*Trichoderma koningii* CABI- 304055	Tk1	Sugarcane rhizospheric soil	Pantnagar, Uttaranchal
NBAIM-F-01759	*Trichoderma koningii* CABI-304059	Tk2	Sugar beet rhizospheric soil	Pantnagar, Uttaranchal
NBAIM-F-01760	*Trichoderma koningii*	Tk3	Tomato rhizospheric soil	Andaman and Nicobar
NBAIM-F-01761	*Trichoderma koningii*	Tk4	Crop rhizospheric soil	Mau, Uttar Pradesh
UNT60	*Trichoderma harzianum*	Th6	Tomato rhizospheric soil	Udham Singh Nagar, Uttaranchal
UNT64	*Trichoderma harzianum*	Th7	Tomato rhizospheric soil	Udham Singh Nagar, Uttaranchal

(continued)

Table 20.1 (continued)

Accession no/culture code	Species name	Code	Biological origin	Geographical region
UNT68	*Trichoderma harzianum*	Th8	Tomato rhizospheric soil	Udham Singh Nagar, Uttaranchal
NAT69	*Trichoderma harzianum*	Th9	Tomato rhizospheric soil	Nainital, Uttaranchal
NAT70	*Trichoderma harzianum*	Th10	Tomato rhizospheric soil	Nainital, Uttaranchal
ALT73	*Trichoderma harzianum*	Th11	Tomato rhizospheric soil	Almora, Uttaranchal
DET89	*Trichoderma harzianum*	Th12	Tomato rhizospheric soil	Dehradun, Uttaranchal
DET94	*Trichoderma harzianum*	Th13	Tomato rhizospheric soil	Dehradun, Uttaranchal
HAT96	*Trichoderma harzianum*	Th14	Tomato rhizospheric soil	Haridwar, Uttaranchal
UNT38	*Trichoderma atroviride*	Tatr1	Tomato rhizospheric soil	Udham Singh Nagar, Uttaranchal
UNS63	*Trichoderma koningii*	Tk5	Tomato rhizospheric soil	Udham Singh Nagar, Uttaranchal
UNT13	*Trichoderma asperellum*	Ta4	Tomato rhizospheric soil	Udham Singh Nagar, Uttaranchal
UNT70	*Trichoderma asperellum*	Ta5	Tomato rhizospheric soil	Udham Singh Nagar, Uttaranchal
UNS28	*Trichoderma virens*	Tvr5	Tomato rhizospheric soil	Udham Singh Nagar, Uttaranchal
UNS30	*Trichoderma virens*	Tvr6	Tomato rhizospheric soil	Udham Singh Nagar, Uttaranchal
NAS46	*Trichoderma virens*	Tvr7	Tomato rhizospheric soil	Nainital, Uttaranchal
ALS47	*Trichoderma virens*	Tvr8	Tomato rhizospheric soil	Almora, Uttaranchal
UNT09	*Trichoderma viride*	Tvd5	Tomato rhizospheric soil	Udham Singh Nagar, Uttaranchal
DET02	*Trichoderma viride*	Tvd6	Tomato rhizospheric soil	Dehradun, Uttaranchal
NAT03	*Trichoderma viride*	Tvd7	Tomato rhizospheric soil	Nainital, Uttaranchal

(Padoni 2000; Rohlf 2000). The heterozygosity (He) and polymorphism information content (PIC) were measured as described by Botstein et al. (1980).

20.2.5.1 Microsatellites as Diagnostic Marker

Microsatellite locus (ThSSR3F/ThSSR3R) as a specific diagnostic marker of *Trichoderma* genus was assessed by performing PCRs as described by Singh et al. (2014). Different *Trichoderma* species (*T. harzianum*, *T. virens*, *T. viride*,

T. asperellum, *T. koningii*, *T. pseudokoningii*, *T. erinaceum*, *T. hamatum*, *T. longibrachitum*, *T. fasciculatum*, *T. atroviride)* and other common fungi (*Alternaria tenuissima*, *A. porri*, *A. sesame*, *A. solani*, *A. brassicae*, *Fusarium udum*, *F. lycopersici*, *F. ciceri*, *F. solani*, *C. falcatum*, *and C. gloeosporioides*) were included in the study.

20.3 Results and Discussion

It is known that microsatellite variation sometimes leads to an alteration in gene expression leading to phenotypic variation and thus microsatellite variations could be implicated as a key player in the process of adaptation and evolution of organisms (Grover and Sharma 2011; Jain et al. 2014). Thus, the study was undertaken to perform comparative in silico survey of conservation and distribution of microsatellites in the transcripts of three sequenced *Trichoderma* species to describe their genetic diversity.

20.4 Abundance and Density of SSRs

Complete sets of transcripts analyzed, TAs, TL and TC were found to contain 20.76% (13,932 transcripts), 23.06% (10,792 transcripts), 33.48% (9397 transcripts) SSRs, respectively (Table 20.2). When total genome sizes were compared for the *Trichoderma* species included in this analysis, the virtual transcripts of TAs (37.46), TL (32.24), and TC (33.48) represented 49.6, 47.8, and 48.6% of genome size, respectively (Table 20.1). Maximum numbers of SSR (2802) were found in TC followed by TAs (2704) and TL (2313). The higher number of SSRs shows the total size covered by transcripts' sequences; maximum size covered by TAs (19.8 Mb) was much higher than transcripts of TL (17.6 Mb). Relative abundance and relative

Table 20.2 Number, length, relative abundance, and relative density of SSRs in transcript sequences of three species of *Trichoderma*

	TAs	TL	TC
Genome size (Mbp)	37.46	32.24	33.48
G + C (%)	52.1	54.0	51.8
No. of transcripts	13,932	10,792	9397
Size covered by transcripts (Mbp) (%)	19.8 (49.6)	17.6 (47.8)	18.4 (48.6)
Total number of SSR identified	2893	2489	3147
Perfect (Max no. of SSR) (%)	2704 (93.4)	2313 (92.9)	2802 (89.03)
Compound (%)	189 (6.6)	176 (8.1)	345 (10.97)
Total length of SSR (%)	44,689 (2.83)	39,654 (2.28)	50,634 (2.39)
Relative abundance of SSR	169.8	186.9	283.6
Relative density of SSR	2685.9	2859.5	3424.7

TAs, TL, and TC represent *T. asperellum*, *T. longibrachiatum*, and *T. citrinoviride*, respectively

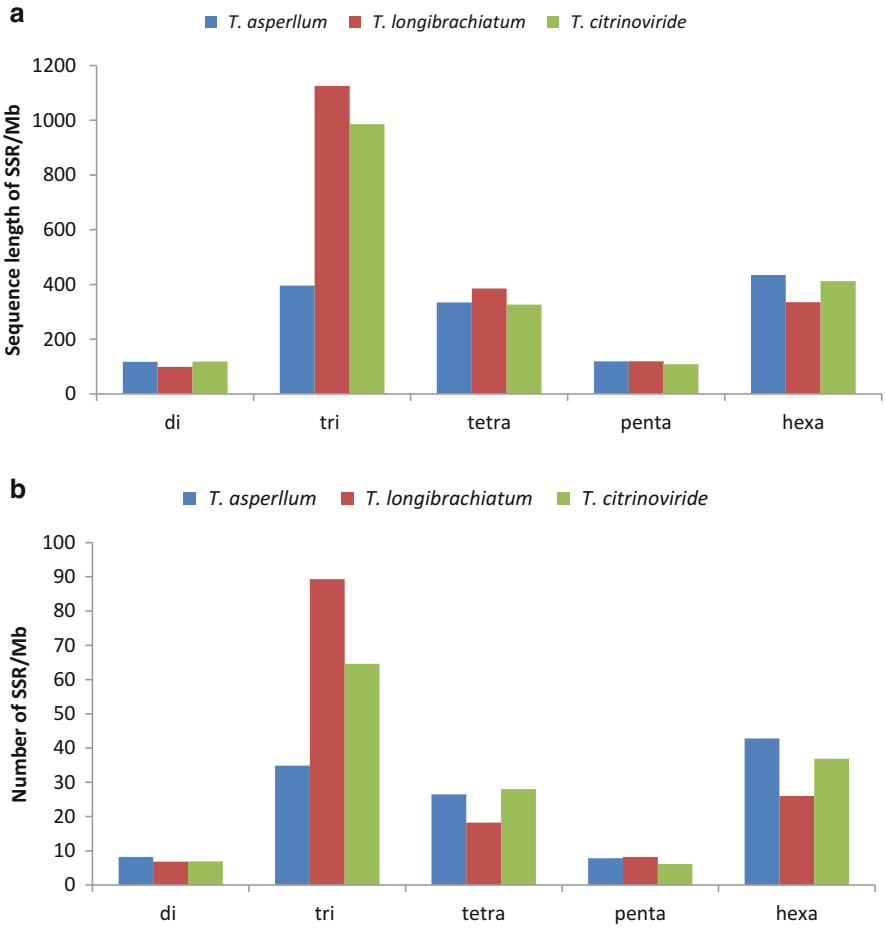

Fig. 20.1 Relative density (A) and relative abundance (B) of SSRs in three different species of *Trichoderma*. TAs, TL, and TC represent *T. asperellum*, *T. longibrachiatum*, and *T. citrinoviride*, respectively

density of SSRs of three *Trichoderma* isolates analyzed were presented in Table 20.2 and Fig. 21.1. The relative abundance of SSR in TC (283.6) was highest followed by TL (186.9) and TAs (169.8). Likewise, the relative density of SSR was higher in TC (3424.7) followed by TL (2859.5) and TAs (2685.9) (Table 20.2; Fig. 20.1). In several species, genome size and GC content have shown to have a certain influence on the occurrence of microsatellites (Coenye and Vandamme 2005). For instance, SSR density tends to have positive correlationship with the genome size in fungal (Karaoglu et al. 2005) and plant genomes (Morgante 2002). Generally it is considered that larger genome contributes to more microsatellites. However, this does not hold true when we compare the relative abundance and density of SSRs in *Trichoderma* sp. This can be explained by the fact that the differences in relative

Table 20.3 Frequency distributions of repeat motifs in the transcripts of *Trichoderma* genomes

Species	Class	Count (C)	Percentage (%)	Relative abundance (RA)	Relative density (RD)
TAs	Di	150	5.1	8.23	116.9
	Tri	634	21.9	34.9	396.1
	Tetra	510	17.6	26.5	334.1
	Penta	138	4.7	7.8	118.8
	Hexa	758	26.2	42.8	434.7
TL	Di	106	4.2	6.8	98.5
	Tri	1467	58.9	89.3	1125.8
	Tetra	357	14.3	18.2	384.9
	Penta	129	5.1	8.19	119.4
	Hexa	415	16.6	26.0	335.3
TC	Di	81	2.5	6.9	118.4
	Tri	1329	42.2	64.6	985.8
	Tetra	513	16.3	28.0	326.3
	Penta	75	2.3	6.1	108.4
	Hexa	759	24.1	36.9	412.1

TAs, TL, and TC represent *T. asperellum*, *T. longibrachiatum*, and. *T. citrinoviride*, respectively

abundance and relative density may be due to the different genomic organizations of analyzed *Trichoderma* sp. The high relative abundance and density of SSRs in TC are expected because of the presence of specific genes which are rare in other species (Singh et al., 2014). Similar explanations have been earlier made by several workers (Coleman et al. 2009; Mahfooz et al. 2015). A very narrow range of G + C % values 51.8 (TC)–54.0 (TL) was observed in analyzed *Trichoderma* genomes. The present study revealed that large transcript sequences do not always have proportionately more SSR loci as observed in *Trichoderma*. These findings are in agreement with the observations of earlier workers (Garnica et al. 2006; Singh et al. 2014).

The frequency distributions of repeat motifs in the transcripts of *Trichoderma* genomes, their counts, percentage, relative abundance, and relative density are shown in Table 20.3. In all three sequence sets, trinucleotide repeats were the most abundant in all species of *Trichoderma*, while dinucleotide repeats were the least common (Table 20.3). The second most common repeats were hexanucleotide repeats followed by tetranucleotide and pentanucleotide repeat motifs. However, the percentage of dinucleotide repeat was higher in *T. asperellum* (5.1%), while pentanucleotide repeat was higher in TL (5.1%). The most abundant trinucleotide repeat motif was AAG/CTT (5.4%) in TL, followed by CAG/CTG (5.2%) in TC and CAG/CTG (4.8%) in TAs, and CCA/TGG was the least abundant motifs (0.8%) in TAs among all three *Trichoderma* species (Table 20.4). These results supported the fact that trinucleotide repeats were found in each genomic region with a significant frequency in eukaryotes (Garnica et al. 2006). In coding regions, the dominance of triplets over other repeats may be due to suppression of non-trimeric SSRs, caused possibly by frame-shift mutations (Mahfooz et al. 2012; Metzgar et al. 2000; Rahim

Table 20.4 Number of longest repeat motifs in transcript sequences of *Trichoderma*

Motif	TAs	TL	TC
Dinucleotide repeat	GA_{16}	CA_{21}	CG_{12}
Trinucleotide repeat	GAA_{51}	CAA_{18}	AAG_{28}
Tetranucleotide repeat	$CCTC_7$	$CTCG_7$	$GTGC_6$
Pentanucleotide repeat	$AGCCA_4$	$TGGGC_5$	$GTGCT_5$
Hexanucleotide repeat	$CCTCCG_9$	$CCAGCA_9$	$CAAGGA_8$

TAs, TL, and TC represent *T. asperellum*, *T. longibrachiatum*, and. *T. citrinoviride*, respectively

2008). The higher occurrence of trinucleotide repeats can be attributed to their translation into an amino acid which contributes to the biological function of the protein (Kim et al. 2008). Because of higher mutation rates of dinucleotide repeats, they may be restricted to maintain the functionality of the transcripts. Interestingly, in the present study, after trinucleotides, dinucleotide repeats were richly distributed in all the analyzed transcripts of *Trichoderma* genome. However, these deviations may be due to differences in defining parameters used in microsatellite screening or may be species-specific characteristic. However, to reach any such concrete conclusion, further research is required.

The sequence composition of repeats is an important factor in determination of abundance of microsatellites. We observed variability in the abundance of repeat types among analyzed *Trichoderma* species (Table 20.4 and Table 20.5). Also, we observed random distribution of microsatellites in the *Trichoderma* genome. The results showed restricted length of SSRs in transcribed sequences. Trinucleotide types were the longest repeat units. GAA was repeated 51 times in TAs, while an 18-time repeat of CAA motif was noticed in TL (Table 20.4). Trinucleotide repeats GAA was found most predominant and common feature in EST-derived SSRs as observed in other fungal species (Kumar et al. 2013; Singh et al. 2014).

Different levels of polymorphism were generated by various dinucleotide repeat primers. In terms of individual longest repeat motif, the most common dinucleotide motif was CA in TL ($n = 21$), TAs ($n = 16$), and TC ($n = 12$) (Table 20.4). On the other hand in TAs and TC, the common SSR motifs were GA_{16} and CG_{12}, respectively. Such species-specific dinucleotide distributions suggest that different forces are acting on the evolution of microsatellites.

20.5 Conservation of SSR Motifs Within Species

To identify unique motif and to study the evolutionary relationship among three species of *Trichoderma*, each motif was analyzed for the presence of its counterpart in the remaining species. Maximum number of repeat motifs shared between all three transcripts was trinucleotide (126, 27%) followed by tetra- (61, 47%), hexa- (32, 6.7%), penta- (12, 5.6%), and dinucleotides (6, 47%) (Fig. 20.2). The sharing takes place between individual transcripts like TL–TAs shared 39 (46%) and TAs–TC shared 96 (45%) trinucleotide motifs. Within all four transcripts, around 47%

Table 20.5 Common repeat motifs identified in *Trichoderma* species

TAs	C	%	TL	C	%	TC	C	%
CAG/CTG	116	4.8	AAG/CTT	134	5.4	CAG/CTG	311	5.2
GCA/TGC	107	4.3	CAG/CTG	128	5.3	GCA/TGC	242	4.9
GCC/GGC	96	3.8	CTC/GAG	111	5.2	GCC/GGC	213	4.8
CTC/GAG	83	3.7	GAA/TTC	98	5.1	CTC/GAG	198	4.7
AGG/CTT	54	3	GCA/TGC	95	5.1	CGC/GCG	186	4.6
AGC/GCT	39	2.8	AGA/TCT	92	5	CCG/CGG	175	4.5
AG/TC	37	2.7	AGC/GCT	84	4.8	CAA/TTG	165	4.4
TA/GA	36	2.7	AGG/CCT	80	4.7	AGA/TCT	162	4.4
TCATCG/ TTTGCA	35	2.7	GAC/GTC	74	4.6	GAA/TTC	145	4.3
CT/AT	32	2.6	CGA/TCG	70	4.5	ACG/CGT	140	4.3
TCAAT/AGCCA	31	2.5	CGC/GCG	63	4.4	TCA/TGA	134	4.2
AGAA/CTTT	30	2.4	CCG/CGG	59	4.3	CAC/GTG	106	4
GAAA/TTTC	30	2.4	CAA/TTG	44	4.2	ATG/CAT	96	3.8
CTCC/GCCC	30	2.4	ACG/CGT	38	3.6	ATC/GAT	78	3.1
ATTC/GGAT	29	2.3	TCA/TGA	34	3.5	CCA/TGG	65	2.4
GCGA/CCGG	25	2.2	CAC/GTG	32	3.2	ACC/GGT	64	2.4
TGGC/CTCC	24	2.1	ATG/CAT	28	3	ACA/TGT	56	2.3
CCT/CAC	24	2.1	ATC/GAT	25	2.7	ACC/GTT	54	2.2
CGC/TTC	24	2.1	CCA/TGG	23	2.4	ATGC/ CACT	36	1.6
CTC/GGC	22	1.8	ACC/GGT	18	2.2	TCT/TTC	24	1
AGA/TCT	21	1.6	ACA/TGT	18	2.2	GC/CG	24	1
GTC/GAG	21	1.6	ACC/GTT	16	2	CCTC/CCGC	18	0.4
TCA/TGA	20	1.4	AG/CT	15	1.8	CCGG/ CCTC	16	0.4
GCG/CAA	20	1.4	GA/TC	14	1.6	CCCA/ CTGG	15	0.4
AAC/AAG	20	1.4	AC/GT	12	1.2	AG/CT	12	0.4
TCA/GCA	14	1	CA/TG	12	1.2	AACC/ AGAA	10	0.3
CAC/GTG	13	0.9	GAAA/ TTTC	10	0.9	CCTG/ GCGG	10	0.3
ATG/CAT	10	0.8	CAGC/ GCTG	10	0.9	GAAA/ TTTC	10	0.3
ATC/GAT	10	0.8				CAGC/ GCTG	10	0.3
CCA/TGG	10	0.8				AAGA/ TCTT	10	0.3

TAs, TL, and TC represent *T. asperellum*, *T. longibrachiatum*, and *T. citrinoviride*, respectively
C = count

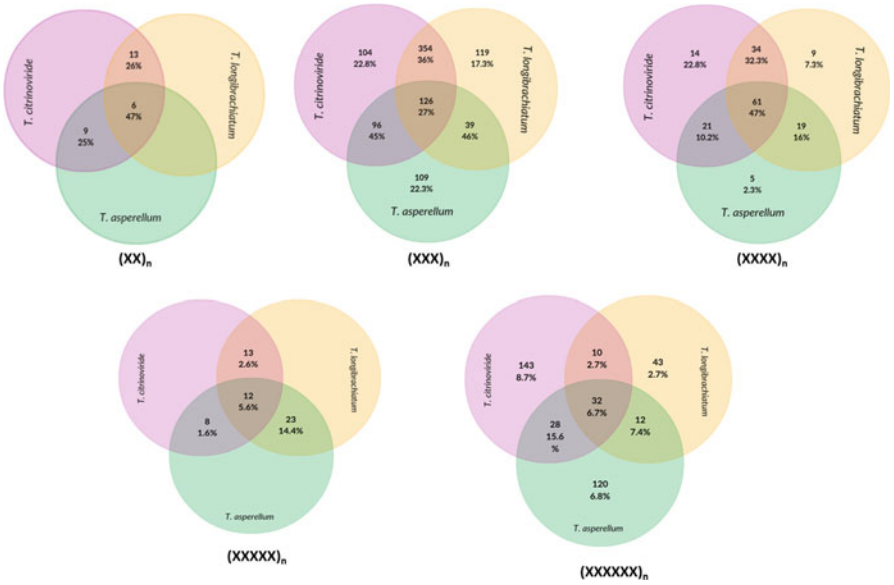

Fig. 20.2 Sharing of different repeat motifs in the transcripts of *Trichoderma* species. TAs, TL, and TC indicate *T. asperellum*, *T. longibrachiatum*, and *T. citrinoviride*, respectively

motifs were found conserved. Probably these motifs lie within the region of core genome which is generally conserved across all the species of *Trichoderma* (Dhusia et al. 2016; Gonthier et al. 2015). We noted that TL–TC and TAs–TC shared the highest percentage (45.0%) of motifs, whereas the least sharing (1.6%) was noted between TAs and TC (Fig. 20.2). The result of this study suggests that the distribution of SSR motifs is strictly species dependent and does not follow a genus-wide pattern.

20.6 Codon Repetition and Distribution of Amino Acid

The trinucleotide microsatellites code for 21 amino acids including the stop codon. Experimentally lysine (AAG) demonstrated the highest percentage of occurrence (98 times) as observed in TC, whereas code for methionine (ATG) was least repeated in TAs (Table 20.6). In multiple combinational codons, arginine (CGA/AGA/AGG/CGT/CGC/CGG) has a maximum frequency of occurrences (432, 17.43%) in TC, whereas tyrosine (4 and 0.63%) has the least frequency in examined TA transcripts. Nonpolar amino acid (1025) had the highest occurrence in TC and the least occurrence (319) in TAs of all three *Trichoderma* species (Fig. 20.3). Positively charged amino acid (22.56%) constituted the second most occurred amino acid followed by polar (18.09%) amino acids and negatively charged (11.36%) amino acids (Fig. 20.3). Glutamine, threonine, and lysine are amino acids that are recorded in

Table 20.6 Distribution of amino acids in genomes of three *Trichoderma* species

Amino acid	Codon	*Trichoderma* species		
		TAs	TL	TC
Alanine	GCT/GCC/GCA/GCG	86	136	341
Arginine	CGA/AGA/AGG/CGT/CGC/CGG	142	178	432
Aspartic acid	GAT/GAC	42	59	106
Cysteine	TGT/TGC	46	58	116
Glutamic acid	GAG/GAA	82	96	142
Glutamine	CAA/CAG	58	98	218
Glycine	GGT/GGC/GGA	46	72	219
Histidine	CAC/CAT	14	33	105
Leucine	CTT/CTG/CTA/CTC/TTA/TTG	156	250	395
Lysine	AAG	12	65	98
Methionine	ATG	4	13	19
Phenylalanine	TTC	16	48	59
Proline	CCT/CCG/CCA	14	15	13
Serine	TCA/TCC/TCT/TCG/AGT/AGC	15	18	25
Asparagine	AAC/AAT	9	11	12
Stop codon	TAA/TAG/TGA	19	28	36
Threonine	ACT/ACA/ACG/ACC	21	34	45
Tyrosine	TAT/TAC	4	10	11
Tryptophan	TGG	18	21	29
Valine	GTC/GTA/GTG/GTT	17	22	57

TAs, TL, and TC represent *T. asperellum*, *T. longibrachiatum*, and *T. citrinoviride*, respectively

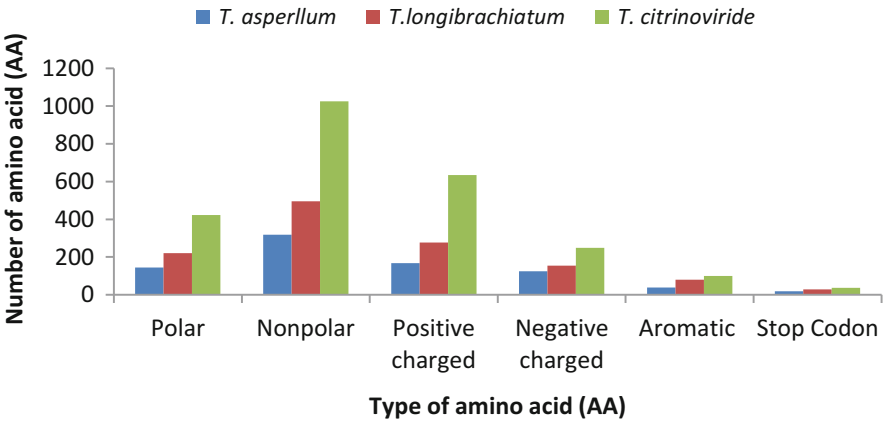

Fig. 20.3 Distribution of amino acids in examined sequences of three genomes of different *Trichoderma* species. TAs, TL, and TC indicate *T. asperellum*, *T. longibrachiatum*, and *T. citrinoviride*, respectively

transcripts of all the seven analyzed *Trichoderma* sp. and are responsible for homeodomain protein. Similarly, cysteine (TGT/TGC) is for zinc-finger-like protein. These observations support the theory that SSRs are randomly distributed in genomes and generally play a direct or indirect role in protein regulation (Gupta et al. 2010; Morgante et al. 2002). As expected we observed higher abundance of arginine as it has a reputation of being major nitrogen sources in fungi (Kaur et al. 2011). Besides this, in conidiation of some fungi, the role of arginine is also reported (Gong et al. 2007). In fungi glutamic acid and glutamine play an important role in nitrogen regulatory pathway, and therefore their abundance is justified. In certain fungal pathogens, metabolic regulation of nitrogen appears to have a significant role in their pathogenicity (Marzluf 1997).

20.7 DNA Polymorphism and Cross-Species Amplification from SSR Markers

Eighteen of the 31 primer pairs were successful amplified in all the 40 isolates of *Trichoderma*. These 18 loci include 5 from TvSSR, 6 from ThSSR, and 7 from TaSSR which were amplified easily with scorable bands ranging from 150 to 600 in all the isolates (Table 20.7). In these SSR loci, 4 (22.2%) were di-, 9 (50.0%) tri-, 3 (16.6%) tetra-, and 2 (13.3%) pentanucleotide repeats. Of these amplified markers, 13 (72.3%) markers were polymorphic and 5 markers showed monomorphic alleles (Table 20.8). Thus, a total of 34 alleles were amplified by 18 markers and 1–5 alleles per microsatellite locus with an average of 1.8 alleles per markers were observed (Table 20.8). A maximum number (5) of alleles were amplified by ThSSR4 marker and minimum of only 1 allele was observed in 5 markers, viz., TvSSR2, TvSSR3, ThSSR3, TaSSR2, and TaSSR6, and by remaining 12 markers, 2 alleles each were amplified (Table 20.7). 100% polymorphism was exhibited by 12 markers, viz., TvSSR1, TvSSR4, TvSSR5, ThSSR1, ThSSR4, ThSSR5, ThSSR6, TaSSR1, TaSSR4, TaSSR9, TaSSR17, and TaSSR19, whereas marker ThSSR2 showed 66% polymorphism (Table 20.7). Five ThSSR (ThSSR1, ThSSR2, ThSSR4, ThSSR5, and ThSSR6), three TvSSR (TvSSR1, TvSSR4, and TvSSR5), and five TaSSR (TaSSR1,TaSSR4, TaSSR9, TaSSR17, and TaSSR19) markers were highly polymorphic with a PIC value ranging from 0.219 to 0.545. TvSSR1,ThSSR4, ThSSR6, TaSSR1, TaSSR4, TaSSR17, and TaSSR19 with PIC values $\geq$0.4 were identified as the most informative SSR markers and heterozygosity values ranging from 0.25 to 0.72 with an average of 0.485 (Table 20.7).

20.8 Diversity and Cluster Analysis

A total of 26 SSR bands from 13 polymorphic primer pairs were used to evaluate genetic diversity and relatedness among 40 *Trichoderma* isolates. Similarity coefficients ranging from 0.17 to 1.0 with a mean of 0.44 among test isolates were observed. For SSR markers developed from *T. virens*, the similarity coefficient

Table 20.7 Evaluation of genetic diversity within *Trichoderma* isolates by amplification of different primers

Primer name/ locus	Primer sequence	Motifs	Tm (°C)	No. of alleles	Expected allele size (bp)	Observed allele size (bp)	Poly	PIC	H_e	Cross-species amplification
TvSSR1	CTATGGTGCCTCTGGTCTTTTC GATGTTGGACTTGATACCACCC	$(CAT)_4$	55.3	2	340	400–500	100	0.469	0.51	*Th*, *Tvd*, *Ta, Tk*
TvSSR 2	CCATCAAATCATCAAGAGTCCA ACAAAGGCCATGAAAGAGAAAG	$(TCT)_4$	54.8	1	391	400	–	–		
TvSSR 3	AACTGGGGAACAACAAACAAAC CCCCTTTACGAGGAGATACGAT	$(AG)_9$	55.4	1	264	250	–	–		
TvSSR 4	CCCCTTTACGAGGAGATACGAT GAGAGAGAAGAAGCGAAAGCAC	$(TC)_9$	54.7	2	194	190–250	100	0.349	0.37	*Th*, *Tvd*, *Ta*, *Tk*
TvSSR 5	GGATCGGCAAGGAATATAAACA CAACTTCCATAAAGACCGAAGC	$(TTCCA)_3$	55.1	2	199	200–290	100	0.320	0.34	*Th*, *Tvd*, *Ta*, *Tk*
ThSSR1	GCGATTGAGAGGAACGAACT AATCAAGTGAGGATTTGCTGCT	$(CTGT)_3$	54.8	2	213	190–230	100	0.398	0.42	*Tvr*, *Tvd*, *Ta*, *Tk*
ThSSR2	CAATCCGGCTCTACCTACTCTC ATATTGGCTTTATTCGTCGTGG	$(TCG)_4$	54.7	2	383	500–600	66	0.288	0.41	*Tvr*, *Tvd*, *Ta*, *Tk*
ThSSR3	AATAGAGACACAGCAGACAGCG TGAACAGACATTAGGATGGGTG	$(AAG)_5$	54.7	1	398	600	–	–		
ThSSR4	GTCGTCGGCCATCATTCC TTTCAAGGGCAGGACTCTCTCT	$(AC)_6$	57.1	5	147	150–300	100	0.545	0.72	*Tvr*, *Tvd*, *Ta*, *Tk*
ThSSR5	AGACGCAGATGAAAAGGAGC GTGGACCTGAGCTTTGATTGTT	$(GAA)_6$	55.0	2	375	450–500	100	0.219	0.25	*Tvr*, *Tvd*, *Ta*, *Tk*
ThSSR6	GCGAATGTCACCATCATCTTC TGAGAGAGCCGGAGTATAGGAG	$(GAAGA)_4$	55.2	2	313	360–400	100	0.445	0.50	*Tvr*, *Tvd*, *Ta*, *Tk*
TaSSR1	AAGCGGTCAGTTGAAAGTAACG AAGGGTTTTGCTTGTCCAGATA	$(ATTA)_3$	55.3	2	142	180–250	100	0.495	0.63	*Th*, *Tvr*, *Tvd*, *Tk*
TaSSR2	AAAGGTAGAGCAAGGGGAAATC CGGCGAAGGGTACTATTTTACA	$(GA)_6$	55.2	1	130	150	–	–		

(continued)

Table 20.7 (continued)

Primer name/ locus	Primer sequence	Motifs	Tm (°C)	No. of alleles	Expected allele size (bp)	Observed allele size (bp)	Poly	PIC	H_e	Cross-species amplification
TaSSR4	CTTGACCTGCTACAACAACTGC TGTCCATCTCTCTCTCTCTTCTCA	$(TGC)_4$	54.8	2	397	400–500	100	0.495	0.56	*Th*, *Tvr*, *Tvd*, *Tk*
TaSSR6	TTTGGAGCTTTACTGGGAGAAG GGTAACAGGAACCTTGGAGTCA	$(TGA)_4$	55.1	1	383	400	–	–		
TaSSR9	ACAGGTCGATTCCAGCACTATT TCTTCTTCTTCTTTGGAGCCAC	$(GAA)_6$	59.6	2	383	320–400	100	0.395	0.36	*Th*, *Tvr*, *Tvd*, *Tk*
TaSSR17	GCTGTTGCTCTCTTACCCTGAT AGGTGATGAAGTATTCGAGCGT	$(CGA)_4$	59.6	2	320	280–390	100	0.469	0.47	*Th*, *Tvr*, *Tvd*, *Tk*
TaSSR19	AATTCCTCTCCGACAACTCCTT CGAATCAGACGAGCTAGAGTCA	$(TATA)_3$	59.2	2	383	300–390	100	0.460	0.43	*Th*, *Tvr*, *Tvd*, *Tk*

Table 20.8 Identification of polymorphism by comparison of TvSSR, ThSSR, and TaSSR markers

	TvSSR markers	ThSSR markers	TaSSR markers	Total markers
Markers used	6	6	19	31
Marker amplified	5 (83%)	6 (100%)	7 (36%)	18 (58%)
No. of monomorphic markers	2 (40%)	1 (16.7%)	2 (28%)	5 (27.7%)
No. of polymorphic markers	3 (60%)	5 (83.3%)	5 (72%)	13 (72.3%)
Average PIC value	0.397	0.343	0.462	0.400
No. of alleles amplified	8	14	12	34
Similarity coefficient value (Avg)	0.55	0.49	0.49	0.51

between isolates ranged from 0.20 to 1.0, with 33.7% genetic diversity. Likewise, for ThSSR markers the similarity coefficients between isolates ranged from 0.11 to 1.0, with 37.8% genetic diversity. For TaSSR markers similarity coefficient value ranged between 0.11 and 1.0 with an average diversity being 37.5% (Table 20.7). On the basis of a similarity index ($\geq$50%), a dendrogram was constructed which resulted in eight clusters (Fig. 20.4). Cluster I is chiefly composed of two *Trichoderma* isolates, namely, *T. harzianum* and *T. viride*, while cluster V involved *T. virens* and *T. koningii*. Clusters II, III, VI, and VII represent a conglomeration of isolates of distinct species.

20.8.1 Microsatellite as a Diagnostic Marker

PCR was performed with DNA from 18 different isolates of *Trichoderma* in order to evaluate the specificity of ThSSR3F/ThSSR3R primer set which resulted in desired amplification of 600-bp amplicon (Fig. 20.5). Also, no amplification was observed in unrelated fungal species (*F. udum*, *F. lycopersici*, *F. ciceri*, *F. solani*, *A. brassicicola*, *A. brassicae*, *A. solani*, *A. porri*, *A. sesame*, *A. arborescence*, *A. alternata*, *A. polenderi*, *A. poneasis*, *A. zinnae*, *C. falcatum*, *C. gloesporioides*, *B. bassiana*, *P. grisea*, *H. oryzae*, *M. phaseolina*, and *R. solani*).

For efficient management of plant pathogens, rapid detection and quantification of *Trichoderma* sp. are very important. However, accurate identification to species level based on morphology is difficult because of similarity of morphological characters and increasing numbers of morphologically cryptic species (Kullnig-Gradinger et al. 2002). Currently, the assays used in *Trichoderma* diagnostic mainly rely on molecular methods and identification tools based on sequence analysis of genes and diagnostic markers; it is now possible to identify *Trichoderma* sp. (Galarza et al. 2015; Solanki et al. 2011). In this study, microsatellite locus "ThSSR3" was identified and characterized in terms of cross-transferability to other related and unrelated taxa. Results of this study confirm that this monomorphic microsatellite marker was highly specific for *Trichoderma* genus, as no amplification signal was observed from other closely related fungi. Therefore, ThSSR3 marker

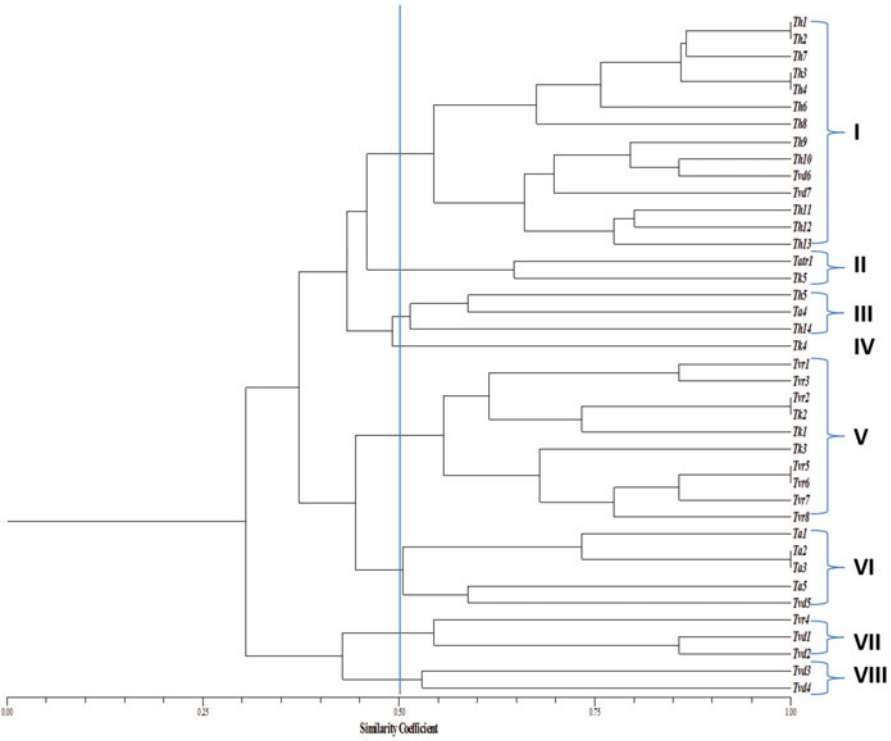

Fig. 20.4 Dendrogram plot representing cluster analysis of 40 antagonistic *Trichoderma* isolates based on polymorphic SSR markers

provides a fast and practical alternative to currently described markers to discriminate and diagnose *Trichoderma* genus from synchronously occurring fungus.

20.9 Conclusion

In summary, five monomorphic and thirteen polymorphic microsatellite markers were identified from different *Trichoderma* species. Moreover, primer sets ThSSR3F (5'-AATAGAGACACAGCAGACAGCG-3′) and ThSSR3R (5′-TGAACAGACATTAGGATGGGTG-3′) amplified a specific amplicon of 600 bp and would be useful as a diagnostic marker to discriminate and diagnose *Trichoderma* species from other closely related fungi. We observed that the high frequency and abundance of SSRs in genus *Trichoderma* are not influenced by the GC content. Higher conservation of repeat motifs in *Trichoderma* suggests its homogeneous genome organization. Thus, this work will provide a better identification of *Trichoderma* strains and development of suitable bio-formulation for sustainable agriculture.

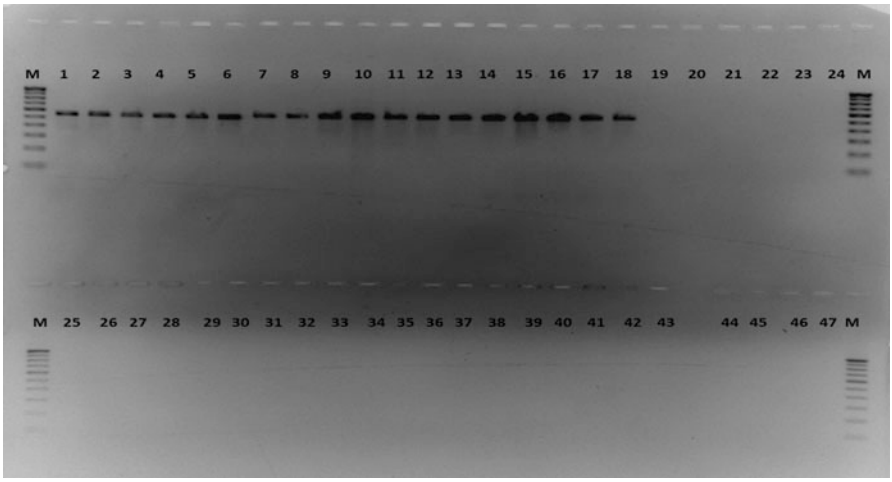

Fig. 20.5 PCR amplification of primer pair ThSSR3F and ThSSR3R showing amplicon of 600 bp. Lane M is a 100-bp DNA marker. Lanes 1–18 represent different *Trichoderma* species, while lanes 19–47 are unrelated fungal species, mentioned in supplementary Table 21.1

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Overview of Biopesticides in Pakistan

21

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Contents

Abstract

Agriculture is a vital sector of Pakistan's economy and accounted for about 30% of GDP according to government estimates. The sector directly supports three-quarters of the country's population, employs half the labor force, and contributes a large share of foreign exchange earnings. Agriculture is badly affected by various pests such as insects, weeds, plant pathogens, and nematodes. In

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Pakistan, the agrochemical industry has been witnessing a relatively steady to high growth, which has been primarily attributed to advancements in the pesticides and fertilizer industries. Nearly 71% of Pakistan's pesticide market is import dependent and annually imports approximately 80,000 tons of pesticides. The current use of pesticides in Pakistan is about 1,30,000 metric tons, of which approximately 90% is applied to cotton, rice, fruits, and vegetables. The wide use of pesticides in agriculture has contaminated the highly value-added commodities like rice, cotton, vegetables, and fruits. Due to contamination and low quality, prices in international markets have shrunken. Recently biopesticides have received much attention as an alternative to chemical pesticides. These comprise living microorganisms (viruses, bacteria, fungi, protozoa, or nematodes) or the metabolites produced by them. These are applied as pesticides in the form of sprays, dust, liquid drenches, liquid concentrates, wettable powders, or granules. The investigation of microbes and their bio-products/metabolites as pesticides dates to the discovery of *Bacillus thuringiensis* (BT) in 1902. Since then, work on the formulation of microbial pesticides has witnessed many ups and downs. This was the time when the use of chemical pesticides was gaining popularity among the farming communities due to their immediate and positive results. This factor visibly influenced the commercial use of microbial biopesticides in agriculture. Several countries are adopting a stringent approach when it comes to imports, with a special focus on regulating the amount of pesticide residues. As a result, the demand for regulated food safety and quality is increasing, which is another reason for growers to adopt biopesticides in their farming practices. The market is segmented based on the product type, formulation type, ingredient type, mode of application, crop and non-crop application, and geography. By the product type, the market is segmented into *bio-herbicides*, *bio-insecticides*, and *bio-fungicides*, with application in both crop-based and non-crop-based categories. Biopesticides are likely to witness faster growth (in double digits) in comparison to synthetic chemicals. The present headline is on the development and use of the biopesticides and the drawbacks of chemical pesticides. Their use is increasing slowly at the rate of 8% per annum, based on the numerous classes of various microbial pesticides. Although microbial pesticides are some of the anciently YIB (yield-increasing bacteria) in China and Russia, nowadays genetically modified microbes and their metabolites are frequently used for the biocontrol of pests and diseases. Major advantages of microbial biopesticides are:

1. The microbes used in preparation of these biopesticides are nontoxic and nonpathogenic to wildlife, humans, and other non-organisms.
2. These are highly specific to a single group of insect pests and thus have no adverse impact on other beneficial living organism.
3. These could be employed where necessary with synthetic pesticides.
4. Their residues are harmless, and these could be used at the time of harvest.
5. Applied microbes may establish in pest population and could be helpful in the maintenance of their population below the threshold level.

Microbial biopesticides are being used successfully on large scales in China, the United States, Australia, and many other countries of the world to break the trust of synthetic pesticides' use in agriculture. But in Pakistan, no efforts have been made to boost their use in agriculture. Commercial production of such kinds of biopesticides may prove a good step toward organic agriculture and help in the production of pesticide contamination-free agriproducts and derived biopesticides.

Keywords
Rhizobacteria · Biopesticides · Biocontrol · *Bacillus thuringiensis* (BT)

21.1 Introduction

Pakistan is an agricultural country, and agriculture in Pakistan is facing the threat of insects and diseases. Human beings started cultivation of plants; the insects and diseases started to cause damage to these plants. Many famines occurred due to disease and insect, e.g., Irish famine occurred due to potato leaf blight. In history, many famines were reported due to insects (locusts). The conventional chemical pesticides have enhanced food production but have also adversely affected the environment and nontarget organisms. In addition, volatile pesticide residues also sometimes raised food safety concern among domestic consumers. Biopesticides are used to control the insect pests, pathogens, and a variety of weeds. Biopesticides act as competitors or inducers of plant host resistance. Biochemical can control the growth, feeding development, or reproduction of an insect pest and pathogen. Biopesticides develop the resistance in plants against an insect pest. The total world production of biopesticides is over 3000 tons per annum, and in Asia, India has a vast potential for biopesticide production and consumption (Gupta and Dikshit 2010). In Pakistan, based on the type of active ingredient, two main research and development institutes are working on bio-fertilizers: (1) the Nuclear Institute for Agriculture and Biology **(NIAB)** and (2) the National Institute for Biotechnology and Genetic Engineering **(NIBGI)**. Biopesticides have been classified into three major classes: (1) biochemical, (2) plant-incorporated protectants, and (3) microbial pesticides.

21.2 Advantages of Biopesticides

- Host specificity
- Ability to multiply in the target cells
- No problem of toxic residue
- No evidence or absence of resistance
- No problem of cross-resistance
- Conventional technique or methods for applications
- Permanent control of a pest or long-persisting effect
- No fear of environment pollution and hence eco-friendly

21.3 Antagonistic Effect of Rhizobacteria

Pseudomonas fluorescens and *Bacillus subtilis*, found in soils, are considered safe for use in the environment and with mammals (Stabb et al. 1994; Zhao et al. 2015). They produce antagonistic activities against several fungal and bacterial pathogens (Ben Abdallah et al. 2015) and can persist in the plant for higher protection (Krebs et al. 1993; Falcao et al. 2014). Their antagonistic activities were frequently related to the production of secondary metabolites with antibiotic properties (Stabb et al. 1994; Asaka and Shoda 1996); most of them have been characterized as dipeptides or cyclic peptides with low molecular weight (Vanittanakom et al. 1986; Nakano and Zuber 1990; Dolej and Bochow 1996).

The metabolites produced by *Bacillus* sp. can also affect the microflora on the rhizosphere, offering an antagonistic environment to the pathogens, or they can activate host defense responses (Garcia et al. 2012; Yamamoto et al. 2015). Several *Bacillus* species had also the ability to promote the growth of the plant through effects on nutrition, root architecture, or the health of the plant (Glick 1995). They could fix molecular nitrogen and possess the considerable capacity to solubilize phosphates. It was established that the exopolysaccharides (EPS) produced by *Bacillus* sp. SCH1 bind to Na^+ ions reducing its concentration and helped the plant's growth (Ashraf 2004). Furthermore, they are among the most widespread bacteria found to colonize plants endophytically, playing important role in the biocontrol of vascular plant pathogens (Hardoim et al. 2008; Tiwari and Thakur 2014). Moreover, their rapid growth in liquid medium and their long-term viability facilitate the development of commercial products (Ongena and Jacquesm 2008). Plant growth-promoting rhizobacteria have the ability to colonize in the rhizosphere and increase the nutrient uptake ability of roots (Frankenberger and Arshad 1995).

21.4 Materials and Methods

The present studies on "use of rhizobacteria and spent mushroom compost for the management of bacterial wilt of potato were carried out in the Department of Plant Pathology, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi.

21.5 Survey and Sample Collection

Diseased samples of potato plant tubers and chickpea were collected from different potato- and chickpea-growing areas of Rawalpindi District for the isolation of bacterial wilt and fungal wilt, respectively. From each field, samples were selected randomly. Field diagnosis of diseased plant samples was done by critically observing the bacterial wilt and fungal wilt symptoms. Simple random sampling technique was adopted for the collection of samples.

Disease samples, showing a variable degree of wilting, were collected on the basis of symptomatology. Samples were labeled and preserved in polythene bags and were stored at 4 °C.

21.6 Isolation and Purification of *R. solanacearum*

Stem segments (about 10 cm in length) from the collar region of wilted potato and chickpea plants were rinsed with sterilized distilled water containing 1% Clorox. The samples were vortexed, and turbid bacterial suspensions were obtained. A loop full of turbid suspension was streaked on 2,3,5-triphenyl tetrazolium chloride (TTC) media from the stem segment of potato and Potato Dextrose Agar (PDA) incubated at 30 °C for 2 days, reddish fluidal colonies were again streaked on TTC plates, and the process was repeated until purified bacterial cultures were obtained with the homogeneity in colony morphology.

21.7 Isolation and Purification of *F. oxysporum*

For the isolation of pathogenic fungi associated with chickpea wilt, infected tissue at the advancing edge of a necrotic area was chopped into pieces of about 2 mm in size. Tissues were surface sterilized in 1% NaClO for 2 min followed by three consecutive treatments with distilled water and damp-dried on paper towels before being plated on the solidify media containing Petri plates. Samples were then placed on Potato Dextrose Agar media (PDA) containing plates. Broad-spectrum antibiotics were added to the media to avoid unwanted bacterial contamination. Plates were incubated at 25 ± 2 °C for 5 to 7 days.

21.8 Isolation of Plant Growth-Promoting Rhizobacteria (PGPR)

21.8.1 Collection of Samples

Soil samples were collected from the major potato- and chickpea-growing areas of Rawalpindi and Chakwal. Plant samples were carefully uprooted, and additional soil was removed by gentle shaking.

21.8.1.1 From Rhizosphere

PGPR were isolated from rhizosphere soil by using a dilution plate technique with nutrient agar medium (Akhtar et al. 2009).

21.8.1.2 From Roots

For isolation of PGPR strains from potato and chickpea rhizosphere, take plant roots, and gently wash twice in sterile water to remove adhering soil, and then root sections of approximately 1 g were added to 200 mL sterile water in flasks and shaken on a

rotary shaker at 150 rpm for 30 min. Then serial dilutions of the root suspension were placed on a nutrient agar medium and incubated at 28 °C for 48 h. When the bacterial colony appeared on the medium, representative isolates were picked and used as a biocontrol agent. Pure cultures of PGPR strains were identified using the morphological and physiological characteristics (Akhtar et al. 2009).

21.8.2 Purification of Rhizobacteria

From the composite culture of bacteria that resulted from the spreading inoculation of dilutions 10^{-2}, 10^{-5}, and 10^{-7}. Using the streaking method, each bacterial colony was purified on a separate nutrient agar plate.

21.8.3 *In Vitro* Evaluation of PGPR for Their Antagonistic Activity

In vitro, antagonistic effects of plant growth-promoting rhizobacteria (*Pseudomonas fluorescens* and *Bacillus subtilis*) against *R. solanacearum* and *F. oxysporum* were carried out by using zone of inhibition method. Nutrient agar medium was used in order to favor the growth of *R. solanacearum*, and the potential growth-promoting rhizobacteria and PDA media were used for the *in vitro* evaluation of antagonistic rhizobacteria against *F. oxysporum*. The Complete Randomized Design (CRD) was used with three replications.

21.8.3.1 Zone Inhibition Method for *R. solanacearum*

To check the antagonistic activity of rhizobacterial isolates, inhibition zone technique was used. The bacterial suspension was prepared by adding a small amount of sterilized distilled water to the media containing the cell of the pathogen and was collected in Petri plates. The sterilized cotton swab was touched to the bacterial suspension and spread evenly over the face of a sterile nutrient agar plate. In the center of the plates, the antagonistic rhizobacterial culture was applied. After 48 h of incubation, the plates were observed to evaluate the antagonistic activity of applied rhizobacterial strains. Zone of inhibition formed by the rhizobacteria was calculated in millimeter (Weller and Cook 1986). Rhizobacterial isolates, which exhibited the considerable antagonistic activity against the pathogen *in vitro*, were selected for further tests.

21.9 Results

21.9.1 Zone Inhibition Method for *F. oxysporum*

The antagonistic potential of six identified rhizobacterial isolates was tested against seven most virulent strains of *Fusarium oxysporum* root-infecting fungal pathogens in dual culture method by Soytong (1992). Both discs of actively growing fungal

hyphae and 24-hour-old culture of rhizobacteria were cultured on the same Petri plate against each other. Data on pathogenic fungal growth inhibition was recorded after 96 h of incubation against control treatment where no rhizobacteria inoculum was applied. The experiment was performed under Complete Randomized Design (CRD) with three replications.

21.10 *In Vitro* Screening of Rhizobacterial Isolates for Their Antagonistic Efficacy Against Root Pathogenic Fungi by Dual Culture Method

Collected data indicates that there is significant difference among all the tested treatments. From all the treatments, PS3 showed maximum growth inhibition (52.34 cm) of pathogenic fungus FOS1 followed by BS1 (48.64 cm), PS2 (47.49), and PS1 (46.43 cm) when compared with the control treatment where there was no growth inhibition of the pathogenic fungi observed. Among all the treatments, BS2 showed the least effective results against test pathogens given in Table 4.16. In all the experiments, PS3 rhizobacteria formed highly effective against all the tested pathogenic isolates of *F. oxysporum*, viz., FOS2, FOS3, FOS4, FOS5, FOS6, and FOS7.

The results of *in vitro* Zone of Inhibition by different Rhizobacterial isolates are described (Fig. 21.1 and Tables 21.1, 21.2, 21.3, 21.4, 21.5, 21.6, 21.7 and 21.8).

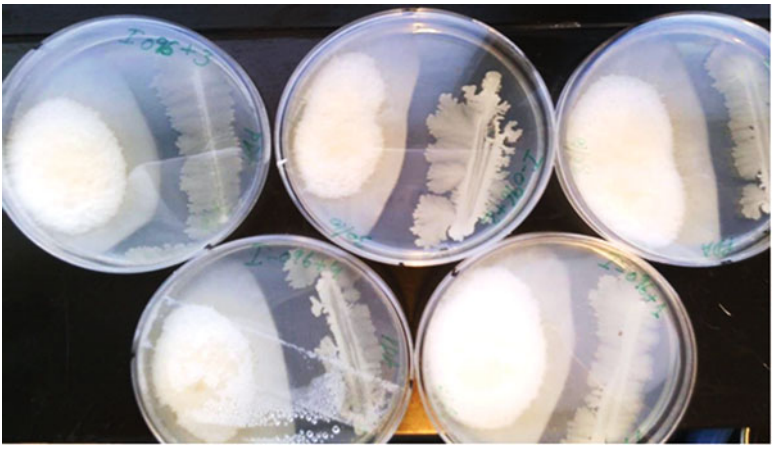

Fig. 21.1 Zone inhibition of fungi by rhizobacteria

Table 21.1 Diameter of zone of inhibition exhibited *in vitro* by rhizobacterial isolates

Isolates	Zone of inhibition (mm)	Isolates	Zone of inhibition (mm)
Rhz 1	2.00	Rhz 11	11
Rhz 2	0.00	Rhz 12	1.00
Rhz 3	1.00	Rhz 13	15
Rhz 4	2.00	Rhz 14	0.00
Rhz 5	0.00	Rhz 15	1.00
Rhz 6	0.00	Rhz 16	9.00
Rhz 7	1.00	Rhz 17	0.00
Rhz 8	0.00	Rhz 18	1.00
Rhz 9	0.00	Rhz 19	0.00
Rhz 10		Rhz 20	0.00

Table 21.2 Antagonistic potential of rhizobacterial isolates against pathogenic FOS1

Treatments	Mean fungal growth inhibition (mm)
FOS1 + PS3	52.33a
FOS1 + BS1	48.63b
FOS1 + PS2	47.48c
FOS1 + PS1	46.43d
FOS1 + BS3	45.66de
FOS1 + BS2	45.57e
Control	0f

LSD 0.05 = 0.517

Table 21.3 Antagonistic potential of rhizobacterial isolates against pathogenic FOS2

Treatments	Mean fungal growth inhibition (mm)
FOS2 + PS3	55.04a
FOS2 + BS1	49.50b
FOS2 + PS1	48.06c
FOS2 + BS3	47.582cd
FOS2 + PS2	47.390cd
FOS2 + BS2	47.19d
Control	0e

LSD 0.005 = 0.5287

Table 21.4 Antagonistic potential of rhizobacterial isolates against pathogenic FOS3

Treatments	Mean fungal growth inhibition (mm)
FOS3 + PS3	55.24a
FOS3 + BS1	50.28b
FOS3 + PS2	49.31c
FOS3 + BS3	48.92c
FOS3 + PS1	48.83c
FOS3 + BS2	46.72d
Control	0e

LSD 0.005 = 0.7669

Table 21.5 Antagonistic potential of rhizobacterial isolates against pathogenic FOS4

Treatments	Mean fungal growth inhibition (mm)
FOS4 + PS3	55.65a
FOS4 + BS2	49.70b
FOS4 + PS2	49.50b
FOS4 + BS3	49.12b
FOS4 + PS2	47.39c
FOS4 + BS1	47.00c
Control	0d

LSD 0.005 = 0.41

Table 21.6 Antagonistic potential of rhizobacterial isolates against pathogenic FOS5

Treatments	Mean fungal growth inhibition (mm)
FOS5 + PS3	53.82a
FOS5 + BS3	49.99b
FOS5 + PS2	49.21bc
FOS5 + BS1	49.12c
FOS5 + PS2	48.73c
FOS5 + BS1	46.05d
Control	0e

LSD 0.005 = 0.4944

Table 21.7 Antagonistic potential of rhizobacterial isolates against pathogenic FOS6

Treatments	Mean fungal growth inhibition (mm)
FOS6 + PS3	54.43a
FOS6 + BS2	50.08b
FOS6 + PS1	49.31c
FOS6 + PS1	48.54c
FOS6 + BS2	47.58d
FOS6 + BS3	47.39d
Control	0e

LSD 0.005 = 0.5968

Table 21.8 Antagonistic potential of rhizobacterial isolates against pathogenic FOS7

Treatments	Mean fungal growth inhibition (mm)
FOS7 + PS3	51.45a
FOS7 + BS2	50.08b
FOS7 + PS1	47.67c
FOS7 + PS2	47.00d
FOS7 + BS1	46.05d
FOS7 + BS3	45.85d
Control	0e

LSD 0.005 = 0.8169

Table 21.9 Effect of treatments on disease incidence and plant growth parameters of potato plants under greenhouse conditions

Treatments	Disease incidence %	Shoot height (cm)	Shoot fresh weight (g)	Shoot dry weight (g)
B28	0f	27.33ab	16.52ab	2.54ab
B28 + Rs	16.4c	26.10bcd	15.65bc	2.45bc
B85	0f	25.45cde	15.39bc	2.25cd
B85 + Rs	14.80d	25.41cde	15.48bc	2.21cd
B28 + B85	0f	28.12ab	15.61bc	2.43bc
B28 + B85 + Rs	11.40e	27.45abc	15.35bc	2.41bc
JA	0f	24.95de	15.12bc	2.01d
JA + Rs	22b	24.45de	15.10bc	2.27cd
SA	0f	25.12de	14.99bc	2.03d
SA + Rs	17c	24.88de	14.95bc	2.21cd
Rs	51a	17.33f	7.98d	1.14e
Control	0f	23.33e	15.26bc	2.11d

Values with different alphabets are significantly different at $p \geq 0.05$
B28, *B. subtilis* B28; B85, *B. pumilus* B85; *Rs*, *R. solanacearum*; JA, jasmonic acid; SA, salicylic acid

21.11 Greenhouse Evaluation of Antagonistic PGPR

In greenhouse evaluation, there was a significant reduction in disease incidence in rhizobacteria-treated plants whether they were alone or in combination. Least incidence (11.40%) was observed in combined application of rhizobacteria (B28 + B85 + Rs). In alone treatments of rhizobacteria, B85 allowed less disease incidence of potato plants as compared to B28 where the incidence was 14.80% and 16.4%, respectively. In treatment with JA and SA, there was also seen significant disease reduction. In the account of plant growth enhancement, there was no significant difference in treatments except the treatment of rhizobacteria in combination, and increased shoot height was observed, while the fresh and dry weight of shoot was observed highest in B28-treated plants. In JA- and SA-treated plants, there was a reduction in disease, but no growth promotion was observed in plants, and consequently, plants turned pale with JA treatment (Table 21.9)

21.11.1 Evaluation of Best Biopesticide Formulation Under Field Conditions

Based on greenhouse results, four antagonistic bacterial-based formulations, viz., BS-1OM, BS-2OM, BS-3OM, and Pf-OM, were selected for their efficacy trial under open-field conditions, and experiments were performed at four locations

Table 21.10 Profile of disease incidence, disease severity, and disease control due to various treatments

Treatments	Disease incidence %	Disease severity %	Percentage disease control
T1	52.67	53.11	35.03
T2	61.67	60.91	30.30
T3	50.67	54.08	34.48
T4	48.00	51.00	41.59
T5	47.00	49.37	43.63
Control	*97.00*	96.25	0
CD ($p = 0.05$)	11.13	10.04	

Table 21.11 Plant growth promotion due to treatment with rhizobacteria

	Plant height (cm)		No of nodules/plant		Dry weight (g/plant)	
Treatments	V1	V2	V1	V2	V1	V2
T1	32.2	60.8	4.66	13.00	1.8	3.0
T2	26.8	80.0	10.33	13.66	1.0	3.2
T3	31.0	71.5	7.33	11.00	2.1	3.5
T4	30.2	48.4	14.00	16.00	2.3	2.8
T5	37.2	91.2	6.00	12.66	2.7	6.5
T6	26.7	24.1	1.60	2.00	0.5	1.4
CD ($p = 0.05$)	7.21		3.31		1.86	

with four replications for each treatment under Randomized Complete Block Design (RCBD). Data regarding disease prevalence (DP%), disease incidence (DI%), and disease severity (DS%) was recorded 90 days after sowing. Data from each location show that BS-3OM efficiently reduced the disease prevalence, incidence, and severity percentages followed by BS-2OM and BS-1OM against control where no biopesticide treatment was applied. Among all the tested treatments, Pf-OM resulted in poor efficacy in controlling the disease.

Effect of antagonistic Rhizobacteria on disease parameters of fungal root disease in chickpea (Table 21.10).

Effect of antagonistic Rhizobacteria on various attributes in chickpea varieties cultivated in wilt-affected field after 60 days (Table 21.11).

Effect of antagonistic Rhizobacteria on various attributes in chickpea varieties cultivated in wilt-affected field after 60 days (Figs. 21.2 and 21.3).

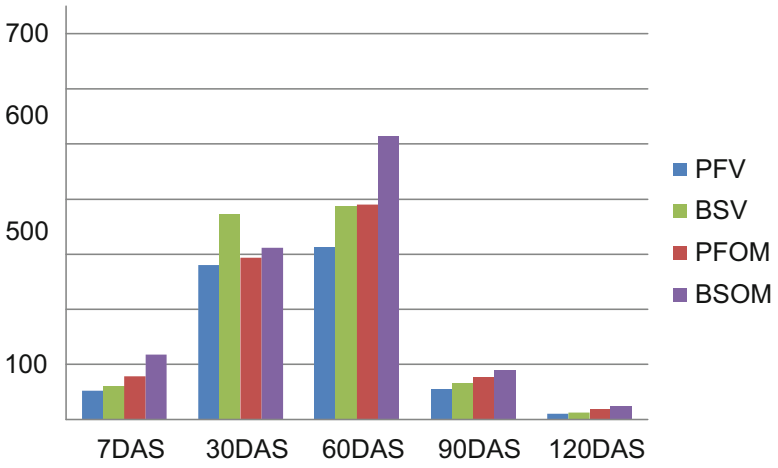

Fig. 21.2 Comparison of rhizobacterial colony-forming units (CFU) on different carrier materials at 28 °C

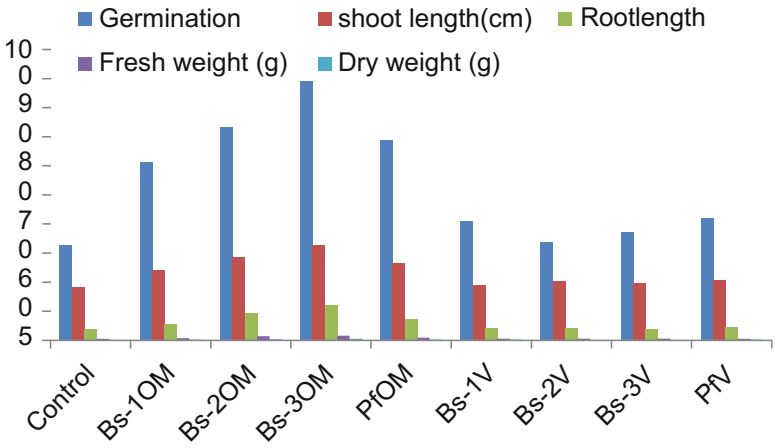

Fig. 21.3 Effect of bio-inoculant formulations on plant growth promotion under glasshouse condition

21.12 Future Directions

Bioformulations are best defined as biologically active products containing one or more beneficial microbial strains in an easy-to-use and economical carrier material. Most formulations are meant for field application; it is essential that suitable carrier materials are used to maintain cell viability under adverse environmental conditions. A good-quality formulation promotes survival of bacteria maintaining available

population sufficient to exude growth-promoting effects on plants. Plant growth-promoting rhizobacterial bioformulation refers to preparations of microorganism that may be a partial or complete substitute for chemical fertilization and pesticides and offer an environmentally sustainable approach to increase crop production and health. Stress should be given to the biological control method which is environmentally safe, and also it can be effective against several pathogens; like in this study we have seen that antagonists also possessed the chitinase production ability, and they can be effective against fungal pathogens too. The isolates proved useful against *Rs*, and *FOS* should also be tested against other viral diseases in potato, chickpea, and other crops if they induce. Also, field applicability of these antagonists should be checked, and proper application methodology, if devised, can be very helpful under field conditions.

21.13 Conclusions

It was concluded that rhizobacterial-based formulations due to their antagonistic and plant growth-enhancing potential can be used in controlling other soilborne plant pathogens as their application has no side effects on human health and environment as well.

In Pakistan present use of pesticides is about 130,000 metric tons which are about 90% apply on cotton, rice, sugarcane, fruits, and vegetables. These pesticides can be made of the products banned in international markets.

WTO (World Trade Organization) suffers many problems due to using pesticidal products. Products have contaminated of pesticides which causes reduce the price in International markets. Then we eliminate the use of pesticides and apply the biopesticides in Pakistan by supporting the microbial pesticides to abolish the pollution.

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22 Evaluation of Indigenous Fluorescent Pseudomonads for the Management of Newly Emerging Wilt of Pomegranate Caused by *Ceratocystis fimbriata*

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Contents

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Abstract

Pomegranate (*Punica granatum* L.) is a commercial fruit crop in India and regarded a vital cash crop in many states including Karnataka. Wilt disease (*Ceratocystis fimbriata* Elli. and Halst.) is a new devastating disease on pomegranate that caused losses estimated up to 30% and becoming a major threat leading to the destruction of several pomegranate orchards. Although suitable wilt management practices including cultural, sanitation, and chemical have been developed, there is still a need to develop more economical, feasible, and effective environmentally friendly alternative control methods. Recently, there is an increased interest for use of plant growth-promoting rhizobacteria (PGPR) for sustainable pomegranate cultivation. Thus, the present investigation is intended to evaluate indigenous fluorescent pseudomonad isolates for biocontrol, PGPR traits, and plant growth promotion. In the present investigation, 35 native isolates of fluorescent pseudomonads from wilt-affected pomegranate orchards were isolated, purified, and characterized. These were screened for their in vitro efficacy using standard dual culture plate culture technique for the inhibition of the growth of *C. fimbriata*. Out of 35 isolates, 11 isolates (PFP-1–PFP-11) significantly reduced the vegetative growth of *C. fimbriata*. These isolates were tested for PGPR traits such as the production of indoleacetic acid (IAA), hydrogen cyanide (HCN), hydrogen sulfide, and siderophore. All the 11 isolates showed bright fluorescence under UV light, and morphological and biochemical tests proved them as *Pseudomonas fluorescens*. The isolates were positive for siderophore production and plant growth-promoting activities. Two isolates PFP-11 and PFP-10 showed remarkable antifungal activity against *C. fimbriata* with the inhibition of 72.04% and 71.20%, respectively. These isolates showed significant producers of HCN, IAA, siderophore, and enzymes. Overall, our results suggest that these fluorescent pseudomonad isolates can be estimated as efficient bioagents for the sustainable management of newly emerging wilt of pomegranate caused by *C. fimbriata* and warrant further evaluations.

Keywords

Fluorescent pseudomonads · PGPR · Biocontrol · Wilt of pomegranate · *Ceratocystis fimbriata*

22.1 Introduction

Application of bioagents for growth promotion and disease management is fascinated in the past few decades due to the hazardous nature of chemicals on ecology (He et al. 1993). Keeping in view, biocontrol agents hold promise to plant growth promotion and the management of the disease. Since biological control is a major component of integrated disease management, it is essential to search for plant growth-promoting rhizobacteria (PGPR) isolates acting against specific pathogens

and their wider application. These microbes induce resistance in plants against the infection by different pathogens. Among the antagonistic bacteria, fluorescent *Pseudomonas* is most preferred because its versatility to control different plant pathogens can suppress pathogen by producing antibiotics (Gupta et al. 2001), lytic enzymes, and siderophores, by inducing systemic defense reactions in the host plant. Effective management of plant diseases by using PGPR depends upon the commercial formulation development with the suitable delivery system followed by field assessment.

Pomegranate (*Punica granatum* L.) is a vital cash crop of India and is commercially cultivated in the major pomegranate growing states. Recently, wilt disease (*Ceratocystis fimbriata* Elli. and Halst.) of pomegranate is becoming a major threat by adversely affecting the yields of the pomegranate fruit crop (Sharma et al. 2008: Sharma 2009). The disease was first reported from Nashik district in Maharashtra in 1978 and subsequently from Kaladgi and Kanamadi areas of Karnataka in 1988 (Somasekhara 1999) and Cudddapah, Andhra Pradesh, in 2002. The disease has also been reported from other countries like Iran, China, and Greece (Tziros and Zavella-Klonari 2008). Based on the damage and loss, the disease has been considered as a nationally important disease in India, and a lot of emphases have been given to tackle this disease problem. However, there is no much research work carried out with regard to biological control of wilt of pomegranate with PGPR mechanisms. The present investigation reports on isolation and characterization of native fluorescent pseudomonads, their bio-efficacy, and elucidating their PGPR mechanisms including estimation defense enzymes for biocontrol control and plant growth promotion induced by PGPR in the management of the newly emerging wilt disease of pomegranate.

22.2 Materials and Methods

22.2.1 Source and Isolation of *Pseudomonas fluorescens*

Pseudomonas fluorescens isolation was carried from rhizosphere soil of wilt-affected pomegranate orchards in Bijapur, Bagalkot, Koppal, Ballari, and Raichur districts. The rhizosphere soil particles loosely adhering to the roots were collected and obtained in standard soil suspension by adding crushed soil sample in a sterile mortar and pestle and shaken with 100 ml of sterile distilled water for 15 minutes. Further, the 1 ml of soil suspension was transferred into water blanks containing 9 ml of sterile distilled water up to 10^{-5}, 10^{-6}, and 10^{-7} dilutions. Later 1 ml of each dilution was transferred on to the Petri plate containing 10–15 ml of King's B medium by pour plate method and kept for incubation to obtain *P. fluorescens*.

22.3 Bio-efficacy of Native *P. fluorescens* Isolates In Vitro

All the 35 isolates were screened for antagonistic activity against *C. fimbriata* using dual culture technique. Mycelial disc (5 mm diameter) from an actively growing *C. fimbriata* culture was taken and placed on the middle of the Petri plates containing fresh potato dextrose agar medium. A loopful of 24-h-old pseudomonas isolates were streaked parallel on either side of the fungal disc. Plates inoculated with *C. fimbriata* and without pseudomonas were considered as control, and each treatment was replicated thrice and kept for incubation at room temperature for 8–10 days. The antagonistic activity was determined by measuring the radial growth of pathogen with bioagent and control. The percent inhibition over the control was calculated by using the formula (Vincent 1947).

22.4 Characterization of Fluorescent Pseudomonads

The native *P. fluorescens* isolates which were found efficient (>50% growth inhibition) on the basis of antagonistic potential were further characterized for their morphological, biochemical characters, PGPR traits, and defense enzymes.

22.5 Morphological Characterization

Eleven *P. fluorescens* isolates were tested for fluorescence under UV light; colony color and cell shape were observed under a microscope; Gram's reaction and growth at different temperatures were recorded (Garrity et al. 2005).

22.6 Fluorescence

Pseudomonas isolates were inoculated onto the sterilized King's B medium contained in test tubes and kept for incubation for 5 days, and yellowish green fluorescent pigment observed under UV light (365 nm) indicated positive results.

22.7 Gram's Staining

Indigenous *Pseudomonas* isolates were tested for Gram's reaction. The result indicated that Gram-negative cells appeared pink in color and Gram-positive cells appeared violet in color.

22.8 Biochemical Characterization

22.8.1 KOH Test

A loopful of young pure culture colony of *Pseudomonas* was taken on a glass slide and mixed by adding 3 percent KOH solution. The result indicates that formation of string with the loop was recorded as positive for the test.

22.8.2 Catalase Activity

Young cultures (24 h) of fluorescent pseudomonads were added to 3 percent hydrogen peroxide (H_2O_2). The gas bubbles production indicates positive for the test (Schaad 1992).

22.8.3 Starch Hydrolysis

Young cultures of fluorescent pseudomonads were spotted on the starch agar plates and incubated at 28 ± 2 °C for 24 h. Later, the incubated plates were flooded with Lugol's iodine solution. The results indicated that a clear zone around the colony was taken as positive for the test (Eckford 1927).

22.8.4 Gelatin Liquefaction

Indigenous *Pseudomonas* isolates were tested for gelatin liquefaction (Blazevic and Ederer 1975). The tubes with cultures that remained liquefied were taken as positive for the test.

22.9 Urease Test

A young colony of native pseudomonas were inoculated to sterilized urea broth and incubated for 24 h at 28 °C. The pink color formation in test tubes indicates positive for the test.

22.10 Hydrogen Sulfide Production

A pure culture of 24-h-old fluorescent pseudomonas colony was stabbed on to the pre-sterilized tubes containing SIM agar and incubated for 48 h at 28 °C. Formation of black color along the line of the stab was indicated as positive for the test (Cappuccino and Sherman 1992).

22.11 Indole Production

A young pseudomonas colony was inoculated to the pre-sterilized SIM agar tubes and kept for incubated for 48 h. at 28 °C. The later incubated tube was added with ten drops of Kovac's reagent. Formation of cherry red color was taken as positive for the indole production.

22.12 Characterization of PGPR Traits

The PGPR traits of efficient (>50% growth inhibition) *P. fluorescens* isolates were carried out with respect to hydrogen cyanide, siderophore, indoleacetic acid, and induced systemic resistance.

22.13 Hydrogen Cyanide Assay

To test for HCN, the cultures of isolates were inoculated into King's B agar plates supplemented with glycine. After this, Petri plates were inverted, and a piece of Whatman filter paper no. 1 impregnated with 0.5% picric acid and 2% of sodium carbonate was placed on the lid. Petri plates were sealed with Parafilm and incubated at 28 ± 2 °C for 96 h. Formation of light brown, brown, or reddish-brown color in the yellow filter paper indicated weak (+), moderate (++), or strong (+++) reaction, respectively.

22.14 Siderophore Production

The selected isolates showing highest biocontrol activity were screened by the CAS method for siderophore production.

22.15 Indoleacetic Acid Production (IAA)

The Production of IAA by the Pseudomonas fluorescence isolates was done by procedure.

22.16 Elucidation of Defense Enzymes by Induced Systemic Resistance

Studies on induced systemic resistance (ISR) were carried out on pomegranate plants by challenge inoculation of the plants with pathogen followed by application of bioagents under pot condition in the glasshouse. The bacterial suspension of the

PFP-11 isolate was applied on pomegranate plants and compared with pathogen *C. fimbriata* alone and plants which were inoculated with distilled water treated as a check. Four plants were inoculated for each treatment, and the inoculated plants were kept in a glasshouse for further studies.

22.17 Treatment Details

T1: *P. fluorescens* (PFP-11) (5 gL^{-1}) – *P. fluorescens* (PFP-11) (5 gL^{-1}) – *P. fluorescens* (PFP-11 (5 gL^{-1}).
T2: *C. fimbriata.*
T3: Control.

The bioagent and pathogen in each treatment combination were applied three times at an interval of 15 days as soil drench at 2l $plant^{-1}$. Estimations of enzymes related to defense were carried out at regular intervals (0, 15, 30, 60, and 90 days after challenge inoculation of the pathogen), and mean was calculated. Leaf samples were taken from responded sides of the pomegranate plants for each treatment, and assays of PO, PPO, and PAL were carried out as per Mayer et al. (1965).

22.18 Results

Biological control of plant diseases using microbial inoculants is receiving increased attention as an environment-friendly alternative to chemical pesticides. Exploitation of plant-microbe interaction in the rhizosphere could promote plant growth and reduce the diseases.

22.19 Isolation and Screening of Native *P. fluorescens* Isolates

In the present investigation, a total of 35 *P. fluorescens* isolates were isolated by collecting the soil samples from the healthy rhizospheric soil of wilt-affected pomegranate orchards. The isolates were identified and confirmed as fluorescent pseudomonads by Gram's reaction, color and shape of bacteria with fluorescence under UV light. Efficacy of 35 indigenous fluorescent pseudomonads was studied in vitro by screening against *C. fimbriata*. The results on inhibition of mycelial growth of *C. fimbriata* was recorded and presented here under Table 22.1 and Plate 22.1. All the isolates were Gram-negative, yellowish green with respect to colony color, rod-shaped bacteria and showed fluorescence under UV light. Among 35 isolates, only 11 isolates showed a high level of antagonistic activity by recording more than 50 percent inhibition of test pathogen. Out of 35 isolates tested, PFP-11 showed the highest inhibition percent of mycelium (72.06%) and the second highest percent inhibition PFP-4 (72.04%), followed by PFP-10 (71.20%), PFP-6 (67.71%), and PFP-7 (66.53%). The remaining isolates showed the moderate inhibition

Table 22.1 Bio-efficacy of native fluorescent pseudomonads against *C. fimbriata*

Isolate	Radial mycelial growth (mm)	Percent mycelial inhibition
PFP-1	32.00	58.00 (49.60)
PFP-2	33.96	56.04 (48.46)
PFP-2	36.06	53.93 (47.25)
PFP-4	17.96	72.04 (58.09)
PFP-5	25.90	64.10 (53.18)
PFP-6	22.83	67.71 (55.38)
PFP-7	23.46	66.53 (54.65)
PFP-8	30.93	59.06 (50.21)
PFP-9	23.83	66.16 (54.42)
PFP-10	18.80	71.20 (57.54)
PFP-11	17.93	72.06 (58.07)
Control	90.00	0
	S.Em±	0.38
	CD @ 1%	1.42

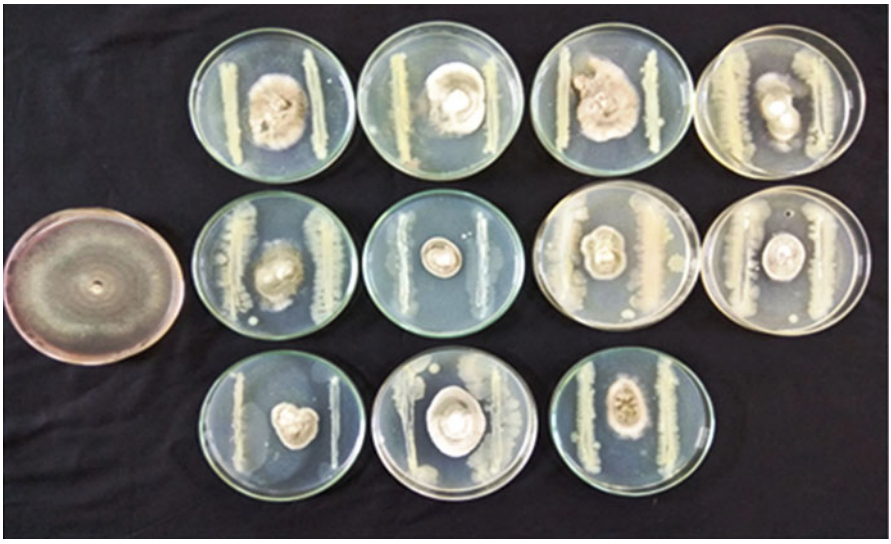

Plate 22.1 Bio-efficacy screening of native isolates of fluorescent pseudomonads against *C. fimbriata*

C. fimbriata. Later, these 11 promising native antagonistic isolates were characterized morphologically and biochemically, tested for plant growth promotion traits.

Table 22.2 Morphological characterization of native fluorescent pseudomonads isolated from pomegranate rhizosphere

Isolate	Fluorescence under UV light	Yellow-green pigmentation	Cell shape	Gram's reaction	Growth at	
					4 °C	44 °C
PFP-1	+	+	Medium rod	Negative	Absent	Present
PFP-2	+	+	Long rod	Negative	Absent	Present
PFP-2	+	+	Long rod	Negative	Absent	Present
PFP-4	+	+	Medium rod	Negative	Absent	Present
PFP-5	+	+	Long rod	Negative	Absent	Present
PFP-6	+	+	Medium rod	Negative	Absent	Present
PFP-7	+	+	Medium rod	Negative	Absent	Present
PFP-8	+	+	Long rod	Negative	Absent	Present
PFP-9	+	+	Long rod	Negative	Absent	Present
PFP-10	+	+	Short rod	Negative	Absent	Present
PFP-11	+	+	Medium rod	Negative	Absent	Present

+ = present; − = absent

22.20 Identification of Isolate Morphological Characterization

Eleven superior native isolates of *Pseudomonas* spp. were cultured in standard medium and incubated to study various morphological characters. The growth in all the isolates initiated after 24 hours of incubation. All isolates showed Gram-negative reaction and fluorescence under UV light and produced slimy, irregular colonies with light yellowish green pigmentation, and they were medium to long rod-shaped that confirmed that all the isolates belong to fluorescent pseudomonads group. Further, the maximum growth of bacteria was noticed at 44 ° C, and there was no growth at 4 ° C (Table 22.2 and Plate 22.2).

22.21 Biochemical Characterization

The biochemical characterization rhizobacterial isolates was done for characterization of PGPR (Table 22.3 and Plate 22.3). All the 11 isolates (PFP-1 to PFP-11) produced positive results with regard to KOH test, catalase test, starch hydrolysis, gelatin liquefaction, urease test, casein hydrolysis, H_2S test, and indole test (Table 22.4).

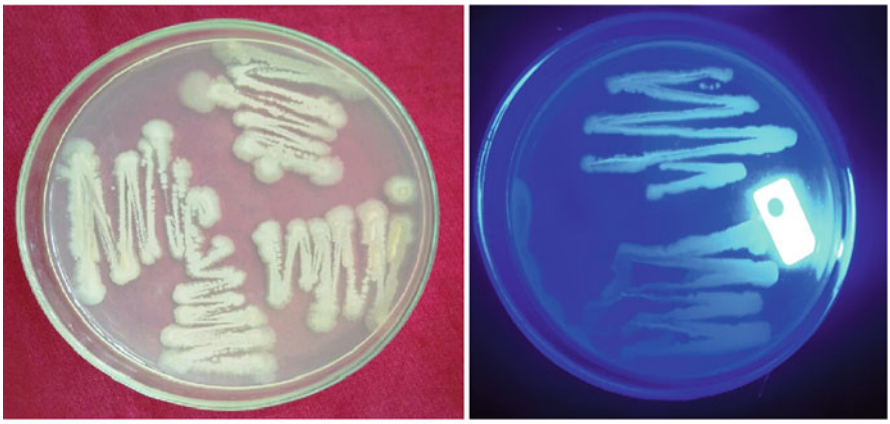

Plate 22.2 Morphological characterization of native fluorescent pseudomonads

22.22 PGPR Traits of the Selected Native Isolates of *P. fluorescens*

The fluorescent *Pseudomonas* isolates reported to produce HCN, hydrogen sulfide (H_2S), and siderophores.

22.23 Production of Hydrogen Cyanide

All the isolates tested were positive HCN production by a change in the color filter paper. Among these, PFP-4 and PFP-11 changed the yellow color of the filter paper to brick red when compared to other isolates and were scored as strong (+++) indicating a higher level of HCN production (Plate 22.4). The isolates PFP-5, PFP-6, PFP-7, and PFP-10 scored as moderate (++) with light brown color, while PPF-1, PFP-2, PFP-3, PFP-8, and PFP-9 produced yellowish brown and were graded as weak (+).

22.24 Siderophore Production

Tested isolates of *Pseudomonas* spp. produced siderophores on CAS agar medium which was indicated by the production of the yellow-/orange-colored zone surrounding the bacterial growth. Among eleven isolates, two isolates (PFP-4, PFP-11) recorded higher siderophore production (4 mm orange color zone), six isolates (PFP-2, PFP-5, PFP-6, PFP-7, PFP-8, PFP-10) produced moderate siderophores (2–4 mm orange color zone), and three isolates (PFP-1, PFP-3, PFP-9) produced fewer siderophores (2 mm orange color zone) (Plate 22.5).

Table 22.3 Biochemical characterization of native fluorescent pseudomonads isolated from pomegranate rhizosphere

Isolates	KOH test	Catalase test	Starch hydrolysis	Gelatin liquefaction	Urease test	Casein hydrolysis	H_2S Test	Indole test
PFP-1	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PFP-2	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PFP-2	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PFP-4	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PFP-5	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PFP-6	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PFP-7	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PFP-8	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PFP-9	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PFP-10	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PFP-11	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive

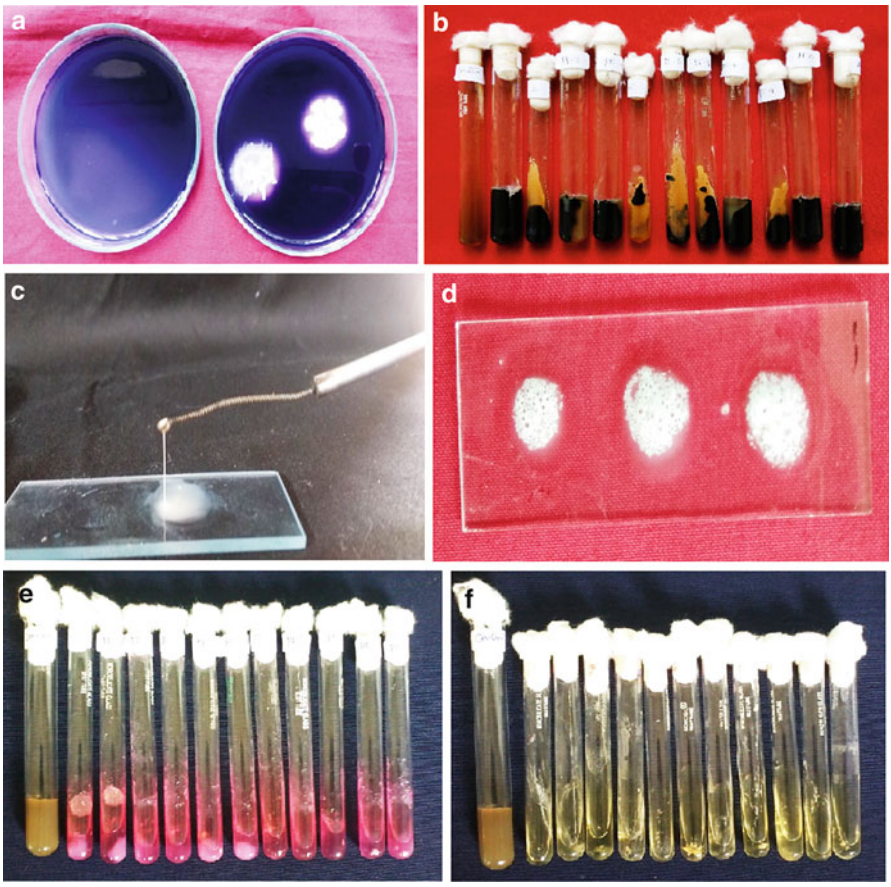

Plate 22.3 Biochemical tests of native fluorescent pseudomonads isolates from healthy pomegranate rhizosphere

22.25 Indoleacetic Acid (IAA) Production

In fluorescent pseudomonad isolates grown in culture medium amended with tryptophan, the isolates produced IAA as detected by the Salkowaski's reagent under spectrophotometer. The results (Plate 22.4) indicated 11 isolates produced indoleacetic acid which varied from 10 µg/ml to 32 µg/ml. The highest concentration of IAA was observed from PFP-11 (32 μgmL^{-1}) followed by PFP-10 (28 μgmL^{-1}) and PFP-4 (25 μgmL^{-1}). The lowest concentration of IAA was produced in PFP-2 (10 μgmL^{-1}).

Table 22.4 PGPR traits of native pseudomonads isolated from pomegranate rhizosphere

Pseudomonads isolates	Production of HCN	Siderophore production	IAA production μg/ml (24 h)
PFP 1	+	+	12.50
PFP 2	+	++	10.00
PFP 3	+	+	12.3
PFP 4	+++	+++	25.00
PFP 5	++	++	20.00
PFP 6	++	++	21.00
PFP 7	++	++	18.50
PFP 8	+	++	16.20
PFP 9	+	+	20.10
PFP 10	++	++	28.00
PFP 11	+++	+++	32.00

+, weak; ++, moderate; +++, strong

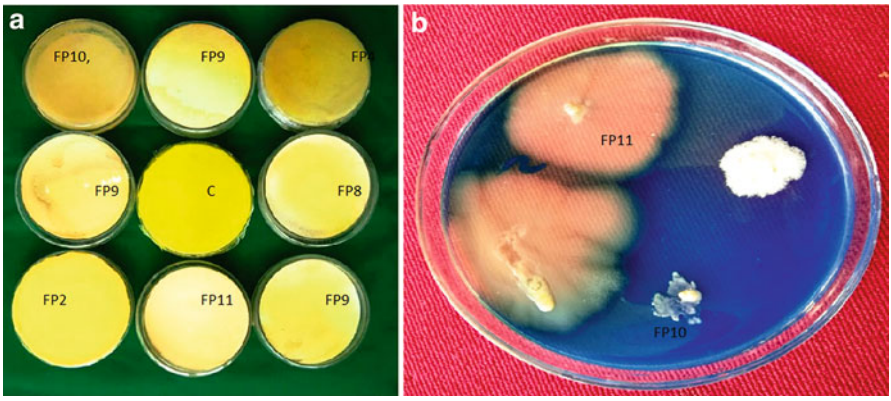

Plate 22.4 Plant growth-promoting traits of fluorescent pseudomonads

Plate 22.5 Siderophore production

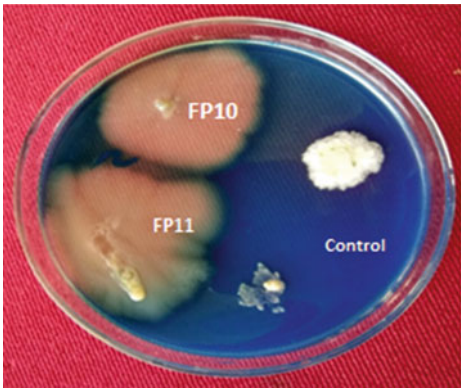

Table 22.5 Elucidation of biochemicals by *P. fluorescens* in pomegranate plants

Treatment	PO (mg/g)	PPO (mg/g)	PAL (mg/g)
T1: *P. fluorescens* (PFP-11)	0.078	0.089	0.071
T2: *C. fimbriata*	0.016	0.016	0.009
T3: Control	0.038	0.045	0.034

22.26 Elucidation of Defense Enzymes by ISR

When compared to pathogen alone and untreated control, the pomegranate plants which were inoculated with *P. fluorescens* showed higher levels of phenolics, PO, PPO, and PAL as compared to pathogen alone and untreated control (Table 22.5). The bioagent increased quantities of PO (0.078), PPO (0.089), and PAL (0.071) activities were recorded under the influence of *P. fluorescens* (T1) compared to *C. fimbriata* alone and untreated control.

22.27 Discussion

Thirty-five native fluorescent pseudomonad isolates were collected from rhizosphere soil samples of pomegranate from different districts of north Karnataka to study the antagonistic activity and plant growth promotion traits. The isolation was done as per the standard procedure. The isolates were purified by the streak plate method. Laha and Verma (1998) isolated six fluorescent *Pseudomonas* spp. from the rhizosphere of healthy cotton seedlings using King's B medium.

In the dual culture test, all the 35 isolates were found effective against *C. fimbriata*. However, only 11 isolates showed a high level of antagonistic activity by recording more than 50 percent inhibition of test pathogen out of 35 isolates. Among the 11 isolates tested, PFP-11 showed the maximum percent inhibition of mycelium followed by PFP-4. Sonyal et al. (2015) confirmed effective inhibition by reducing mycelial growth of *C. fimbriata*. Several authors have also reported that *P. fluorescens* inhibited several plant pathogens including *C. fimbriata* (Raja 2017).

Eleven isolates of *P. fluorescens* were studied in detail for morphological and biochemical characters. It was evident from the observations that all the 11 isolates (PFP-1 to PFP -11) showed Gram-negative reaction and fluorescence under UV light and produced slimy, irregular colonies with light yellowish green pigmentation and they were medium to long rod-shaped. These characteristics were regarded as taxonomically useful characteristics for *Pseudomonas* identification (Cartwright and Benson 1995). Similar findings were reported by earlier workers (Shivangi et al. 2017).

Further, biochemical characterization of fluorescent pseudomonads showed that all the isolates were positive for biochemical characteristics such as KOH test, catalase test, starch hydrolysis, gelatin liquefaction, urease test, casein hydrolysis, hydrogen sulfide, and indole production. These biochemical tests further confirmed that all the 11 isolates were fluorescent pseudomonads as reported by earlier workers (Vinay et al. 2016).

The highly efficient isolates which were selected after screening against the target pathogen were used for the elucidation of major mechanisms, which aids in antimicrobial activity and leads to suppression of the target pathogen. It is a prerequisite for selecting the potential biocontrol agents against phytopathogens. The fluorescent pseudomonads were known to express different mechanisms, viz., HCN, H_2S, siderophores, and indoleacetic acid (IAA). Fluorescent pseudomonads reported inducing the systemic resistance in plants. The production of phytohormones, suppression of deleterious organisms, production of IAA, activation of phosphate solubilization, and promotion of the mineral nutrient uptake are believed to be involved in plant growth promotion by PGPR. All the 11 selected isolates were positive for plant growth promotion traits such as the production of HCN, siderophore, and indoleacetic acid. *Pseudomonas* spp. are known for their plant growth promotion and disease control (O'Sullivan and O'Gara 1988).

In quantitative estimation, PFP-4 and PFP-11 recorded as strong producers of HCN. *Pseudomonas* releasing HCN were reported in the rhizosphere of different crop soils suppressive to various plant pathogens (Ramette et al. 2006). In our study, PFP-4 and PFP-11 were also identified as strong producers of HCN although other isolates were able to produce siderophores. Saranraj et al. (2013) reported that *Pseudomonas fluorescens* (PS-8) showed maximum siderophore production and the least siderophore production was showed by the isolate PS-4. All the isolates have produced the IAA; it ranged from 10 to 32 μg/ml. The highest concentration was in PFP-11 while lowest in PFP-2. The results are similar to the reports of earlier workers. IAA may function as an important signal molecule in the regulation of plant development.

In our study, the aim was on biotic inducers for generating the defense molecules against *C. fimbriata* in pomegranate. The ISR is mainly focused on the defense-related proteins, viz., PO, PPO, and PAL. Findings revealed that there was an increase in the activity of PO, PPO, and PAL in pomegranate plants treated with PGPR strain *P. fluorescens* when compared to pathogen alone and inoculated and control (Distilled water). Radjacommare et al. (2004) also reported increased activity of PO and PPO.

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Comparative Study of Indigenous and Nonindigenous Rhizobacterial Isolates to Induce the Resistance of Bunching Onion Against *Spodoptera exigua* (Hübner)

23

Trimurti Habazar, Arneti Arneti, Trizelia Trizelia, Ilma Juita, and Viner Elviona Putri

Contents

Abstract

Spodopter aexigua (Hübner) is known as a cosmopolitan pest of many crops, including bunching onion. Infestation by *S. exigua* is often serious in tropical regions, and insecticide resistance is a major problem in the management of this pest. Treating crops with a group of naturally occurring beneficial root bacteria, termed plant growth-promoting rhizobacteria (PGPR), has been shown to increase plant growth and enhance plant health. The effects of PGPR on plant–insect interactions have been reported in very few studies. The aim of this study was to compare the effect of indigenous and nonindigenous PGPR to induce the resistance of bunching onion against *S. exigua* and to increase plant growth and yield. The study was designed as a field experiment at endemic area for *S. exigua*. Treatments were 12 indigenous rhizobacterial strains from bunching onion rhizosphere, 14 nonindigenous rhizobacterial strains from the *Cyperus esculentus* rhizosphere, a control (without PGPR), and an insecticide (imidacloprid). PGPR strains were inoculated on bunching onion at two periods: at the time of planting as a seedling treatment, and on 2-week-old plants as a soil

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drench. The randomized complete block design was replicated three times. During the experiment, the population density of *S. exigua* and the damage to bunching onion and its growth and yield were evaluated. Our results showed that inoculation with the indigenous rhizobacterial strains was more effective to induce the resistance of bunching onions against *S. exigua* and to increase the growth and yield of bunching onion compared to nonindigenous rhizobacterial strains, control, and insecticide.

Keywords
Rhizobacteria · Indigenous · Nonindigenous · Plant growth promotion · Resistance · Bunching onion · *Spodoptera exigua*

23.1 Introduction

The beet armyworm, *Spodoptera exigua*, is known as a pest of the Welsh onion or green bunching onion, an important fresh vegetable in many Asian countries (Ueno 2006). All growing stages of the onion were attacked by the armyworm, although there was a positive relationship between plant height and infestation level (Ueno 2015). *S. exigua* can cause as much as 100% yield loss in the onion (Abdi 2003). Pest control is crucial to the stable production of marketable bunching onions. Current methods for controlling *S. exigua* are the use of sex pheromone, pesticides, and cultural measures (Zheng et al. 2000). Chemical control, that is, the use of synthetic pesticides, is currently the sole measure (except 'hand-picking') for controlling pests of the Welsh onion in Vietnam (Ueno 2006). However, relying exclusively on chemicals can cause the resurgence of pest populations and the development of pesticide resistance, causing chemical control to become ineffective (Dent 2000).

Biological control of pest insects in agriculture is the focus of many studies because of the risks in the continuous use of synthetic insecticides, which can cause resistant pests or the occurrence of secondary pests (de Oliveira Araújo 2015). The interest in the use of microorganisms such as plant growth-promoting rhizobacteria (PGPR) in agriculture has increased significantly in past years, because in both plant growth promotion and insect biological control, among other applications, they are potential substitutes for chemical products, thus favoring environmental preservation (Peixoto et al. 2002; Souza 2001).

The potential effects of PGPR on a variety of insect herbivores, compared with those on plant pathogens, remain sparsely explored. So far, a few species belonging to the selective genera of PGPR such as *Pseudomonas*, *Bacillus*, and *Serratia* have been studied against insect herbivores (Gange et al. 2012). *Stenotrophomonas maltophilia* affects the growth of the larval stage of *Helicoverpa zea*, the corn earworm, leading to more than 60% reduction in adult emergence, and the pupae and adults that emerged from bacteria-infected larvae were smaller (Bong and Sikorowski 1991). Qingwen et al. (1998) reported that *Pseudomonas gladioli* treatment to cotton plants reduced the growth, consumption rate, and digestive ability of the cotton bollworm, *Helicoverpa armigera*. In rice, different strains of *Pseudomonas fluorescens* effectively suppressed

infestation of the leaffolder *Cnaphalocrocis medinalis* in field conditions by the increased accumulation of defense molecules, chitinase and proteinase inhibitors (Saravanakumar et al. 2007). Zehnder et al. (1997) showed that a PGPR mixture (*Pseudomonas putida* 89B-61, *Serratia marcescens* 90-166, *Flavimonas oryzihabitans* INR-5, and *Bacillus pumilus* INR-7) significantly reduced populations of cucumber beetles. In *Arabidopsis*, *P. fluorescens* positively affects the weight gain of *Myzus persicae* (Pineda et al. 2012), but the same microbe–plant combination negatively affects the development of *Spodoptera exigua* (van Oosten et al. 2008). Irrespective of pest resistance, the majority of studies with *Pseudomonas* spp. and *Bacillus* spp. have shown the multiple advantages to plants through elevated crop yield, conservation of natural enemies in the crop ecosystem, and alteration of plant defense signaling (Gadhave 2015).

Two recent studies reported the negative effects of individual species inoculation of *Bacillus* on the growth and development of generalist insect herbivores (Vijayasamundeeswari et al. 2009; Valenzuela-Soto et al. 2010). The bioformulation containing *B. subtilis* showed detrimental effects against *Helicoverpa armigera* in cotton (Vijayasamundeeswari et al. 2009) and against virus-free *Bemisia tabaci* in tomato (Valenzuela-Soto et al. 2010). However, in contrast to pseudomonads, a mixture of two *Bacillus* species showed no effects against plant herbivores. A soil amendment base application of pepper plants with two *Bacillus* species, *B. subtilis* and *B. amyloliquefaciens*, significantly increased pepper yields, but failed to suppress green peach aphid (*Myzus persicae*) populations (Herman et al. 2008). These studies with *Bacillus* spp. highlighted that the use of individual and mixtures of microbial species can have varied effects on invading insects. Furthermore, these effects are likely to be specific to plants, pest species, and the degree of insect specialism.

The diversity of rhizobacteria has been studied by many researchers to determine their functional role in the management of soil-borne diseases, soil health improvement, and yield enhancement in major food crop systems (Johri et al. 2003; Lugtenberg and Kamilova 2009), but very few studies have explored how the use of PGPR may affect plant–insect interactions. The aim of this study was to compare the efficacy of indigenous and nonindigenous PGPR to induce the resistance of bunching onion against *Spodoptera exigua* and to increase plant growth and yield.

23.2 Materials and Methods

23.2.1 Source, Isolation, and Multiplication of Rhizobacteria

Samples were collected from the rhizosphere of healthy bunching onion as indigenous rhizobacteria and from the rhizosphere of yellow nutsedge as nonindigenous rhizobacteria in an endemic area of *Spodoptera exigua* at Cingkariang Village, Banuhampu Residence, Agam District, West Sumatera Province (Indonesia). Soil and plant samples were tagged with the location, date of collection, and type of crop and then brought to the Microbiological Laboratory at Faculty of Agriculture,

Andalas University. Samples were stored in a refrigerator (at 5 °C) until isolation of rhizobacteria, which was done 1 day after transport to the laboratory. For root suspensions, 0.1-g aliquots of side-roots of bunching onion or yellow nutsedge were homogenized with 1 ml sterilized tap water and shaken with an electric shaker. From this suspension, a dilution series up to 10^{-6} was prepared and 1 ml root suspension was placed in a Petri dish to which 10 ml nutrient agar (NA) (37.5 g NA in 1 l DW) was added and stirred well. Petri dishes were allowed to stand for 1 h to allow for solidification of the medium. Plates were placed for 2 days at room temperature (about 30 °C). Thereafter, dominant bacterial colonies were purified on NA medium as a suspected biocontrol agent (BCA). A single colony of bacteria then was transferred aseptically to a microtube that contained 1 ml sterilized aquadust as stock culture and stored in the refrigerator.

The rhizobacterial strains from stock cultures were first grown on NA medium to verify their purity. The inoculum was produced by transferring one loopful from the culture to 100 ml NB in a 250-ml Erlenmeyer flask and incubating at room temperature (28 ± 2 °C) on a shaker at 150 rpm for 48 h (preculture). The main culture was produced by transferring 1 ml preculture to 50 ml coconut water in a 250-ml flask and incubating in the same manner as the preculture: after 48 h incubation, the broth contained approximately 9×10^8 CFU ml^{-1} (Habazar et al. 2012). The bacterial suspensions were determined for bacterial density by comparing with scale 8 of McFarland's solution (approximately 10^8 cells ml^{-1}) (Klement et al. 1990).

23.2.2 In Planta Evaluation of Indigenous and Nonindigenous Rhizobacterial Strains for Control of *Spodoptera exigua* on Bunching Onion

Field studies were conducted as pot experiments at Taluak IV Suku Village, Banuhampu District, Agam, West Sumatera. This experiment was aimed to assess the effects of indigenous and nonindigenous rhizobacterial treatments for control of *S. exigua* infestation on bunching onion and to compare rhizobacterial treatment with weekly applications of insecticide for the control of *S. exigua.* The experiment used a randomized complete design consisting of 28 treatments and three replicates. The treatments were 12 indigenous rhizobacterial strains from a bunching onion rhizosphere, 14 nonindigenous rhizobacterial strains from the rhizosphere of yellow nutsedge, control (no rhizobacteria), and insecticide treatment (with imidacloprid).

Rhizobacterial strains were inoculated on bunching onion in two steps: at planting date as seedling treatment by a root-dipping technique before transplanting and on 2-week-old plants as a soil drench. Plants were grown in the field with humidity and temperature depending on natural conditions. A complete fertilizer (nitrogen:phosphate:potassium, 15:15:15) was applied at two phases of plant age: 1.09 g $plant^{-1}$ (175 kg ha^{-1}) at 21 days after planting (DAP) and 1.09 g $plant^{-1}$ (175 kg ha^{-1}) at 42 DAP (Setiawati et al. 2007).

The parameters observed were the diversity of bacterial colonies in the rhizosphere of bunching onion and yellow nutsedge; the development of armyworm

damage on bunching onion; and the first symptoms of armyworm, number of egg clutches, larval population, pest incidence, and pest severity. The efficacy of rhizobacterial treatment on bunching onion as a biocontrol to reduce the damage of armyworm has been used by the formula according to Sivan and Chet (1986) as follows:

$$E = (T - C)/C \times 100\%.$$

where E = the efficacy of treatment, T = treated plant, and C = control plant.

To examine the effect of rhizobacteria on plant growth characteristics, plant height, number of leaves, fresh weight, and total biomass were counted. The efficacy of rhizobacterial treatment on bunching onion as a biocontrol to reduce the damage of armyworm was used by the formula according to Sivan and Chet (1986, modified) as follows:

$$E = (C - T)/C \times 100\%.$$

where E = the efficacy of treatment, T = treated plant, and C = control plant.

23.3 Results

23.3.1 Diversity of Rhizobacterial Strains from Rhizospheres of Bunching Onion and Yellow Nutsedge

Isolations yielded 12 indigenous rhizobacterial strains from the bunching onion rhizosphere and 14 nonindigenous rhizobacterial strains from the rhizosphere of yellow nutsedge. The bacterial diversity in healthy rhizospheres of bunching onion and yellow nutsedge varied with regard to the morphological colony and the gram reaction (Table 23.1).

The bunching onion rhizosphere was dominated by a gram-positive bacteria population with white, irregular, flat colonies, and all rhizobacterial strains from yellow nutsedge rhizosphere were gram positive. All rhizobacterial strains showed negative by the hypersensitive response (HR); thus, those rhizobacterial strains are not plant pathogens. Based on the morphological colony, the gram reaction, and HR, we have found 18 rhizobacterial types from 26 rhizobacterial isolates.

23.3.2 In Planta Evaluation of Indigenous and Nonindigenous Rhizobacterial Strains to Control *Spodoptera exigua* on Bunching Onion

Rhizobacterially treated and insecticide-treated bunching onion plants had reduced numbers of egg clutches, larval populations, incidences on leaves, and severity of pest damage compared to untreated plants as a control. The total number of egg

Table 23.1 Morphological characters, gram reaction, and hypersensitive reaction of rhizobacterial strains from bunching onion rhizosphere (AGRZ) and yellow nutsedge rhizosphere (CRT)

	Morphological character of the colony						Hypersensitive reaction
Rhizobacterial strains	Form	Elevation	Edge	Diameter (cm)	Color	Gram reaction	
AGRZ 13a	Circular	Raised	Lobate	0.5	White	+	–
AGRZ 22b	Circular	Raised	Entire	1.0	White	+	–
AGRZ 21b	Circular	Raised	Entire	0.8	White	+	–
AGRZ 43c	Circular	Raised	Entire	0.3	Transparent	–	–
AGRZ 23d	Circular	Flat	Lobate	0.7	White	+	–
AGRZ 31d	Circular	Flat	Lobate	0.8	White	+	–
AGRZ 11f	Circular	Convex	Entire	0.4	Red	+	–
AGRZ 52g	Circular	Convex	Entire	0.5	White	+	–
AGRZ 14h	Circular	Umbonate	Filamentous	1.1	White	+	–
AGRZ 51i	Irregular	Flat	Lobate	1.2	White	+	–
AGRZ 12j	Irregular	Convex	Filamentous	0.6	White	+	–
AGRZ 32k	Rhizoid	Flat	Rhizoid	1.6	White	+	–
CRT 4.2k	Rhizoid	Flat	Rhizoid	1.5	White	+	–
CRT 1.2aa	Irregular	Umbonate	Undulate	0.4	White	+	–
CRT 1.1aa	Irregular	Umbonate	Undulate	0.5	White	+	–
CRT 2.1bb	Irregular	Umbonate	Undulate	2.0	White	+	–
CRT 5.1bb	Irregular	Umbonate	Undulate	2.0	White	+	–
CRT 2.5cc	Irregular	Umbonate	Undulate	3.3	White	+	–
CRT 1.9dd	Irregular	Flat	Entire	2.3	White	+	–
CRT 1.6gg	Filamentous	Flat	Filiform	0.9	White	+	–
CRT 1.4hh	Circular	Flat	Entire	0.4	White	+	–
CRT 1.7hh	Circular	Flat	Entire	0.5	White	+	–
CRT 1.3jj	Circular	Raised	Entire	0.3	White	+	–
CRT 1.8jj	Circular	Raised	Entire	0.6	White	+	–
CRT 1.5kk	Rhizoid	Flat	Rhizoid	1.2	White	+	–
CRT 2.2kk	Rhizoid	Flat	Rhizoid	1.5	White	+	–

clutches and larvae of armyworm on rhizobacterially inoculated onion during the growing season showed considerable variation between indigenous and nonindigenous rhizobacterial strains. The armyworms deposited most of their eggs on bunching onion in the control plants. On the bunching onions inoculated with indigenous rhizobacteria, fewer armyworm egg clutches were deposited than on nonindigenous rhizobacterially inoculated plants (Fig. 23.1), with their development checked every week (Fig. 23.2).

The number of egg clutches on indigenous rhizobacterially inoculated bunching onion varied from 0.98 to 3.31 and were lower than on nonindigenous rhizobacterially inoculated bunching onion and insecticide treatment (3.17–8.00). The best rhizobacterial strains to reduce the egg oviposition of armyworms on bunching onion were AGRZ21b, AGRZ22b, AGRZ23d, and AGRZ12j.

Generally, the numbers of larvae during the growing season of bunching onion were also lower on indigenous rhizobacterially inoculated bunching onion than on nonindigenous inoculated bunching onion, insecticide-treated plants, and control plants (Figs. 23.3 and 23.4). Three rhizobacterial strains were the best for reducing the number of armyworm larvae on bunching onion: AGRZ 14h, AGRZ 21b, and AGRZ 13a.

Armyworm incidence on leaves was lower at 70 days after planting (DAP) on indigenous reduced armyworm incidence on leaves of bunching onion (AGRZ 13a, AGRZ 14h, AGRZ 11f), rhizobacterially inoculated bunching onion than nonindigenous rhizobacterially inoculated bunching onion, insecticide treatment, and control plant (Fig. 23.5). The incidence of leaves on indigenous rhizobacterially inoculated bunching onion varied from 29.98 to 50.73%, whereas on nonindigenous rhizobacterial inoculated bunching onion varied from 41.20–60.70%. Four of the indigenous rhizobacterial strains showed the best ability to reduce armyworm incidence on leaves of bunching onion, namely, AGRZ 43c, AGRZ 21b, AGRZ 52g, and AGRZ 31d.

All indigenous rhizobacterially inoculated bunching onion showed lower pest severity caused by the armyworm at 70 DAP than nonindigenous rhizobacterially inoculated bunching onions and control plant (Fig. 23.6); insecticide-treated bunching onion also showed low pest severity. Pest severity on indigenous rhizobacterially inoculated bunching onion varied from 19.20–31.18%, whereas on nonindigenous rhizobacterially inoculated bunching onions this varied from 41.20% to 60.70%. Three indigenous rhizobacterial isolates showed the best ability to reduce the severity of armyworm damage on leaves of bunching onion (AGRZ 11f, AGRZ 12j, AGRZ 43c). Based on the efficacy of rhizobacterial treatment on bunching onion to reduce the deposition of egg clutches and the number of larvae during the growing season, pest incidence and pest severity compare than control plant, it has found four indigenous rhizobacterial strains as the best to control armyworm, namely AGRZ 13a, AGRZ 43c, AGRZ 11f, and AGRZ 14h.

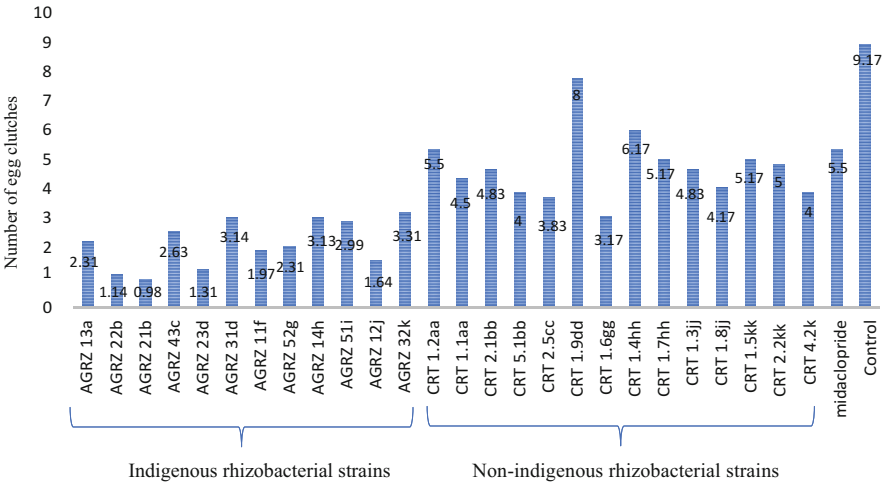

Fig. 23.1 Comparative analysis of a a number of egg clutches deposited by the armyworm between indigenous rhizobacterially inoculated bunching onion and nonindigenous rhizobacterially inoculated bunching onion during growing season

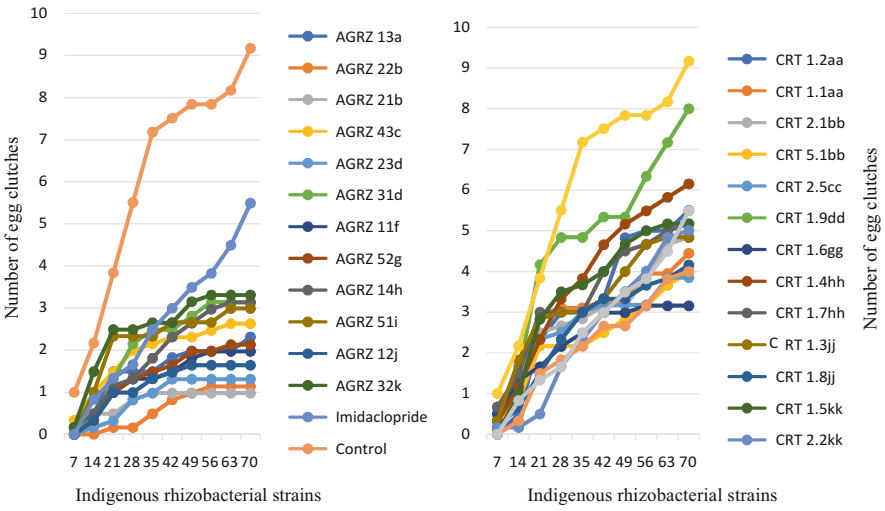

Fig. 23.2 Comparative development of a number of egg clutches deposited by the armyworm on indigenous rhizobacterially inoculated bunching onion and nonindigenous rhizobacterially inoculated bunching onion during the growing season

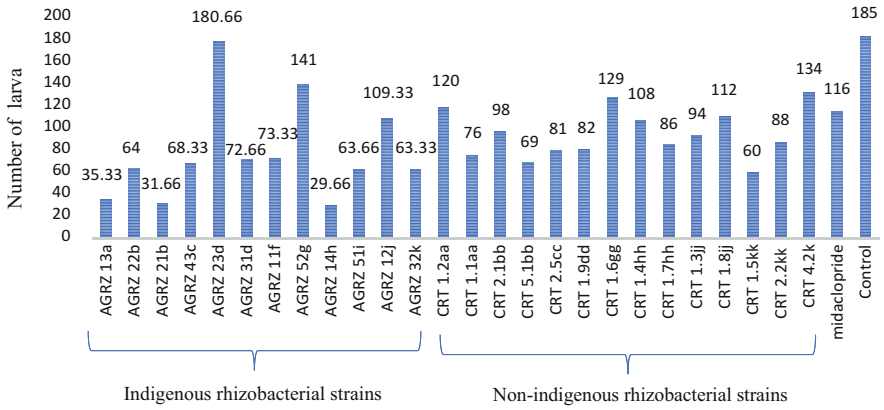

Fig. 23.3 Numbers of armyworm larvae from indigenous rhizobacterially inoculated bunching onion and nonindigenous rhizobacterially inoculated bunching onion during the growing season

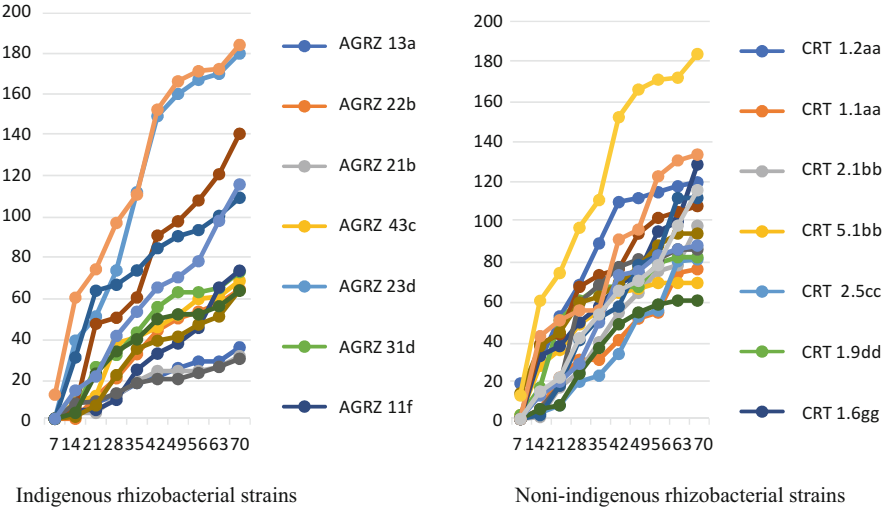

Fig. 23.4 Comparison of the number of armyworm larvae between indigenous rhizobacterially inoculated bunching onion and nonindigenous rhizobacterially inoculated bunching onion during growing season

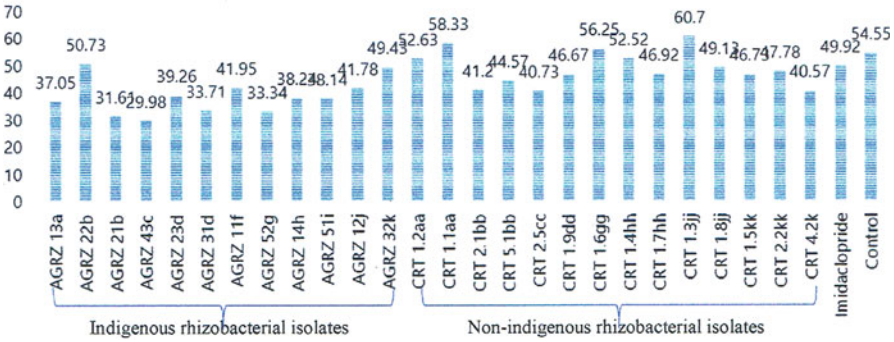

Fig. 23.5 Incidence of infested leaves by armyworm on indigenous rhizobacterial inoculated bunching onion and nonindigenous rhizobacterial inoculated bunching onion at 70 days after planting (DAP)

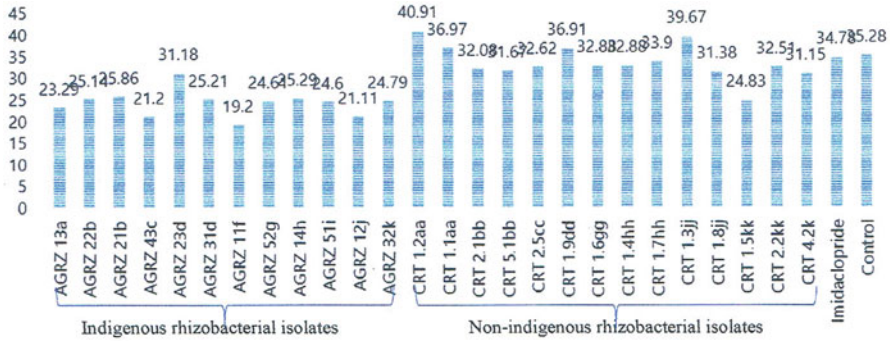

Fig. 23.6 Severity of armyworm infested leaves on indigenous rhizobacterial inoculated bunching onion and nonindigenous rhizobacterial inoculated bunching onion (70 DAP)

23.3.3 In Planta Evaluation of Indigenous and Nonindigenous Rhizobacterial Strains for Increasing Growth and Yield of Bunching Onion

Results revealed that plant height increased in plants treated with indigenous rhizobacteria over uninoculated control plants. Bunching onion plants were taller when inoculated with indigenous rhizobacteria and treated with insecticide than when inoculated with nonindigenous rhizobacteria or untreated (Fig. 23.7). The height of bunching onion plants inoculated with indigenous rhizobacteria varied from 44.00 to 53.66 cm, whereas those plants inoculated with nonindigenous rhizobacteria varied from 34.73 to 45.23 cm in height. Three indigenous rhizobacterial isolates of bunching onion showed the best increase in number of leaves (AGRZ 21b, AGRZ 14h, AGRZ 23d).

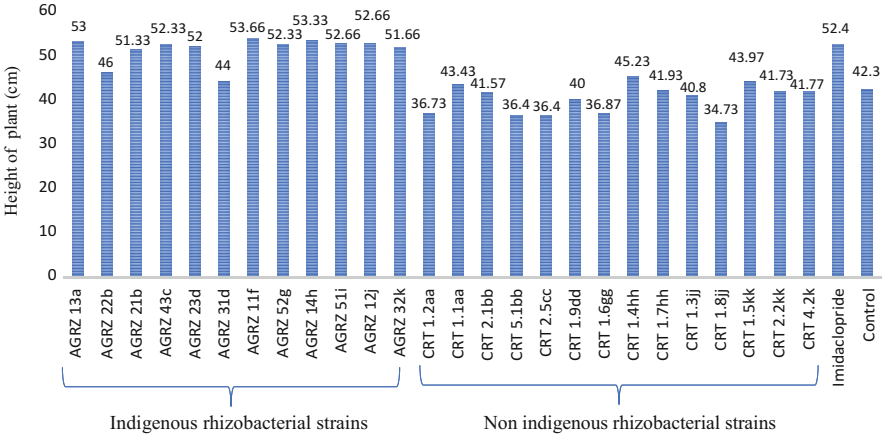

Fig. 23.7 Height of bunching onion plants on indigenous rhizobacteria and nonindigenous rhizobacterial treatment (70 DAP)

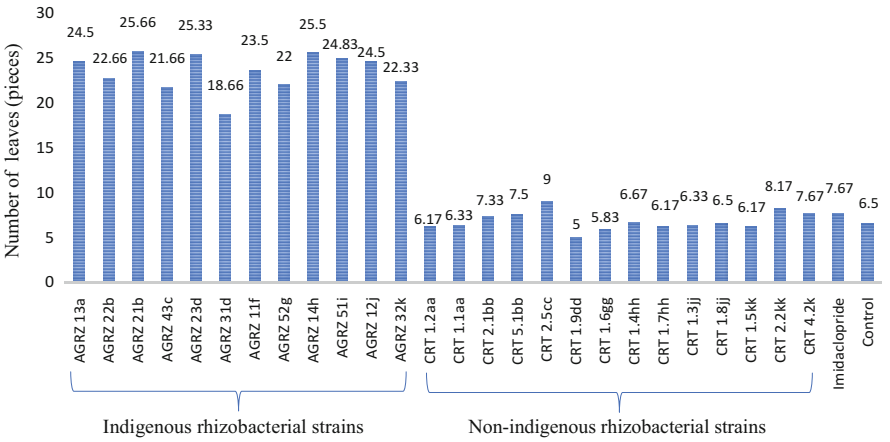

Fig. 23.8 Number of leaves on bunching onion treated with indigenous rhizobacteria and nonindigenous rhizobacteria (70 DAP)

The number of leaves on indigenous rhizobacterially inoculated bunching onion varied from 18.66 to 25.66 pieces, whereas on nonindigenous rhizobacterially inoculated plants this varied from 5.00 to 8.17 pieces. The same result for number of leaves was higher on indigenous rhizobacterially inoculated bunching onion than on nonindigenous rhizobacterially inoculated, insecticide-treated, and untreated plants (Fig. 23.8).

Generally, the yield of bunching onion was higher in rhizobacteria-treated plants and insecticide-treated plants than for untreated plants (Fig. 23.9). The yield in the control plant was 5.60 ton/hectare (ha). Nevertheless, not all the rhizobacterial

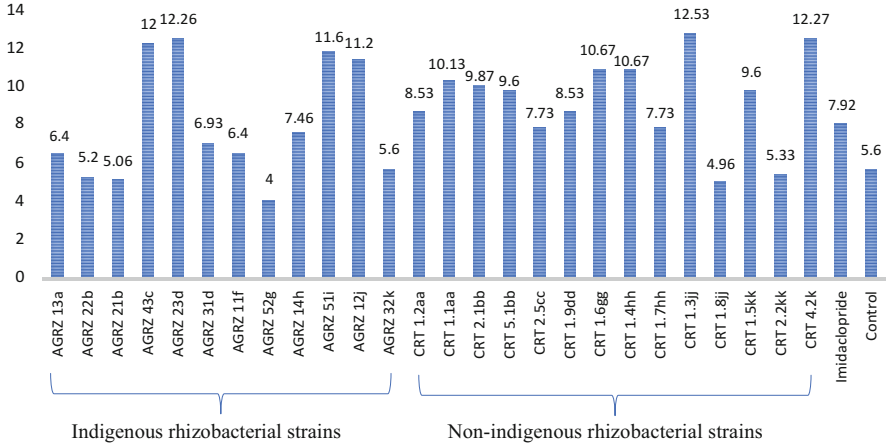

Fig. 23.9 Yield of bunching onion inoculated with indigenous rhizobacteria and with nonindigenous rhizobacteria

treatments could enhance the yield of bunching onion. Five rhizobacterial strains showed lower yield than control plants (4.00–5.33 ton/ha), which did not differ from the yield of indigenous rhizobacterially inoculated bunching onion. Two indigenous rhizobacterial strains (AGRZ 23d, AGRZ 43c) and two nonindigenous rhizobacterial strains (CRT 1.3jj, CRT 4.2k) showed the best increase in the yield of bunching onion. Although the yield was not different between indigenous and nonindigenous rhizobacterial treatments, the quality of the product was quite different. For example, the height of the plants and the number of leaves were higher with indigenous rhizobacterial treatment of bunching onion.

Based on the efficacy of rhizobacterial treatment to enhance the growth and yield of bunching onion compared with control plants, it was found that three indigenous rhizobacterial strains and two nonindigenous rhizobacterial strains were the best for increasing the growth and yield of bunching onion, namely, AGRZ 43c, AGRZ 23d, AGRZ 51i, CRT 42k, and CRT 13jj. One rhizobacterial strain (CRT 13jj) was found which could increase the yield of bunching onion but that had pest incidence and pest severity higher than that of control plants.

23.4 Discussion

Pot experiments in the field showed that bunching onions grown from seedlings treated with PGPR showed lesser deposition of egg clutches and populations of the armyworm, *Spodopter aexigua*, and lower incidence and severity of armyworm infestations (Figs. 23.1, 23.2, 23.3, 23.4, 23.5, and 23.6) compared with nontreated control plants. Similar results have been reported by Zehnder et al. (1997) in a cucumber experiment which demonstrated that plants grown from seed treated with PGPR sustained significantly lower populations of the cucumber beetles *Diabrotica*

undecimpunctata howardi and *Acalymma vittatum*, and lower incidence of bacterial wilt disease compared with nontreated control plants and plants sprayed weekly with the insecticide sevin valerate.

The results showed that inoculation of indigenous rhizobacteria on bunching onion can reduce the number of egg clutches of *S. exigua* in the growing season (10.7–36.1% compared with control plants), whereas the nonindigenous rhizobacteria-treated bunching onion showed a higher number of egg clutches (34.6–67.3% compared with control plants) (Figs. 23.1 and 23.2). It does mean the indigenous strains were more effective to control the armyworm than nonindigenous rhizobacterial strains. The armyworms deposited most of their eggs on bunching onion in the control plants compared with rhizobacterially inoculated plants. According to Nangle (2012), PGPR treatment can reduce oviposition by females. *S. exigua* females are capable of distinguishing between PGPR-treated and untreated cotton plants, as well as among some of the PGPR-treated plants. More notably, the results showed that PGPR treatments influence the oviposition behavior of *S. exigua*. Similarly, Coy (2014) has reported that female fall armyworms (FAW), *Spodoptera frugiperda*, deposited eggs mostly on the grass in the control containers (55%) with the remaining eggs on the enclosure. Less than a third ($\leq$29%) of eggs were deposited on the grass by female FAW in any of the PGPR-treated Bermuda grass pots.

Our results showed fewer eggs deposited by armyworm on indigenous rhizobacterially treated bunching onion (AGRZ 21b, 10.7%). Average counts of larval armyworm in this study were lower in the PGPR treatments compared with the nontreated control (Figs. 23.3 and 23.4). Three of the indigenous rhizobacterial strains were the best to reduce the number of armyworm larvae on bunching onion, namely, AGRZ 14h, AGRZ 21b, and AGRZ 13a (29.66, 31.66, and 35.33 compared with control, 180 larvae/plant during the bunching onion growing season). Reduction of the number of larvae on bunching onion treated by these rhizobacterial strains varied from 16% to 19%. This result is also lower than found in an experiment by Zehnder et al. (2001) in which PGPR-treated cucumber showed fewer cucumber beetles (0.44–0.73, reduced 2542%) as compared with the nontreated control (1.73). This finding suggested that rhizobacterial treatment for some plants is extendable to induced plant systemic resistance that can overcome the development and damage of armyworm on bunching onion.

Beneficial effects of PGPR and bioprotectants against insect pests on plants have been reviewed. Several species of nonpathogenic root-inhabiting microbes that have systemic effects on aboveground insect communities involving organisms at several trophic levels can trigger physiological changes and induction of defense in the host plant (Pineda et al. 2010; Katayama et al. 2011; Pineda et al. 2013). Induced plant defense against plant pathogens or insect herbivores can alter concentrations of secondary metabolites in the shoots and roots that influence plant interactions with nonpathogenic soil microbes. During the past few years, evidence has accumulated that plants have a sophisticated defense mechanism by actively recruiting nonpathogenic root-associated microbes following an attack by pathogens or insects (Rudrappa et al. 2008; Lakshmanan et al. 2012; Lee et al. 2012). The indirect effects

of microbes on herbivores via plant-mediated mechanisms, compounds produced by nonpathogenic root microbes, could also have a direct effect on insect attraction (Pangesti 2015). We have not found any information on the influence between indigenous and indigenous PGPR on the behavior of insect pests in general, but this study demonstrates that four indigenous rhizobacterial isolates, such as AGRZ 13a, AGRZ 43c, AGRZ 11f, and AGRZ 14h, from healthy bunching onion in an endemic armyworm area reduced egg deposition and the number of armyworm larvae. This result confirmed our previous research to control bacterial wilt on chili in which 13 selected rhizobacterial isolates from healthy chili rhizosphere in an endemic area of bacterial wilt disease (indigenous) reduced disease incidence caused by *Ralstonia solanacearum* on chili (0%) compared with control plants (100%) (Yanti et al. 2017). Habazar et al. (2011) also screened 41 rhizobacterial isolates from the healthy ginger rhizosphere in an endemic area of bacterial wilt disease in which no bacterial wilt disease was observed on ginger plants (0%) compared with control plants (100%).

This study revealed that bunching onion that was grown with indigenous rhizobacterial isolates had greater value in the monitored growth parameters such as height of the plant and number of leaves than indigenous rhizobacterial isolates, and the control, which was not treated with any biofertilizer, had the lowest value (Figs. 23.7 and 23.8). These results confirmed our previous research that showed all rhizobacterial isolates from healthy ginger rhizosphere increased the growth and yield of ginger compared to control plants (Habazar et al. 2011). Our results are in agreement with the findings of earlier research. Study with *Bacillus subtilis* and *B. amyloliqufaciens* showed increased plant growth and yield on different plants. PGPR treatments increased shoot weight, shoot length, and stem diameter of muskmelon and watermelon transplants (Kokalis-Burelle et al. 2003). Egamberdieva (2008) observed the positive effect of PGPR on the growth of wheat and pea. It was determined that PGPR applications could increase plant growth and seed germination rate, improve transplant emergence and response to stress conditions, and protect from disease (Elkinci et al. 2014). Such species as *Azospirillum*, *Pseudomonas*, and *Azotobacter* have significant impact on seed germination and transplant growth (Shaukat et al. 2006a, 2006b; Nezarat and Gholami 2009). Application of strains BSCBE4 and PA23 at the rate of 20 g kg^{-1} of seed significantly increased the growth of hot pepper seedlings (Nakkeeran et al. 2006). The highest fresh shoot and dry root weights of cabbage transplants were obtained from the application of *Bacillus megaterium* KBA-10, and root diameter, root length, and fresh root weight were obtained from the application of *B. megaterium* TV-91C (Elkinci et al. 2014). According to Nguyen and Ranamukhaarachchi (2010), rhizobacterially isolated TR6 treatments resulted in the highest pepper and tomato fruit weights compared with control plants. The ability to increase growth and yield of chili by rhizobacterial isolates was, presumably, that PGPR are effective root colonizers that survive and proliferate along with plant roots, resulting in enhanced plant growth (Whipps 2001). Yanti et al. (2017) obtained two indigenous rhizobacterial isolates that can control bacterial wilt diseases and increase the growth of chili.

23.5 Conclusion

Our results showed that inoculation with indigenous rhizobacterial strains was more effective to induce the resistance of bunching onions against *Spodoptera exigua* and to increase the growth and yield of bunching onion compared to nonindigenous rhizobacterial strains. Control of *S. exigua* by these indigenous rhizobacterial strains may help reduce chemical applications and their environmental impacts in agricultural systems where bunching onion crops are grown and will lower the cost of disease management. The use of rhizobacteria for increasing the yield and crop protection is a promising approach in modern systems of sustainable agriculture. Future research should be directed towards detailing the mode of action of these strains to control *Spodoptera exigua*.

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Use of Bioinoculants in the Modulation of Volatile Organic Compound Emission Under Environmental Stresses for Sustainable Agriculture

24

Poulami Chatterjee, Sandipan Samaddar, Aritra Roy Choudhury, Denver Walitang, Sunyoung Jeon, and Tongmin Sa

Contents

Abstract

Long-term effects of chemical applications in agroecosystems have brought about the rise in awareness with greater emphasis on sustainable development and eco-friendly agriculture. This has opened up the advancement and increased utilization of biofertilizers as key components acting as nutrient suppliers and lower agricultural burden that lead to sustainable agriculture and conservation of the environment. Predominant biofertilizers are plant growth-promoting rhizobacteria (PGPR) with plant growth-promoting functional traits. Bacteria containing ACC deaminase are well-known to provide plant stress tolerance by reducing the ethylene level in stress-elicited plant; on the other hand, the physiological responses and mechanism of improved plant stress resistance by bacteria are poorly understood. As a part of plant defence against biotic and abiotic stresses, plants release a range of volatile organic compounds (VOC). Though

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VOC emission is a part of plant defence, it has a large impact to the environment and climate change. The higher atmospheric reactivity of VOC leads to generation of ozone, secondary aerosol formation and cloud formation. However, the emission of VOCs from different plant species is well studied in this respect, but the control of VOC emission from plants is a complex and open question. This chapter aims to focus on the function of the ACC deaminase-containing bacteria in controlling VOC emission along with other plant growth-promoting characteristics.

Keywords
Bioinoculants · ACC deaminase · Volatile emission · Environmental stresses

24.1 Introduction

The shift in paradigm from the use of toxic chemical fertilizer to biofertilizer is one of the main objectives of agricultural scientists all over the world. Biofertilizers can be defined as any substance containing microorganisms (PGPB, AMF, etc.) used primarily in agriculture to increase plant growth under normal and stressed conditions (Vessey 2003). Biofertilizers lessen environmental burden originating from the chemical compounds, and its role in biotechnological advancements can be taken into account for the betterment of the environment.

Ethylene, an important phytohormone, is a central regulator when plants experience stress. The stress ethylene is synthesized in response to different stresses (Abeles et al. 1992; Morgan and Drew 1997). The 1-aminocyclopropane-1-carboxylate (ACC) deaminase-containing bacteria can regulate the ethylene emission by cleaving ACC into α-ketobutyrate and ammonia, thereby reducing ethylene emission in plants.

Biofertilizers are a key feature for sustainable development and eco-friendly agriculture. In this chapter, an overview is given on specific groups of PGPB with promising potential to be developed as biofertilizers. Their features, biological importance and mechanisms employed for plant growth promotion are also discussed. Further, we review the interesting role of PGP in modulating emissions of volatile organic compounds when plants experience stress conditions.

24.2 Plant Growth-Promoting Microorganisms

Agriculture is largely affected in response to biotic and abiotic stress; a routine use of various physical and chemical treatments is carried out against these factors to maximize yield. Nodule-forming bacteria or free-living bacteria in soil or plant tissues mainly increase plant growth and development by fixing nitrogen from atmosphere, secretion of phytohormone and detoxification of ROS or by producing siderophores and chelating agents to protect plant from abiotic and biotic stresses (Kloepper et al. 1988; Kamnev and van der Lelie 2000). Biofilm formation is another

phenomenon governed by quorum sensing, where microbes produce and sense signalling molecules to create a microbial population (Burd et al. 2000; Daniels et al. 2004; Mayak et al. 2004) nearby plant parts and contaminated soils or to help in the detoxification of the soil. Lucy et al. (2004) mentioned that along with solitary application of bacteria, co-inoculation of arbuscular mycorrhizal fungi and bacteria can be the better option for phytoremediation.

24.3 Bioprospecting PGPB as Biofertilizers

Nitrogen-fixing bacteria particularly from graminaceous plants are grouped into rhizosphere organisms, facultative endophytes and obligate endophytes (Baldani et al. 1997). Beijerinck first reported *Spirillum lipoferum* as diazotroph; later *Azotobacter paspali-Paspalum notatum* association and the rediscovery of *Spirillum lipoferum* (Döbereiner et al. 1972; Döbereiner and Day 1976) increased the use of diazotroph as a bioinoculant. However, it has been noted that *Acetobacter diazotrophicus* directly transfers N to host sugarcane, which was evidenced by (Sevilla et al. 2001).Bacteria belonging to various genera isolated from different plants under microaerophilic conditions possessed higher nitrogenase activities (measured by their acetylene-reducing activity (ARA)) revealing that the property of nitrogen fixation is widely spread among PGPB. The genera included *Azospirillum*, *Enterobacter*, *Methylobacterium*, *Burkholderia*, *Stenotrophomonas*, *Bacillus* and *Pseudomonas*. These bacteria also proved their potential for fixing nitrogen as measured by their nitrogenase activities. However, *A. brasilense* has shown better nitrogenase activity in roots of wheat plants (Kim et al. 2005). In addition, these bacteria also improved seedling vigour index and higher accumulations of plant hormones (Lee et al. 2005).

Phosphate-solubilizing microbes can efficiently secrete organic acids and acid phosphatase, required for the mineralization and solubilization of phosphorus in the soil (Gerretson 1948; Pikovskaya 1948; Sperber 1958; Illmer and Schinner 1995; Rodriguez and Reynaldo 1999; Chung et al. 2005), and it has been observed that phosphate-solubilizing microbe application improved the phosphate availability in plants (deFreitas et al. 1997). Strains isolated from Chinese cabbage primarily were affiliated to the genus *Pseudomonas* implying the versatility of the microorganisms. Previous investigations on the identification of pseudomonads from different soils of Korea revealed 53 genotypes separating them into ten distinct clusters including two groups that were not previously described (Kwon et al. 2005). The pseudomonads from Chinese cabbage had their close relationship with *Pseudomonas poae* and *Pseudomonas trivialis*, and these are isolated from grass leaves (Behrendt et al. 2003). There are some reports that endobacteria associating with ectomycorrhiza of scots pine share a close linkage with *P. poae* and *P. trivialis* (Izumi et al. 2006), which were reported as phosphate solubilizers. Sundara et al. (2002) mentioned that use of PSB reduced 25% in the application of P fertilizer and enhanced the availability of P in soil and also the status of P in sugarcane. In contrast, inoculation of maize plants with phosphate-solubilizing *Enterobacter agglomerans* had no effect

on uptake of P by plants (Laheurte and Berthelin 1988). The results of other studies also commemorate that inoculation of bacterial strains to canola had no effect on P content of the plants (Poonguzhali et al. 2008). On the other hand, when bacteria were inoculated in plants, it contributed to elongation of roots and increase in dry weight of plants. The characteristics observed imply plant growth-promoting potential demonstrated by bacterial isolates can be attributed to other characteristics rather than only P solubilization. Furthermore effect of acid phosphatase activities of the root extracts of canola showed acid phosphatase activity has nothing to do with solubilizations of inorganic phosphates; the synthesis of the phosphatases is stimulated when the level of inorganic P in the growth medium is limiting (Torriani 1960; Richardson 2001). Thus the relationship shared between P solubilization and activities of phosphatase enzyme cannot be established always. From the observations it can be said that the inoculation of PSB did not solubilize the rock phosphate or produced phosphatases from hydrolysing organic P. However, the availability of P was always higher after bacterial inoculation on root solutions compared to plants without inoculation.

24.3.1 *Azospirillum*

Azospirillum species are free-living, aerobic and heterotrophic bacterial species which has the ability to fix atmospheric nitrogen (N_2) under microaerobic conditions (Roper and Ladha 1995). They have been documented to have extensive interactions with crop plants either by growing in the rhizoplane or colonizing the intracellular crevices (Sumner 1990; Kennedy and Tchan 1992; Kim et al. 2005). *Azospirillum* species have mostly been regarded as an epiphyte which has the ability to grow in the vicinity or on root surfaces of wide range of plant species. The widely known *Azospirillum lipoferum* and *Azospirillum brasilense* have been isolated from both the root and stems of rice plants (Ladha et al. 1987), whereas *Azospirillum amazonense* has only been reported to colonize the root system (Pereira et al. 1989). Among all the heterotrophs in rice soils, *Azospirillum* was reported to contribute 1% of it as estimated by International Rice Research Institute (IRRI). Among the isolates of *Azospirillum,* 85% of it belonged to the *A. lipoferum* implying its essential role in colonization of rice plants (Ladha et al. 1987). *A. lipoferum* inoculation has been shown to increase rice yield significantly up to 6.7 g plant^{-1} under greenhouse conditions (Mirza et al. 2000). Nayak et al. (1986) have also reported the increase in plant height and tiller number of rice plants after inoculation of plants by *A. lipoferum*. The inoculation of *A. lipoferum* has also been reported to increase the yield by 22% in field conditions (Balandreau 2002). *Azospirillum* inoculation has been shown to enhance P and NH_4^+–N uptake by rice plants (Murty and Ladha 1988). On the other hand, it has also been reported that *Azospirillum* was able to reduce the infection caused by *Xanthomonas oryzae* with enhancement of various yield components (Islam and Bora 1998).

Studies showed when N fertilizers are applied at a lower rate (50–60 kg N ha^{-1}), *A. brasilense* inoculation enhanced the yield components and also the yield of wheat

grains by 30% in field soils (Okon and Labandera-Gonzalez 1994), whereas the higher rate of application (110–170 kg N ha^{-1}) didn't show any statistical significance (Dobbelaere et al. 2001). Hence, the report prospects to supplement a substantial amount of urea-N for wheat while maintaining yields by inoculating *Azospirillum*. The inoculation of *Azospirillum* has shown to increase N and P uptake in field trials (Galal et al. 2000) by stimulating the enhancement of growth of plant root. There have been other reports which validated the beneficial effect of *Azospirillum* inoculation on wheat yields under greenhouse as well as field conditions (El-Mohandes 1999; Ganguly et al. 1999). The initial supplementation of soil by malate and subsequent inoculation of NH_3-excreting strain of *A. brasilense* has shown increased N uptake by wheat plants under greenhouse conditions (Islam et al. 2002). The inoculation of wheat plants with different types of *Azospirillum* sp. has shown significant differences in plant growth promotion of wheat plants (Han and New 1998; Saubidet and Barneix 1998). Malik et al. (2002) had established that *A. brasilense* and *A. lipoferum* contributed about 7% and 12% of wheat plant N by biological nitrogen fixation (BNF), respectively. This contribution can form a major part for obtaining a greater crop yield with less application of N fertilizers. Lower amounts of N supply to wheat plants should not be under evaluated as it may play a crucial role in increasing the capacity of the plant to assimilate soil-N. As the wheat plants cannot release much C to the rhizosphere soil, it can be a major problem for *Azospirillum* to perform the BNF activity. The supplementation of malate into the soil can alleviate this problem under laboratory conditions (Wood et al. 2001). An ammonium-excreting mutant of *A. brasilense* demonstrated much of N fixed in wheat plants were performed by BNF after several days' growth of seedlings. Similarly, other studies showed similar capabilities of *Azospirillum* to fix more biological nitrogen and also to express *nifH* considerably (Deaker and Kennedy 2001). Conjugation experiments with various strains of *Azospirillum* with *E. coli* S17.1 transformed with the plasmid *pLA-lacZ* which expresses *lacZ* gene in the conjugated strains was confirmed by β-galactosidase assay. *A. brasilense* CW301 had shown the highest β-galactosidase activity followed by *A. lipoferum* CW1503 (Kim et al. 2005) under aerobic conditions. These conjugants were used for the colonization studies on wheat roots. The inoculation of these strains has been shown to adhere to the roots of wheat plants soon after inoculation and didn't remain in the hydroponic solution (Zeman et al. 1992). Therefore, sufficient time was allowed for the bacteria to differentiate completely during the 10 days following inoculation. Vande Broek et al. (1993) and Arsene et al. (1994) had concluded that β-galactosidase activity and X-gal staining could be correlated with the extent of colonization. A correlation between β-galactosidase activity and association of bacterial population with plant roots was validated with such type of assays. The expression of β-galactosidase activity was significantly higher in the case of *A. brasilense* compared to *A. lipoferum* (Kim et al. 2005). This report validated the adaptability of *A. brasilense* to the particular plant culture conditions. On the other hand, Katupitiya et al. (1995) had shown that *A. lipoferum* are more efficient than *A. brasilense* on the same wheat genotypes.

In sand culture and wet soils, *A. brasilense* cells were reported to move towards wheat roots (Bashan 1986). This implies the significant chemotactic ability

demonstrated by *Azospirillum* strains in different environments. *Azospirillum* transformed with *pLA-lacZ* plasmids enabled visualization in roots of wheat plants by examination of root segments by staining with X-gal (Kim et al. 2005). Studies utilizing *nodG-lacZ* fusions which express lacZ gene showed localization of bacteria to the roots implying their colonization potential (Katupitiya et al. 1995). Vande Broek et al. (1993) proposed a mechanism involving chemotaxis for the preferential colonization of *Azospirillum* sp. in lateral roots and root hair zones of wheat plants.

A. brasilense inoculation with cotton plants by dipping the roots of seedlings in a suspension of bacterial cells for 30 mins (Fayez and Daw 1987) showed significant increase in root hair growth and number of lateral roots, which has subsequently enhanced plant dry weight under greenhouse conditions (Bashan 1998). The inoculation of *Azospirillum* sp. with cotton plant seedlings significantly increases the N content of the plant up to 0.91 mg plant^{-1} (Fayez and Daw 1987). *Azospirillum* inoculation with cotton plants has also shown to downregulate fungal and bacterial infections by producing antifungal and antibacterial compounds. It has also shown to produce growth regulators and fix iron from rhizospheric zone by producing siderophores (Pandey and Kumar 1989). The facultative diazotrophs, *A. brasilense* and *A. lipoferum*, generally colonize roots, stems and leaves of plants, while *Azospirillum amazonense* is found in roots and stems (Reis et al. 2000). The inoculation of *Azospirillum* enhanced cane yield in both plant (9 t ha^{-1}) and ratoon crops (5 t ha^{-1}) in the field (Shankariah and Hunsigi 2001). Muthukumarasamy et al. (1999) also reported the increase in N content of sugarcane leaves after inoculation with *Azospirillum* sp.

24.3.2 *Burkholderia*

The genus *Burkholderia* consists of 29 species, including *Burkholderia vietnamiensis*, *Burkholderia kururiensis*, *Burkholderia tuberum* and *Burkholderia phynatum* which have been reported to be potential N_2 fixer (Estrada-de los Santos et al. 2001; Vandamme et al. 2002). *Burkholderia vietnamiensis* was the first isolate of this genus to be reported by Gillis et al. (1995). It was isolated from the rhizosphere of young rice plants cultivated on a Vietnamese soil (Trân et al. 1994). The field trial experiments with rice by inoculation of *B. vietnamiensis* enhanced grain yield significantly (up to 0.8 t ha^{-1}) (Trân et al. 2000). The inoculation of *B. vietnamiensis* in these field trials reduced the usage of 25–30 kg N ha^{-1} inorganic fertilizer. The ^{15}N tracer technique revealed that *B. vietnamiensis* contributes to 19% of rice plant N (152 μg N plant^{-1}) under gnotobiotic conditions (Baldani et al. 2000). *B. vietnamiensis* had been isolated from the rhizospheric soil of rice plants, and hence it is regarded as an epiphyte (Baldani et al. 1997). However, Baldani et al. (2000) had reported the isolation of an endophytic *Burkholderia* sp. from the interior parts of roots, stems and leaves of rice from Brazil. It has been shown to have the capability to fix 31% of rice plant N (372 μg N plant^{-1}) from the atmosphere and subsequently increased the plant biomass by 69% (22 mg plant^{-1}) under gnotobiotic conditions (Baldani et al. 2000). On the other hand,

several *Burkholderia* spp. such as *Burkholderia glumae* and *Burkholderia cepacia* cause diseases in plants (Balandreau 2002). Hence, appropriate care and risk-reducing techniques should be employed while isolating and culturing *Burkholderia*.

Burkholderia brasiliensis is an endophytic bacteria which can colonize root, stem and leaves of sugarcane plants, whereas *Burkholderia tropicalis* has been reported to colonize roots and stems (Reis et al. 2000). These bacterial strains have the ability to produce antagonistic substances against nematodes (Meyer et al. 2000). Our previous study focuses on *Burkholderia* isolated from roots and rhizosphere soil of rice, and they are utilizing methanol as carbon source. It produced indole-3-acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC) deaminase and possessed antagonism against the phytopathogens *Erwinia carotovora* subsp. *Carotovora* and phosphate-solubilizing ability. *Burkholderia* strains originally isolated as phosphate-solubilizing bacteria also produced IAA, ACC deaminase and siderophores. Furthermore, these strains produce cell to cell communicating quorum sensing signal molecules under in vitro and in planta conditions (Poonguzhali et al. 2007).

24.3.3 *Methylobacterium*

Methylobacterium is strictly aerobic, facultative methylotrophic, gram-negative, rod-shaped bacteria that are able to grow on one-carbon compounds (e.g. methanol or methylamine), as well as on a variety of C_2, C_3 and C_4 substrates (Green 1992). *Methylobacterium organophilum* is reported to use methane as sole carbon source for energy metabolism (Patt et al. 1976). The genus of these bacterial species is classified in α2 subclass of the *Proteobacteria* and has been reported to consist 14 species (Madhaiyan et al. 2007). The distribution of *Methylobacterium* has been reported from wide range of natural and man-made environments such as soil, air, dust, fresh and marine water sediments, water supplies, bathrooms, air-conditioning systems and masonry (Hiraishi et al. 1995; Trotsenko et al. 2001). There have been reports of some *Methylobacterium* species which are opportunistic human pathogens (Truant et al. 1998; Hornei et al. 1999). *Methylobacterium* are widely associated with terrestrial as well as aquatic plants, effectively colonizing roots and leaves (Austin et al. 1978; Yoshimura 1982; Corpe and Rheem 1989; Trotsenko et al. 2001; Lidstrom and Chistoserdova 2002). The symbiotic relation of *Methylobacterium* with plants relies on the utilization of methanol produced by plants during cell wall degradation and deposition of the same in the stomatal opening (Trotsenko et al. 2001). These groups of bacterial strains can stimulate plant growth by production of phytohormones (cytokinins and auxins) (Ivanova et al. 2001; Koenig et al. 2002), by fixation of atmospheric nitrogen (Sy et al. 2001a, b) or by their antagonistic properties against plant pathogens (Holland and Polacco 1994) through induce systemic resistance (Madhaiyan et al. 2004). They have a characteristic property of being pinkish in colour due to the presence of carotenoids and hence widely termed as pink-pigmented facultative methylotrophs. *Methylobacterium* has been reported to be a highly robust class of bacterial species as they are resistant to high or low temperatures and freezing conditions and few of

them are also shown to be resistant to UV and ionizing radiations (Trotsenko et al. 2001). *Methylobacterium* has been known to metabolize methyl chloride (McDonald et al. 2001), methyl bromide (Goodwin et al. 2001), methyl iodide (Schaefer and Oremland 1999), dichloromethane (Doronina et al. 2000), ethylated sulphur-containing compounds (de Zwart et al. 1996), methylated amines (Trotsenko et al. 2001), methyl tert-butyl ether (Mo et al. 1997) and cyanate and thiocyanate, which are regarded as toxic organic chemicals. Madhaiyan et al. (2005a, b) reported *Methylobacterium* sp. strains capable of producing considerable amounts of phytohormones such as IAA and cytokinins which subsequently stimulate seed germination and plant development. The cytokinin production helps to induce greater cytokinins in the host plants (Butler et al. 2000). The *Methylobacterium* sp. was also able to show ACC deaminase activity which in turn reduced the amount of "stress ethylene" produced by plants during abiotic and biotic stress conditions. The reduction of "stress ethylene" levels on plants enhanced the growth and development of the host plant after inoculation with *Methylobacterium* sp. under greenhouse conditions.

Production of auxins and cytokinins by *Methylobacterium* constitutes their importance in promoting plant growth. For instance, they have been shown in some cases to stimulate seed germination, root growth and morphology and plant development, possibly by the production of phytohormones (Holland 1997; Freyermuth et al. 1996). Production of IAA auxin and cytokinins by *Methylobacterium* strains is also reported (Koenig et al. 2002; Omer et al. 2004). Also, the induced systemic resistance against the rice sheath blight pathogen and groundnut rot pathogens through the production of pathogenesis-related proteins in *Methylobacterium* has also been reported (Madhaiyan et al. 2004, 2006b). Recent evidence also suggests the occurrence of plant growth-modulating enzyme ACC deaminase in *Methylobacterium* and the role of *Methylobacterium* ACC deaminase in lowering the ethylene levels in canola (Madhaiyan et al. 2006a).

24.4 Going Global: The Future Prospect of PGPB as Mitigators of Environmental Stress

The activity of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, an enzyme that can lower plant ethylene levels, is typically increased by a wide variety of environmental stresses such as flooding, drought, heavy metals, organic contaminants, pathogen attacks and salt stress. Our lab bioinoculants already showed and reported to have different stress-tolerant abilities under adverse environmental conditions. Our work has demonstrated that different halotolerant bacteria, isolated from soils obtained in a barren field and from the rhizosphere of halophytes, are able to withstand high salt concentrations (1.75 M NaCl) and pH (6.5 to 8.5) and can facilitate plant growth promotion in the presence of growth-inhibitory levels of salt. We also stated that arsenic (As)-tolerant bacteria, possessing PGP traits identified from the As-polluted environment, were found to enhance root growth and tolerance index of maize in the presence of As (Shagol et al. 2014). Moreover, our data also

reported that ACC deaminase-producing *Methylobacterium* sp. helped tomato plants, challenged with *Ralstonia solanacearum* (RS), to survive under biotic stress, and also significantly reduced disease symptoms and lowered ethylene emission under greenhouse condition (Yim et al. 2013). Subramanian et al. (2015) also reported the major role of ACC deaminase to control chilling stress.

Plant responses to different levels of temperatures are commonly considered antagonistic. Moreover, membrane fluidity and membrane rigidity, these two major factors, mainly provide resistance to low and high temperature stress to plant (Sung et al. 2003). A group of antioxidants such as glutathione, water-soluble ascorbate and lipid-soluble tocopherols (Camejo et al. 2006; Havaux and Kloppstech 2001) mainly control the enhanced production of reactive oxygen species after chilling and heat stress. Though there are some points of convergence of responses to heat and chilling stresses, it is observed that higher concentrations of sugars can give more tolerance to both heat and cold stress resistance (Gusta et al. 1996). Additionally, excluding a group of non-volatile antioxidants, a wide range of biogenic volatile organic compounds (BVOCs) is generated by plants during and after stress. Conversely, products of the lipoxygenase pathway – LOX products containing various C6 aldehydes and alcohols (also called green leaf volatiles) and emissions of short-chained alcohols and aldehydes such as methanol – constitute one of the first stress responses of plants (Loreto et al. 2006).

Many researchers reported elicitation of lipoxygenases under a group of stresses, including cold stress, mainly in leaves (Fall et al. 2001; Nemchenko et al. 2006; Copolovici and Niinemets 2010). Besides that, lipoxygenase (LOX) product emissions have been observed to be elicited in response to pathogen (Jansen et al. 2009; Steindel et al. 2005), light (Staudt and Lhoutellier), heat (Loreto et al. 2006), ozone (Beauchamp et al. 2005) and herbivory (Copolovici et al. 2011) and in fruits under cold stress (Mao et al. 2007). The LOX product emission rate is directly related to the severity of stress responses (Beauchamp et al. 2005; Copolovici and Niinemets 2010; Fall et al. 1999; Niinemets 2010).

Apart from stress volatiles, emissions of a number of specific volatiles are repeatedly induced from stressed plants, including mono- and sesquiterpenes and homoterpenes, along with volatiles depending on the stress type (Niinemets 2010). Isoprene accounts for 40% of total global biogenic volatile organic compounds' (BVOCs) emissions with the total amount emitted of 440–660 Tg carbon per year (Guenther et al. 2006). As a dominant and highly reactive BVOC, isoprene reacts rapidly with hydroxyl radicals (•OH), nitrogen oxides (NOX) and ozone in the atmosphere after release from vegetation; this strongly impacts tropospheric ozone production and aerosol formation and partly controls the methane lifetime. Thereby, isoprene emissions potentially influence large-scale Earth system processes; thus it is essential to investigate the influence of environmental factors on isoprene emissions from plants to predict global climate change and provide useful guidelines on environmental management policies for the future.

Stress-induced volatile is mostly involved in plant-plant and plant-insect communications (Arimura et al. 2009; Loreto and Schnitzler 2010); besides that increased level of terpenoids also reported to play a major role as lipid-soluble

antioxidants in plant stress resistance (Vickers et al. 2009). Many scientists reported that monoterpenes increase plant heat stress resistance; surprisingly more heat exposure to the plant has shown to significantly heighten terpenoid emissions. It is known that the response of plants to abiotic stress and pathogen response is at least partly mediated by ethylene. It has been reported that increased biosynthesis of isoprene is directly associated with abiotic stress and is a signal of herbivore infestation (Arimura et al. 2002, 2009; Matsui 2006; Vickers et al. 2009).

Primary and secondary metabolism of the plant is differently regulated by abiotic stress. BVOC emission is mainly dominated by the inhibition of photosynthesis by reducing CO_2 uptake and regulating the diffusion rate in leaves to control biochemical reactions of the photosynthetic cycle under stress conditions. Terpene biosynthesis requires a high amount of photosynthetic carbon (Loreto et al. 1996), and some unidentified photosynthates are the main suppliers of carbon that causes stimulation of terpene biosynthesis. However, various researchers reported from their carbon labelling studies that isoprene may also be formed from xylem-transported glucose and chloroplastic starch (Schnitzler et al. 2004). Some other reports (Brilli et al. 2007) pointed out that, because of starch depletion, extra-chloroplastic sources of carbon may be activated and feed carbon to volatile terpenes. Stress-induced damage mainly induces the emission of volatiles, thus giving protection to plant under stress condition (Vickers et al. 2009).

Photosynthesis is the main regulator of isoprene biosynthesis because increased drought stress cannot reduce its emission until its photochemical cycle shuts down completely (Sharkey and Loreto 1993).The sustained emission of isoprene to salt and drought (Teuber et al. 2008; Loreto and Delfine 2000) is made possible by the induction of carbon sources alternative to photosynthesis, possibly related to respiration (Loreto and Delfine 2000) or starch breakdown (Schnitzler et al. 2004). Isoprene and monoterpenes are produced through a dedicated metabolic pathway that is stimulated by several abiotic stresses. This tightly regulated terpene biosynthesis and the observation that emission of volatile terpenes represents a significant loss of photosynthetic carbon led to the proposition that these compounds play important physiological and ecological roles in the protection of plants from environmental constraints.

However, some ACC deaminase-producing bacteria have already been reported to provide stress tolerance to plant (Siddikee et al. 2010, 2011; Yim et al. 2013). Moreover, it is stated that an extracellular matrix formed by bacterial biofilm can provide an almost infinite range of macromolecules beneficial for plant development and growth. Biofilms contain sugars and oligo- and polysaccharides that can play various roles in bacteria-plant interactions, e.g. in improving water availability in root medium. The water retention capacity of some polysaccharides can exceed severalfold their mass (Timmusk et al. 2014).

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Salinity Resistance of *Azotobacter* Isolated from Saline Soil in West Java

25

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Contents

Abstract

The important barrier for food crop production in saline soils is high electrical conductivity as well as low nitrogen availability. Biofertilization through the application of a nonsymbiotic nitrogen-fixing rhizobacterium which is resistant to the saline condition is one of the ways to increase nitrogen uptake in such soil. The objectives of this experiment were to evaluate the resistance of *Azotobacter* for sodium chloride and their effect to enhance the early growth of tomato seedling. *Azotobacter* was isolated in Ashby's medium from paddy soil with the electrical conductivity of 4 dS m^{-1}. Sensitivity test to saline condition was conducted in sterile Ashby liquid medium enriched with 0.85%, 1.7%, and 3.4% of sodium chloride for three isolates with higher N-fixing capacity. For pot trial, tomato seedling was grown in saline soil and inoculated with single strain bacterial liquid culture. The pot experiment was setup in split-plot design to test the combination treatment of two most sodium-resistant *Azotobacter* isolates and their concentration. The results showed that *Azotobacter* isolates S2 and K4 were more resistant to 3.4% sodium chloride although their generation time was slightly higher. In the pot trial, shoot height of a 4-week-old tomato seedling increased after *Azotobacter* S2 and K4 mixed inoculation. *Azotobacter*

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inoculation increased leaf number and root's dry weight of tomato. The results suggested that saline-resistant *Azotobacter* has plant growth-promoting activity.

25.1 Introduction

Intensive agricultural land use change affected the availability of suitable lands for food crops production in Indonesia, especially in Java Island where the annual loss of agricultural area is nearly 80.000 ha. Food production such as vegetable cultivation is forced to be carried out in a marginal land dominated by unfertile soil. In the coastal area of Java, farmers try to grow vegetable in saline lowland soil, but the yield was low. The USDA system defines that saline soils have an electrical conductivity of the saturated paste (EC_e) > 4 dS m^{-1}, exchangeable sodium percentage (ESP) < 15 or sodium adsorption rate (SAR) < 13, and pH < 8.5 (Yan et al. 2015).

High salinity changes soil physical, chemical, and microbiological properties. Soil particles were flocculated because soluble salts like Ca^{2+} and Mg^{2+} ions in soil colloids increased EC of soils, lowered bulk density, and increased porosity (Srivastava et al. 2014) due to the processes of swelling and dispersion (Worku and Bedadi 2016). High salt content in soil lowers osmotic potential of soil water and their availability to plants (Bhatt et al. 2008). Excess sodium inhibits potassium uptake by roots and cationic potassium which plays an important role in maintaining cell turgidity and enzyme activities (Xiong and Zhu 2002). The high content of sodium disturbs the photosynthetic process by impairing in both biochemical and photochemical process (Munns and Tester 2008).

Level of ammonium (NH_4^+) in saline soil was low which can be explained by reduced mineralization (Walpola and Arunakumara 2010). Sources of available nitrogen in soil among others are organic N mineralization into inorganic N and nitrogen fixation via the bacterial enzymatic process. Reduced level of ammonium threats the availability of nitrate (NO_3^-), a major form of nitrogen available for root uptake. Chemical nitrogen fertilization is common to induce plant productivity in the saline soil to fulfill plant requirements rapidly but excessive and intensive use of inorganic fertilizers can decrease soil health. Long-term use of inorganic fertilizers may lead to soil organic matter reduction and inadequate level of nutrients under farming management with no nutrient turnover from crop residues (Su et al. 2006).

High soil salinity reduced microbial activity as well as threats to plant growth. Salinity reduced microbial activity through osmotic stress and toxic ion (Yan et al. 2015) and could change the microbial community structure. A significantly increased content of archaea was demonstrated in the sample with the highest salinity (Andronov et al. 2012). Higher concentration of sodium chloride (3000 mg kg^{-1}NaCl) decreased plant length in common bean (*Phaseolus vulgaris* L.), but root length development was inhibited by 1000–3000 mg kg^{-1} of sodium chloride (Beltagi et al. 2006). Increased salt concentrations up to 250 mmol

ceased the germination percentage of leafy vegetable pak choi (Memon et al. 2010) but sodium chloride at low and medium concentration increased plant height although sodium chloride above 240 mM decreased *Vicia faba* L. height (Qados 2011).

Plant growth-promoting rhizobacteria (PGPR) are widely reported to reduce the use of inorganic fertilizer. Well-known PGPR *Azotobacter* sp. is commercialized in the form of either liquid- or carrier-based inoculant. This biofertilizer serves available nitrogen by fixing dinitrogen gas in soil (Jiménez et al. 2011). *Azotobacter* produced growth regulators such as cytokinin, auxin, and gibberellin which are important to stimulate cell enlargement and division (Salmeron et al. 1990). Regulatory circuits of abscisic acid, indole acetic acid, cytokinins, gibberellic acid, salicylic acid, brassinosteroids, jasmonates, ethylene, and triazole produced when the plant is exposed to abiotic stresses (Fahad et al. 2015). Certain *Azotobacter* isolates exhibited a high tolerance to abiotic stress due to their ability to produce exopolysaccharides (Sivapriya and Priya 2017; Hindersah et al. 2017).

There is a significant role of *Azotobacter* as plant growth promotor in saline soil. *Azotobacter* sp. grows and fixed nitrogen in medium with 0.3–1.5 M sodium chloride and showed plant growth-promoting activity (Sangeeta et al. 2014). Some *Azotobacter* are able to proliferate in medium containing 6–10% sodium chloride (Akhter et al. 2012). Positive growth response of sorghum grown in saline soil following 17 of 22 *Azotobacter* inoculation was reported (Aly et al. 2012).

In Indonesia, *Azotobacter* biofertilizer application increased in the attempt to reduce nitrogen fertilizer in vegetable production. The effect of *Azotobacter* liquid inoculum on plant growth and yield of tomato is reported elsewhere (Mahato et al. 2009; Ramakrishnan and Selvakumar 2012; Sharafzadeh 2012; Astari et al. 2014), but research concerning *Azotobacter* in saline soil is still limited. The first step to develop *Azotobacter* biofertilizer resistant to high salinity is by collecting *Azotobacter* isolates that adapt to salt and osmotic stress. We have *Azotobacter* isolates from saline soil in West Java Indonesia, but their resistance to salt especially sodium chloride has not been tested. The objective of this experiment was to evaluate three isolates of *Azotobacter* (K4, S1, and S2) resistant to some level of sodium chloride and to demonstrate the effect of sodium-resistant *Azotobacter* on early growth of tomato.

25.2 Materials and Methods

The experiment was divided into two steps. The first experiment was in vitro salinity resistance test, and the second one was bioassay for tomato early growth, conducted each at Soil Biology Laboratory and the greenhouse of Faculty of Agriculture, Universitas Padjadjaran, Indonesia, from July to October 2016.

25.2.1 Source of *Azotobacter*

Three isolates of *Azotobacter* coded as K4, S1, and S2 were the collection of Soil Biology Laboratory Faculty of Agriculture, Universitas Padjadjaran. *Azotobacter* K4 was isolated from rice rooting zone (rhizosphere) grown in saline soil at Karawang District, West Java, Indonesia; while S1 and S2 were obtained from Subang District, West Java, Indonesia. Nitrogenase activities of *Azotobacter* K4, S1, and S2 were 183.2 nmol/g/h, 64.1 nmol/g/h, and 172.5 nmol g^{-1} h^{-1}, respectively. All *Azotobacter* isolates were grown on Ashby's mannitol agar slant at 30 °C for 72 h before used in both experiments.

25.2.2 Source of Tomato Seed

Tomato variety, "Permata" F1 Hybrid, was developed by East-West Seed, Indonesia, for the lowland area located less than 200 m above sea level. The potency of cumulative total harvested fruit was about 50–70 t ha^{-1}, and generally, individual fruit weight was 50 g and measure about 4.5 cm in width and 6.5 cm in length. The taste of the fruit was sour with the brix scale of 4.5. This variety was tolerance to *Fusarium oxysporum* race O, *Fusarium oxysporum* race I, tomato mosaic virus, *Pseudomonas solanacearum*, and *Alternaria solani*.

25.2.3 Salinity Resistance Test

Salinity resistance test has been performed by use of nitrogen-free Ashby's mannitol medium containing gL^{-1} mannitol, 10; K_2HPO_4, 0.2; $MgSO_4.7H_2O$, 0.2; $CaSO_4.2H_2O$, 0.1; NaCl, 0.2; and $CaCO_3$, 5. Each *Azotobacter* pure culture was grown in Ashby's liquid medium at pH 7 with 0.85%, 1.7%, and 3.4% of sodium chloride and replicated three times each. As much as 1% of 3-day-old bacterial pure culture was suspended in 50 mL sterilized Ashby's medium in 250 ml Erlenmeyer flask with and without sodium chloride. All culture was incubated for three consecutive days on a gyratory shaker at 115 rpm at room temperature (23–26 °C).

The growth of each bacterial culture has been determined by their cell count at days 1, 2, and 3. *Azotobacter* cell density in the liquid culture was count by the direct method by counting chamber or hemocytometer. The growth rate of *Azotobacter* culture between two points of sampling time which is stated as generation time was determined by the following equation:

$$\mathrm{GT} = t/n$$
$$n = (\log n_t - \log n_o)/\log 2$$

where:

GT: generation time
n: number of generation

n_t: cell density at t sampling time
n_o: cell density at the beginning of the experiment
t: time (hour) between two sampling times

25.2.4 Bioassay on Tomato

The greenhouse experiment site is located in a tropical area at 752 m above sea level. Saline soil with an electrical conductivity of 5.95 dS m^{-1}collected from the topsoil of paddy field at Pusakanagara Sub District, Subang District, West Java. The soil was clayed Ultisols composed of 79% clay, neutral in acidity (pH was 6.75), very low in organic-C 0.65%, and very low in C/N. Cation exchange capacity of soil was 13.52 cmol kg^{-1}, and the base saturation was 98.86%. Total nitrogen was moderate, while total P_2O_5, available P_2O_5, and total K_2O content were high due to intensive use of inorganic fertilizer. The experimental treatments consisted of six treatments with two bacterial isolates and the concentration of liquid inoculum. The treatments were:

A: control, without inoculation
B: 0.5% of *Azotobacter* K4 liquid culture
C: 1.0% of *Azotobacter* K4 liquid culture
D: 0.5% of *Azotobacter* S2 liquid culture
E: 1.0% of *Azotobacter* S2 liquid culture
F: 0.5% of mixed *Azotobacter* K4 and S2 liquid culture
G: 1% of mixed *Azotobacter* K4 and S2 liquid culture

All treatments were replicated four times and arranged in a randomized complete block design.

In laminar flow with the aseptic method, 10 mL of physiological sodium chloride was poured to one slant of 3 days old *Azotobacter*. The bacterial colony on the surface of slant were mixed with the sodium chloride solution and homogenized by a magnetic stirrer. The suspension was poured into 50 mL of Ashby's medium in 250 mL Erlenmeyer flask and incubated 3 days in room temperature inside a reciprocal shaker at the speed of 115 rpm.

The soil was collected from topsoil of Ultisols soil order and mixed with cow manure equal to 20 ton/hectare, 7 days before transplanting. Black polyethylene bag was filled with 1 kg of growth medium and placed in the greenhouse. Individual 21-day-old tomato transplant was grown on each pot. Ten milliliters of *Azotobacter* liquid inoculum was diluted with 10 mL of groundwater and poured evenly into soil surface 3 days after transplanting. All plant was maintained in the greenhouse for 30 days and watered equally to the soil field capacity. Plant height and leaf number were measured once a week. At the end of experiment, shoot of the individual plant was separated from the root. Individual shoots and roots were heated separately at 70 °C before their dry weight was measured.

25.2.5 Statistical Analysis

All data except that of the in planta experiment was subjected to variance analysis (5% F-test) using SPS 17.0 to determine the effect of *Azotobacter* inoculation on plant and soil parameters in the bioassay. Duncan's multiple range tests at $\alpha \leq 0.05\%$ was carried out to determine the significant differences among treatment factor if F-test was significant.

25.3 Results

25.3.1 Salinity Resistance of *Azotobacter*

The *Azotobacter* isolates K4, S1, and S2 all showed positive growth at a narrow range of salinity (Table 25.1), and all isolates proliferate in the presence of relatively high sodium chloride concentration. At the beginning of the experiment, cell density in bacterial culture was 10^4 CFU mL^{-1}. Three days after incubation, all liquid culture contained 10^8 CFU mL^{-1}*Azotobacter's* cell irrespective of isolates or salinity, with exception of S1 which demonstrated slower growth at day three in the presence of 3.4% of sodium chloride. At the end of the experiment, the cell density of all *Azotobacter* isolates in culture with sodium chloride addition was slightly lowered in the presence of 1.7% and 3.4% of sodium chloride compared with control during 3 days of incubation period. The greater bacterial population in liquid cultures with 3.4% sodium chloride was observed in K4, 1.75×10^8 CFU mL^{-1}.

Slow growth of all *Azotobacter* isolates was evident in lag phase, between the day of inoculation and day 1. At logarithmic phase, sodium chloride level in cultures

Table 25.1 Effect of sodium chloride concentration on cell growth of *Azotobacter* isolates in nitrogen-free medium

Isolate code	NaCl (%)	Cell density[a] (CFU mL^{-1}) at day				Generation time at log phase (h)
		0	1	2	3	
K4	Control	4.57×10^4	9.90×10^4	3.60×10^7	7.30×10^8	3.74
	0.85	3.19×10^4	8.85×10^4	3.20×10^7	2.90×10^8	3.45
	1.70	3.92×10^4	5.25×10^4	2.55×10^7	2.70×10^8	3.76
	3.40	1.61×10^4	3.25×10^4	1.80×10^7	1.75×10^8	3.58
S1	Control	2.82×10^4	6.10×10^4	2.00×10^7	3.05×10^8	3.58
	0.85	1.80×10^4	5.60×10^4	1.60×10^7	2.80×10^8	3.45
	1.70	1.15×10^4	3.60×10^4	1.45×10^7	1.51×10^8	3.51
	3.40	6.55×10^4	2.60×10^4	1.10×10^7	1.20×10^7	6.38
S2	Control	2.10×10^4	9.45×10^4	2.65×10^7	3.65×10^8	3.46
	0.85	1.89×10^4	5.85×10^4	2.30×10^7	3.00×10^8	3.44
	1.70	1.54×10^4	4.35×10^4	1.55×10^7	1.85×10^8	3.54
	3.40	1.32×10^4	3.10×10^4	1.30×10^7	1.45×10^8	3.58

[a]The data are from three replicate plates

slightly affected the generation time (GT) of all isolates (Table 25.1). The isolates K4, S1, and S2 all grew the same rate in the presence of salt with a GT around 3, but 3.4% sodium chloride in liquid cultures decreased the GT of isolate S1 which grew almost two times slower with a GT of 6.38 h. Among the three isolates, the GT of *Azotobacter* K4 in control treatment was relatively higher. Of the three isolates, *Azotobacter* S2 demonstrated constant growth irrespective of salt level.

25.3.2 Bioassay on Tomato

Azotobacter inoculation was not significantly increased plant height and leaves a number of tomato transplants at first week to fourth week (Fig. 25.1). The pattern of plant growth from the point of view of plant height was similar irrespective of inoculation treatments. Effective treatment to increase shoot height was inoculation of *Azotobacter* K4 liquid culture at 1% dilution rate and of mixed K4 and S2 isolates at the same dilution rate. Both of the treatments produced 10.22 cm and 10 cm shoot height, respectively, at the final week of pot experiment, whereas the least effective treatment was 0.5% of mixed K4 and S2 liquid culture which produced a plant with only 7.27 cm heights.

Analysis of variance showed that no significant effect of *Azotobacter* inoculation on leaf number at all sampling time. Leaf number average of tomato transplants at final week was 14.5–19.7 (Fig. 25.2.). Irrespective of statistical analysis leaves, a number of plants treated with *Azotobacter* liquid culture were greater than that of un-inoculated one which had 11.1 leaves on average. The most effective inoculation treatment to increase leaf number of 4-week-old tomato transplant was *Azotobacter* K4 liquid culture at a dilution rate of 0.5% (Fig. 25.2).

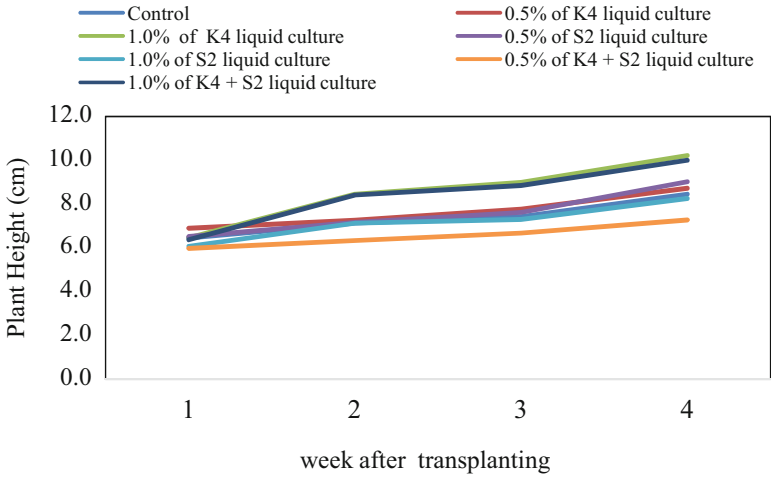

Fig. 25.1 Effect of different concentration and an isolate of *Azotobacter* inoculum on plant height of tomato 4 weeks after transplanting

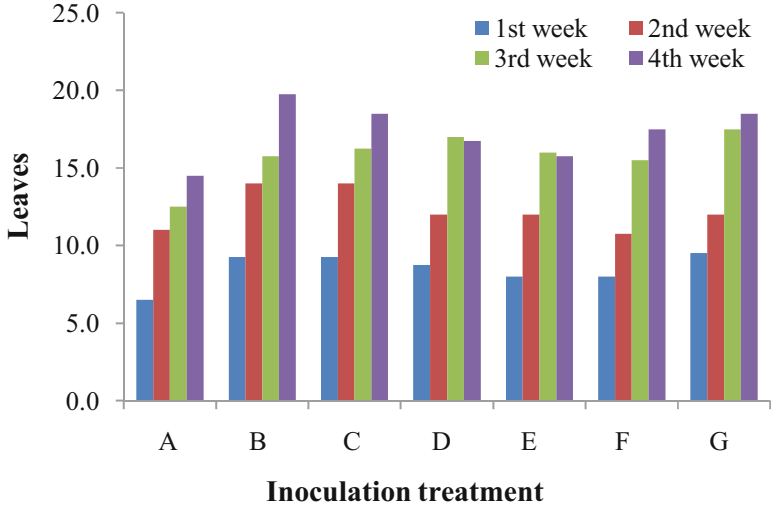

Fig. 25.2 Effect of different concentration and an isolate of *Azotobacter* liquid inoculum on leaf number of tomato 4 weeks after transplanting. *A*, control; *B*, 0.5% of K4; *C*, 1.0% of K4; *D*, 0.5% of S2; *E*, 1.0% of S2; *F*, 0.5% of mixed K4 and S2; and *G*, 1.0% of mixed K4 and S2

Table 25.2 Effect of different concentration and isolate of *Azotobacter* inoculation on the shoot and root dry weight of tomato fruit at 4 weeks after transplanting

Azotobacter inoculation			Dry weight (g)		Nitrogen uptake (mg/plant)
			Shoot	Root[a]	
A	:	Control, without inoculation	0.09	0.02 a	0.71
B	:	0.5% of K4 liquid culture	0.13	0.03 ab	1.08
C	:	1.0% of K4 liquid culture	0.18	0.05 b	1.71
D	:	0.5% of S2 liquid culture	0.13	0.04 ab	1.03
E	:	1.0% of S2 liquid culture	0.20	0.05 b	1.90
F	:	0.5% of K4 + S2 liquid culture	0.15	0.05 b	1.41
G	:	1.0% of K4 + S2 liquid culture	0.22	0.07 c	1.88

[a]Numbers followed by the same letter were not significantly different based on Duncan's multiple range test at $\alpha = 0.05\%$

Azotobacter inoculation caused a significant increase of root dry weight at 30 days after transplanting but did not on shoot weight (Table 25.2.). It was found that root dry weight was significantly increased when seedling inoculated with and mixed inoculation at any dilution rate, compared to the control (Table 25.2). Root dry weight of plant received either 1.0% of K4 or 1.0% of S2 liquid culture increased up to 0.05–0.07 g, while that of control was 0.02 g. The highest root dry weight, 0.07 g, was shown by transplant received 1.0% of a mixture of *Azotobacter* K4 and S2 liquid culture.

The shoot dry weight varied from 0.09 to 0.22 g at 4 weeks after transplanting (Table 25.2). The highest shoot dry weights (0.22 g) showed by the plant with mixed

inoculation of K4 and S2 at 1% dilution rate of bacterial liquid culture although statistically was not significant with control and other treatment. This shoot dry weight was more than twice higher than that of control.

Azotobacter inoculation did not change nitrogen uptake; irrespective of statistical analysis, if transplant grew without *Azotobacter*, then nitrogen uptake was lowest (Table 25.2). Inoculation treatment caused 45.1–164.8% more nitrogen uptake at 4 weeks after transplanting compared to the control. Highest uptake of nitrogen was reached by tomato transplant after the application of *Azotobacter* S2 liquid culture at a dilution rate of 1%. Regardless of *Azotobacter* isolates, the effect of *Azotobacter* inoculation on nitrogen uptake was more pronounced in plant received liquid inoculum with 1% dilution than 0.5%.

25.4 Discussion

All isolates grow in either 0.85% physiological sodium chloride (isotonic condition for nonmarine microbes) or higher level of sodium chloride. The ability of *Azotobacter* to proliferate in saline condition proved that the bacteria might require sodium cation for growth. *Azotobacter* may require sodium cation for growth. Sodium-dependent strain 184 of *Azotobacter chroococcum* which has very low catalase activity has been studied (Page et al. 1988). They concluded that strain 184 was more susceptible to H_2O_2 when grown under low aeration in nitrogen-fixing conditions than when it was grown in the presence of NH_4^+. Two years before that finding, *A. chroococcum* absolutely dependent on Na^+ for growth has been isolated from soils of Alberta, Canada (Page 1986). Most sequenced bacteria possess mechanisms to import choline and glycine betaine into the cytoplasm and act as a potent osmoprotectant, such as in *Pseudomonas aeruginosa* (Wargo 2013). Glycine betaine is an important osmoprotectant for many species in all domains of life. To protect cell function and turgor under osmotic stress conditions, osmoprotectants are imported or synthesized and accumulate in the cytosol (Wood 2011). Our experimental results support the evidence that two bacterial species were able to adapt up to 10% of sodium chloride in nutrient broth growth medium (Mendpara et al. 2013).

In our research, *Azotobacter* isolated from saline soil may have counteracted osmotic stress by synthesizing osmoprotectant to maintain cell turgor and hence normal metabolism. Adding the specific media with osmoprotectant 2% glycerol for *Azotobacter* sp. resulted in the highest population densities in liquid culture with 0.4% sodium chloride (Dayamani and Brahmaprakash 2014) proving that osmoprotectant was required to survive. *Azotobacter salinestris* produced osmoprotectant substances as1-aminocyclopropane-1-carboxylate deaminase enzyme, salicylic acid, proline, and exopolysaccharide (Omer et al. 2016). All isolate of *Azotobacter* used in our experiment has not been tested for exopolysaccharide production; we proved that *Azotobacter* in an agricultural area in West Java produces EPS as a protectant to heavy metal stress (Hindersah and Sudirja 2009). In subsequent research, the role of sodium in EPS synthesis should be clarified.

The generation time at log phase of *Azotobacter* K4, S2, and S1 were generally between 3.44 and 3.74 h, but higher generation time showed by *Azotobacter* K4 which grown in media with 3.40% NaCl. The generation time of the same genus of bacteria dictates among other by medium composition and species. The generation time of *Azotobacter vinelandii* wild type and their mutants were not different, 2.3 h (Martin et al. 1989). Later finding showed that generation time of *A. vinelandii* in bulk medium enriched by ferric citrate at early exponential phase was 2.48 h (Sandercock and Page 2008). Our experimental results provided the recent base data concerning the adaptation capability of nitrogen-fixing rhizobacteria *Azotobacter* in the tropical saline environment. Intensive research with higher levels of sodium chloride to obtain the saline resistance profile of *Azotobacter* is required.

Azotobacter inoculation enhanced root dry weight of 4-week-old tomato plant but the increase depended on *Azotobacter* isolate and concentration of liquid inoculum. Regardless of statistical analysis, bacteria increased shoot weight and nitrogen uptake. *Azotobacter* promotes plant growth via at least two different mechanisms. The most known mechanism is nitrogen fixation which increases NH_4^+-N available for root uptake. *Azotobacter* produced secondary metabolites and synthesizes auxins, cytokinins, and gibberellin-like substances (Kukreja et al. 2004). In our experiment, saline-resistant *Azotobacter* did not increase shoot height and leaf number significantly although data in Fig. 25.1 showed that a slight increase of shoot height was evident following some dilution rate of two *Azotobacter* isolates' inoculation.

Low increase in shoot might be due to hard soil texture which in turn lowered soil aeration; the clay content of the soil was 79% with a pH of 6.75. However, root dry weight significantly increased by certain *Azotobacter* inoculation which is similar with the results of *Azotobacter* inoculation on herbaceous thyme (Sharafzadeh et al. 2012) grown in lighter soil, clay loam soil.

A slight increase of nitrogen uptake following *Azotobacter* inoculation might be caused by the change of mineralization pattern in saline soil. In saline soil, NH_4^+ content was lower compared to the non-saline soil (Walpola and Arunakumara 2010). In saline soils with EC of 0.2, 4.1, and 11.4 dS m^{-1}, nitrification of NH_4^+ to NO_3^- was reduced by salinity treatments, but total mineralized N was highest in the urea treatment (Irshad et al. 2005). Salinity with EC of 9–27 dS m^{-1} inhibited the second step of nitrification, causing nitrite (NO_2^-) accumulation in soil (Akhtar et al. 2012).

The second step of nitrification is enzymatically transformation of nitrite to nitrate, salinity inhabitation reduced NO_3^- in the soil which is a good environmental condition for nitrogen fixation to be processed. High level of NO_3^- is a limiting factor to transform dinitrogen to ammonia enzymatically. The nitrogenase enzyme catalyzes the reduction of atmospheric dinitrogen to ammonia; however, the enzyme activity is suppressed by an ample or excess supply of fixed N. In the presence of excess fixed N, the diazotrophic nitrogenase enzyme is no longer active, either due to downregulation of protein synthesis or inactivation of the protein (Rudnick et al. 1997).

Nitrogen is most often the limiting factor for plant growth in the saline soil since the low level of nitrate limited nitrogen uptake by roots. Collecting saline-resistant *Azotobacter* to support agriculture in seawater-affected soil in the northern part of Java will be important to support sustainable agriculture. Inoculation of *Azotobacter* in saline soil is one of the ways to ensure nitrogen supply. Limited fixed N in soil induces nitrogenase activity which is, in turn, enabling to increase fixed N through nitrogen fixation of this symbiotic rhizobacterium.

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