ISOLATION AND CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) TO IMPROVE PLANT GROWTH

THESIS

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September 2015

Dedicated To

The Loving Memory of my Mother

DECLARATION

I hereby declare that Ph.D. thesis entitled, **"Isolation and Characterization of Plant Growth Promoting Rhizobacteria (PGPR) to improve plant growth"** is an independent work carried out by me under the supervision of Dr. Ashok Pandey, Head, Biotechnology Division, National Institute for Interdisciplinary Science and Technology, CSIR, Trivandrum-695 019, India and this work has not been submitted elsewhere for the award of any other degree.

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CERTIFICATE

This is to certify that the work presented in the thesis entitled **"Isolation and Characterization of Plant Growth Promoting Rhizobacteria (PGPR) to Improve Plant Growth"** is based on the original research done by **Ms. Deepa CK** under my guidance and supervision in the Biotechnology Division, CSIR-National Institute for Interdisciplinary Science and Technology, Trivandrum-695 019, and that no part of this work has been submitted previously for the award of any degree.

Ashok Pandey
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PREFACE

The Thesis entitled “Isolation and Characterization of Plant Growth Promoting Rhizobacteria (PGPR) to improve plant growth” has been framed into 6 chapters. Chapter 1 gives a brief introduction about PGPR and the outline of mechanisms involved in plant growth promotion. Chapter 2 presents an in depth of the PGPR mechanisms as well as the up-to-date research view on PGPR technology. Chapter 3 describes the standard materials and methods followed in this research work. Chapter 4 deals with the results obtained from laboratory and net house studies with PGPR. Chapter 5 deals with the discussion of the results with reported works of other PGPR researchers. Chapter 6 finally gives a brief summary and conclusion of the work. Appendix include list of media compositions, equipments, and publications involved in this work. The primary screening strategy adopted for the selection of rhizospheric isolates includes phosphate solubilisation and IAA hormone production. Then the isolates with dual attributes of phosphate solubilisation and IAA production were then screened for nitrogen fixation, chitinase activity, siderophore, ammonia, HCN production, phytase activity, ACC deaminase activity, antifungal activity and finally growth promoting ability of various crop plants in green net house conditions. On the basis of highly significant growth promotion of all the test plants, three best isolates were selected and characterized for phenotypic and genotypic characteristics. Talc based formulation of the selected three isolates were prepared and their population dynamics on storage of 180 days. The comparative effects of PGPR consortium and single isolate based PGPR bioformulation on growth of black pepper were also studied. HPLC analysis of organic acids secreted into the medium the best 3 isolates was performed. The study clearly demonstrated that the beneficial interactions between the PGPR with multiple growth promoting attributes and the test plants used in this study. Over all the selected best isolates promoted growth of tomato, cowpea, black pepper and cassava crops at different levels of efficacy for each test plants.

Deepa CK

Table of Contents

List of Tables

List of Figures

Abbreviations

ACC	1-aminocyclopropane-1-carboxylate
CAS	Chrom azurol sulphonate
LSD	Least Significant Difference
TSB	Trypticase soya broth
NA	Nutrient agar
PVK	Pikovskaya
TCP	Tricalcium phosphate
NBRIP	National Botanical Research Institute's phosphate liquid growth medium
PDA	Potato dextrose agar
PDB	Potato dextrose broth
GA	gluconic acid
CA	citric acid
FA	formic acid
LA	lactic acid
PA	propionic acid
SA	succinic acid
TA	tartaric acid
$FeCl_3$	Ferric chloride
IAA	indole-3-acetic acid
HPLC	High performance liquid chromatography
PGPR	plant growth promoting rhizobacteria
HCl	Hydrochloric acid
LAI	Leaf area index
HCN	Hydrogen cyanide
CFU/ml	Colony Forming Units per millilitre
SEM	Scanning Electron Microscopy
mg	milligram

Chapter 1

INTRODUCTION

The "green revolution", which took place in 1960's and 1970's increased the food production to several -folds in India, Pakistan and several countries in south East Asia, thereby feeding three times as many people. However, this revolution has already been exploited to its limits and alternative solution are required to embark upon to further increase the food production, which could be attained the through integration of classical breeding and advanced techniques in crop production and biotechnology. This could create "evergreen revolution" for food production and crop management on sustainable basis. One of the approaches in this regard is the use of biologically based pest and nutrient management strategies that are naturally occurring and environmentally safe products. In this regard, the use of plant growth promoting rhizobacteria (PGPR) has found a potential role in developing sustainable systems in crop production (Sturz *et al.,* 2000; Shoebitz *et al.,* 2009; Glick, 2012). A variety of symbiotic (*Rhizobium* sp.) and non-symbiotic bacteria (*Azotobacter, Azospirillum, Bacillus, Pseudomonas, Klebsiella* sp., etc.) are now being used worldwide with the aim of enhancing plant productivity (Cocking 2003; Ahemad and Kibret., 2013).

In recent years, there has been an upsurge in the selection of beneficial microbes for developing plant growth promoting formulations. Rhizobacteria exert beneficial effects on the plants by enhancing the nutrient status of soil and availability of nutrients to the plants, secreting growth-promoting hormones, and suppressing soil-borne pathogens (Klöepper and Schroth, 1981; Glick, 1995; Vessey, 2003; Loon, 2007; Rodriguez *et al*., 2008; Yang *et al*., 2009; Bashan and de- Bashan., 2010; Glick, 2012). There are numerous reports on plant growth promotion by bacteria that have the ability to solubilize inorganic and/or organic

phosphorous from soil after their inoculation in the soil or plant seeds **(**Richardson, 2001; Rodriguez *et al*., 2006; Khan *et al*., 2007; Battacharya and Jha, 2012)

PGPR also influence plant growth and development by the production of phytohormones such as auxins, gibberellins, and cytokinins (Van Loon, 2007; Glick 2012; Ashraf *et al*., 2013). The production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, studied for some PGPR, lowers the level of ethylene known to inhibit the root growth by cleaving the ethylene precursor ACC to α-ketobutyrate and ammonia (Glick *et al*., 2007). Another important trait of PGPR is the production of siderophores, which suppress fungal pathogens and solubilize iron-bound phosphorus by chelating iron (Buysens *et al.,* 1996; Duponois *et al*., 2006; Sayyed and Chincholkar, 2009; Perez-Montano *et al*., 2014).

A diverse array of bacteria, including *Rhizobium, Bradyrhizobium Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia, Pseudomonas, Klebsiella, Enterobacter*, *Xanthomonas* and *Serratia* have been reported to augment the plant growth by increasing seed emergence, plant biomass, and crop yield (Glick, 1995; Lugtenberg *et al*., 2002; Joseph *et al*., 2007; Selvakumar *et al*., 2008b; Akhtar and Siddiqui, 2009; Glick 2012; Perez-Montano *et al*., 2014). The selection of strains with multiple plant growth promoting activities could be more beneficial than the strains with a single, or a few plant growth promoting attributes for developing microbial inoculants. Combinations of beneficial bacterial strains that interact synergistically are currently being devised and numerous recent studies show a promising trend in the field of inoculation technology (Figueiredo *et al*., 2010; Nandal and Hooda 2013) However, the majority of microbial inoculants have suffered from inconsistent field performance limiting the full potential benefits offered by PGPR, mainly due to the stress generated by environmental factors such as moisture status, temperature, alkalinity/acidity and salinity in the soil (Rangarajan *et al*., 2002; Vriezen *et al*., 2006). The PGPR strains must be rhizosphere competent to survive, colonize, establish and influence plant growth. Therefore, the selection of stress-tolerant and rhizosphere-competent strains is essential for consistency in field performance for application as the microbial inoculants. PGPR revealing huge potential for enhancing crop production must be mass-produced and formulated into cost-effective, uniform and readily applicable forms for commercial application. The first step in such processes is to design the

medium with optimum growth conditions. Effective population density of active cells in plant rhizosphere is one of the prerequisite for the efficient activity of the inoculated PGPR; hence, high cell density cultivation is required for improving the microbial biomass production (Riesenberg and Guthke, 1999; Martinez-Viveros *et al.*, 2010).

However, the effectiveness of PGPR in increasing crop productivity has been limited by the variability and inconsistency in their field performance. The selection of stress-tolerant and rhizosphere-competent PGPR with multiple plant growth-promoting attributes and broad-spectrum plant growth-promoting activity has been advocated by many researchers. Though the beneficial effects of inoculation of such PGPR have been well documented at several locations, there is continued need to evolve the region specific strains that could be able to positively influence plant growth in prevailing soil and edaphic conditions (Pandey *et al*., 1998).

The Western *Ghats* is one of the world's richest sources of bio-resources. The part of the Western *Ghats* in Kerala state eco-region is considered as the richest in endemic species and is the most diverse area in the entire Western Ghats assessment area. To- date, only limited information exists on microbial exploration for plant growth promoting activity from rhizospheric soil of silent valley, which is an evergreen tropical rain forest in India. In the present investigation, attempts were directed towards the selection of region-specific strains exhibiting the highest number of traits associated with the PGPR ability under *in vitro* conditions and pot experiments of regional crop black pepper and tapioca, as a prelude to their use at field level.

Thus, from the above (and also literature given in Chapter 2) it is apparent that there is continuous efforts in isolating and characterizing plant growth promoting rhizobacteria which could be used to improve the growth of the plants. The isolates should have phosphate solubilisation capacity, IAA hormone production and also several other attributes such as nitrogen fixation, chitinase activity, etc. Other important aspect is bio-compatibility of the isolates for developing formulations. Furthermore, it is also apparent that isolates from different soils/places have different attributes and newer isolates with better characteristics and bio-compatibility are always needed. Thus, it was towards this direction, the

present study was undertaken, focusing novel isolates from the Western ghats in Kerala.

Hence, the proposed work was planned with following objectives.

1. Isolation and screening of PGPR for multiple plant growth-promoting attributes from the silent valley region of Western *Ghats* of Kerala.
2. Evaluation of selected PGPR strains alone as well as in combination for improving the growth of black pepper and cassava under pot conditions.
3. Optimization of growth medium for selected PGPR strains.
4. Formulation of PGPR inoculants and testing the shelf-life of talc-based bio-formulation.
5. Identification and characterization of selected PGPR strains.

Chapter 2

REVIEW OF LITERATURE

The literature pertinent to the present research work is summarily reviewed under the following heads:

2.1 Plant rhizosphere and rhizodeposition

The rhizosphere is defined as a thin zone of soil around living roots, notified by the presence of most microbially diverse and metabolically active region, stimulated by plant root exudates (Grayston *et al.*, 1997) and extends a few millimeters from the root surface where the soil are bound by plant roots (Bhattacharya and Jha 2012). The bacteria colonizing this root environment are termed as rhizobacteria (Kloepper 1991). The rhizosphere are known as an intense interactive zone as the root exudates consists of phenolics, various types of sugars, proteins, organic acids, mucilages, amino acids, and various other organic metabolites that can be utilized by the soil microbes for their survivability. (Lambers *et al.,* 2009). This nutritious environment results in a much higher population of bacteria in the rhizosphere but lowers the diversity/species richness than in the bulk soil (Lugtenberg and Kamilova, 2009; Ahmad *et al.,* 2011).

Rhizobacteria have profound effects on plant health and nutrition and the chemical signals released by both partners mediate the interaction or communication between the plants and rhizobacteria. As a nutrient rich environment, the rhizosphere supports a diverse population of micro- and macro-organisms, which form complex

interactions with the plant root (Richardson *et al.,* 2009), ranging from the competitive to mutualistic (Roesti *et al.,* 2006; Bais *et al.,* 2006; Narula *et al*., 2009). The associations involves of the plants with parasitic, or non-parasitic deleterious bacteria and fungi are among the detrimental ones, while beneficial relationships are those observed with the non-symbiotic, or symbiotic beneficial rhizosphere bacteria and fungi (Barea *et al.,* 2005). The structural community of rhizobacteria is determined by the soil type and fertility as well as the amount and composition of root exudates, which is depended on the plant genotype (Marschner *et al.,* 2011; Pii *et al.,* 2015). In return, the rhizobacterial community may influence this interaction by exuding the compounds as a means of communication that is recognizable by neighboring bacteria and root cells of the host plants (Gray and Smith 2005).This mutual interactive communication can affect plant growth, nutrient status and also susceptibility to stress and pathogens in the host plant (Morgan *et al*., 2005; Jha and Saraf 2015).

The rhizodeposition includes the release of organic and inorganic root products into the soil (Gobat *et al*., 2004). Rhizodeposits involves a wide variety of compounds (Table 2.1) derived from the sloughed-off root cells and tissues, mucilages, and exudates originating from intact roots, and soluble lysates and volatile compounds released from the damaged cells (Curl and Truelove, 1986; Dakora and Phillips 2002; Uren 2001).

Table 2.1 Organic compounds released by the plant roots.

Sugars:	Arabinose, fructose, galactose, glucose, maltose, mannose, mucilages of various compositions, oligosaccharides, raffinose, rhamnose, ribose, sucrose, xylose, deoxyribose
Amino acids:	α-Alanine, β-alanine, g-aminobutyric, α-aminoadipic, arginine, asparagine, aspartic, citrulline, cystathionine, cysteine, cystine, deoxymugineic, 3-epihydroxymugineic, glutamine, glutamic, glycine, histidine, homoserine, isoleucine, leucine, lysine, methionine, mugineic, ornithine, phenylalanine, praline, proline,

	serine, threonine, tryptophan,tyrosine, valine
Organic acids:	Acetic, aconitic, ascorbic, aldonic, benzoic, butyric, caffeic, citric, pcoumaric, erythronic, ferulic, formic, fumaric, glutaric, glycolic, lactic, glyoxilic, malic, malonic, oxalacetic, oxalic, p-hydroxybenzoic, piscidic propionic, pyruvic, succinic, syringic, tartaric, tetronic, valeric, vanillic
Fatty acids:	Linoleic, linolenic, oleic, palmitic, stearic
Sterols:	Campesterol, cholesterol, sitosterol, stigmasterol
Growth factors and vitamins:	p-Amino benzoic acid, biotin, choline, N-methyl nicotinic acid, niacin, pathothenic, thiamine, riboflavin, pyridoxine, pantothenate,
Enzymes:	Amylase, invertase, peroxidase, phenolase, acid/alkaline phosphatase, polygalacturonase, protease
Miscellaneous:	Auxins, scopoletin, hydrocyanic acid, glucosides, unidentified ninhydrinpositive compounds, unidentifiable soluble proteins, reducing compounds, ethanol, glycinebetaine, inositol, and myo-inositol-like compounds, Al-induced polypeptides, dihydroquinone, sorgoleone, isothiocyanates, inorganic ions and gaseous molecules (e.g. CO_2, H_2, H1, OH^-, HCO_3), some alcohols, fatty acids, and alkyl sulphides.

Adapted from Uren (2001), Curl & Truelove (1986) and Dakora & Phillips (2002).

The rhizodeposition can be influenced by many biotic and abiotic factors of plant and soil (Fig. 2.1). Of the total photosynthetic production of the plant, the rhizodepositon corresponds to 15-60% of photosynthate leakage and provides an

important carbon and energetic source towards the microorganisms of the rhizosphere (Marschner, 1995).The microbial communities of rhizosphere can be either stimulated, or inhibited by the components of root exudates (Hartmann *et al.,* 2009) and different microbial species vary in their ability to utilize and compete for the substrates. The qualitative as well as the quantitative compositions of root exudates are affected by various environmental factors, including light intensity, soil pH, soil type, oxygen status, soil temperature, nutrient availability and the presence of microorganisms exerting a greater impact on root exudation than differences due to the plant species (Singh and Mukerji 2006). The rhizosphere being the largest source of carbon supply within soil, houses a rich microbial community, consisting of 10^{10} bacteria per gram of soil (Gans *et al.,* 2005; Roesch *et al.,* 2007) as well as encompass a large diversity of taxa (Kyselková *et al.,* 2009; Gomes *et al*., 2010).The root exudate richness in diversity (organic acids, phytosiderophores, sugars, vitamins, amino acids, nucleosides, mucilage) attracts microbial populations, especially those able to metabolize plant-exuded compounds and proliferate in this microbial habitat (Bais *et al.,* 2006; Pothier *et al.*, 2007; Badri *et al.,* 2009; Shukla *et al.,* 2011; Drogue *et al.,* 2013). The microbial community associated to plant roots are referred as the rhizomicrobiome (Chaparro *et al.,* 2013), characterized by the uniqueness from that of the microbial community of the surrounding the soil, a direct consequence of bacterial competition for the nutrients liberated in the vicinity of plant roots (Bulgarelli *et al*., 2013; Chaparro *et al*., 2013).

In addition to root exudates, the rhizobacteria also utilize the secretions from the epidermal cells, root cap cells and root hairs besides those derived from the microbial degradation and modification of the dead epidermal cells. These serves as a source of nutrients and microsites that permit niche exclusivity to rhizobacteria during root colonization and sustenance.

Deleterious rhizobacteria without parasiting the plant tissues (Schippers *et al*., 1987) produce phytotoxins, pectinolytic enzymes and/or phytohormones and compete with the plant, or with the beneficial microorganisms for the uptake and metabolism of nutrients (Suslow and Schroth, 1982 a; Nehl *et al.,* 1997).The beneficial microorganisms in the rhizosphere mainly include plant growth promoting rhizobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF).

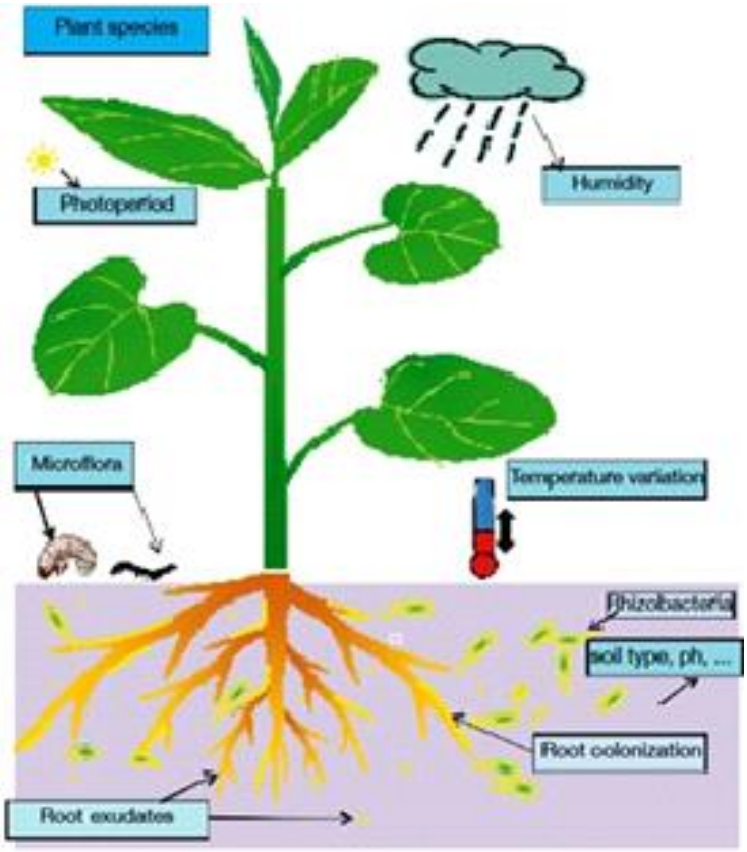


Fig. 2.1 Ecological factors influencing the root exudation process and effect on rhizosphere colonization by PGPR (Nihorimbere *et al*., 2011)

2.2 Plant growth promoting rhizobacteria (PGPR)

In recent years, the exponential increase in fertilizer consumption throughout the world has caused serious environmental problems, including accumulation of heavy metals in the soil and plant system, which in turn affect the food chain. Further, fertilization also leads to water, soil and air pollution (Serpil *et al.*, 2012). It has also been estimated that more than 100 million tonnes of nitrogen, potash and phosphate-chemical fertilizers have been used annually in order to increase plant yield (Glick *et al*., 1999). The dangerous negative effects of the chemical fertilizers on the global environment and the cost associated with their production have given impetus to the research on bioinoculants. Biofertilizers are more commonly known as microbial inoculants, which are artificially multiplied cultures of certain soil organisms that can improve soil fertility and crop productivity.

Plant-growth-promoting rhizobacteria (PGPR) are free-living bacteria in the rhizosphere that exerts beneficial effects on the growth of the plants (Kloepper *et al*., 1988; Lugtenberg *et al*., 2001; Saraf *et al*., 2010). PGPR mainly comprise of a broad range of soil bacterial taxa (Vessey 2003; Lucy *et al.*, 2004), isolated and screened for plant growth promoting attributes (Chanway and Holl, 1993; Cattelan *et al*., 1999; Goswami *et al*., 2014) and evaluated for plant growth promotion (Lifshitz *et al*., 1987; Chanway *et al*., 1989; Bashan 1998; Mayak *et al*., 1999; Bent *et al*., 2001; Salamone 2000; Rodriguez *et al*., 2008). Some of the common and well identified genera are *Azospirillum*, *Pseudomonas*, *Azotobacter*, and *Bacillus.* PGPR reduce excessive use of chemical inputs in agriculture, and therefore, offer an environmentally-friendly alternative for maintaining crop productivity in intensive agricultural practices, and thus, reduces the environmental degradation caused by the chemical fertilizers.

The leaching of chemical nutrients results in increase of nutrient content in environmental water, thereby promote algal growth and decreased dissolved oxygen levels creating harmful conditions in water eco-systems. PGPR provides essential nutrients for plant growth, or enhance nutrient availability, play a role in pathogen suppression, offer environmental sustainability and improvement of soil health in the long term (Vessey 2003; Lucy *et al.*, 2004; Lugtenberg and Kamilova 2009), thereby potentially reducing the use of chemical fertilizers and pesticides.

2.3 Mechanisms of Plant Growth Promotion by PGPR

PGPR have direct as well as indirect effect on plant growth and yield (Kloepper *et al*., 1989; Glick 1995; Rodriguez *et al*., 2008). The direct growth promoting mechanisms involve the solubilization of phosphorus, nitrogen fixation, sequestering of iron by the production of siderophores, phytohormones production such as auxins, cytokinins, gibberellins and lowering of ethylene concentration (Kloepper *et al*., 1989; Glick *et al*., 1999; Glick *et al*., 2007; Jha *et al*., 2009). The indirect mechanisms of plant growth promotion by the PGPR include depletion of iron from the rhizosphere, synthesis of antifungal metabolites such as volatile and non-volatile antibiotics, production of fungal cell wall lysing enzymes, competition

for the sites on the roots and induced systemic resistance (Kloepper *et al*., 1988 a, b; Glick *et al*., 1999; Persello-Cartieaux *et al*., 2003; Sayyed and Chincholkar 2009). Numerous PGPR possess multiple plant growth promoting attributes, which influence plant growth at different developmental stages. PGPR stimulate cell division through the early plant development by producing phytohormones. Further, the plant growth may be enhanced by the PGPR by providing sufficient amount of iron and phosphorus from the soil.

2.3.1 Phosphate solubilization

2.3.1.1 Phosphorus (P) in the soil system and its availability to plants

Phosphorus is the second most important key element after nitrogen as a macronutrient in terms of plant requirement. Even though P is abundant in the soils, in both organic and inorganic forms, its availability is restricted as it occurs mostly in insoluble forms (Khan *et al.,* 2010; Sharma *et al.,* 2013 a). The P content in average soil is about 0.05% (w/w) but only 0.1% of the total P is available to the plant because of poor solubility and its fixation in soil (Illmer and Schinner, 1995). An optimum availability of phosphorus is very important for the proper formation of primordia of plant reproductive parts during the early phases of plant development. It also plays significant role in seed formation, early maturation of crops, increasing root ramification and strength, thereby imparting vitality and disease resistance capacity to the plant (Sharma *et al.,* 2013a). P deficiency in the plants results in stunted growth, wilting of leaves, delayed maturity and reduced yield (Sawyer and Creswell, 2000).

A majority of the portion of soluble inorganic phosphates applied as chemical fertilizers to the soil are rapidly fixed into the forms that are unable to utilize by the plants (Kim *et al*., 1998a; Richardson 2001; Fernández *et al*., 2007). The fixation and precipitation of P is largely dependent on the pH and soil type. P becomes fixed in the acidic soils by free oxides and hydroxides of aluminium and iron, while in the alkaline soils, it gets fixed by calcium (Jones *et al*., 1991; Khan *et al.,* 2010; Sharma *et al,* 2013a). A second major component of soil P is organic matter, present largely in the forms of inositol phosphate (soil phytate), which comprises of 30- 50% of the

total organic P in majority of the soil (Paul and Clark 1996). Other organic P compounds in soil are in the form of phosphomonoesters, phosphodiesters, including phospholipids and nucleic acids, and phosphotriesters. The organic substrates in the soil are also a source of phosphorus for plant growth. Phosphorus in the labile organic compounds is slowly mineralized as available inorganic P, or immobilized into more stable organic materials as a part of the soil organic matter (Mckenzie and Roberts 1990; Gyaneshwar *et al*., 2002).

2.3.1.2 Phosphate solubilizing microorganisms

The use of microbial inoculants possessing P-solubilizing activities in agricultural soils is considered as an environmental-friendly alternative to further the applications of chemical- based P fertilizers. Microorganisms are an integral component of the soil P cycle and are important for the transfer of P between the different pools of soil (Fig. 2.2). Phosphate-solubilizing microorganisms belonging to diverse actinomycetes, bacteria and fungi have been isolated from the rock phosphate deposits and rhizosphere of several crops (Pareek and Gaur 1973; Illmer and Schinner 1992; Whitelaw *et al*., 1999; Nautiyal *et al*., 2000; Gyaneshwar *et al*., 2002; Patel *et al*., 2008; Park *et al*., 2009). Bacterial genera such as *Achromobacter, Azospirillum, Aerobacter, Azotobacter, Agrobacterium Bacillus, Ralstonia Beijerinckia, Burkholderia, Enterobacter, Rhizobium, Erwinia, Flavobacterium, Gluconoacetobacter, Microbacterium, Pseudomonas, Serratia, and Micrococcus* are reported as the most significant phosphate solubilizing bacteria (Sturz and Nowak 2000; Sudhakar *et al*., 2000; Mehnaz and Lazarovits 2006, Poonguzhali *et al*., 2008; Kundu *et al*., 2009; Linu *et al*., 2009; Bhattacharyya and Jha, 2012). Studies have shown that *Bacillus* and *Pseudomonas* are amongst the most efficient bacteria solubilizing different phosphate substrates (Rodriguez and Fraga 1999; Tilak *et al*., 2005; Jha *et al*., 2009).

In addition to the inorganic phosphate solubilization, bacteria also play an important role in organic phosphate solubilization in the soil. The predominant form of organic phosphates present in the soil is phytate (myo-inositol 1, 2, 3, 4, 5, 6,-hexakis-dihydrogen phosphate). Phytases are a subgroup of phosphatases, which catalyzes the partial, or complete hydrolytic removal of orthophosphate from phytate (Vohra and Satyanarayana 2003). The commercially promising nature of microbial

phytase as well as the production of extracellular phytase producing *Enterobacter* sp. 4 from the rhizospheric region of leguminous plants have been reported (Pandey *et al*., 2001). The complete hydrolysis of phytate results in the production of one molecule of inositol and six molecules of inorganic phosphate (Singh and Satyanarayan 2011).The phytase producing plants are unable to utilize soil phytate due to the lack of extracellular phytase activity (Greiner and Alminger, 2001, Idriss *et al*., 2002). The phosphate solubilization is a complex phenomenon, which depends on several factors such as nutritional, physiological and growth conditions of the microorganisms (Reyes *et al*., 1999). *Bacillus amyloliquefaciens*, *B. subtilis*, *B. laevolacticus*, *B. licheniformis*, *Burkholderia cepacia*, *Citrobacter braakii*, *C. freundi*, *Enterobacter aerogenes*, *E. cloacae*, *Klebsiella terrigena*, *K. aerogenes*, *Proteus mirabilis*, *Pseudomonas fluorescens*, *P. mendocina*, *P. putida*, *P. syringae*, *Pseudomonas* sp., *Serratia marcenscens* and *Yersinia rohdei* have been reported to produce phytases (McGrath *et al*., 1995; Thaller *et al*., 1995; Greiner *et al*., 1997; Skrary and Cameron 1998; Richardson and Hadobas 1997; Kerovuo *et al*., 1998; Rodriguez and Fraga 1999; Richardson 2001; Idriss *et al*., 2002; Kim *et al*., 2003; Cho *et al*., 2005; Gulati *et al*., 2007; Huang *et al*., 2008).

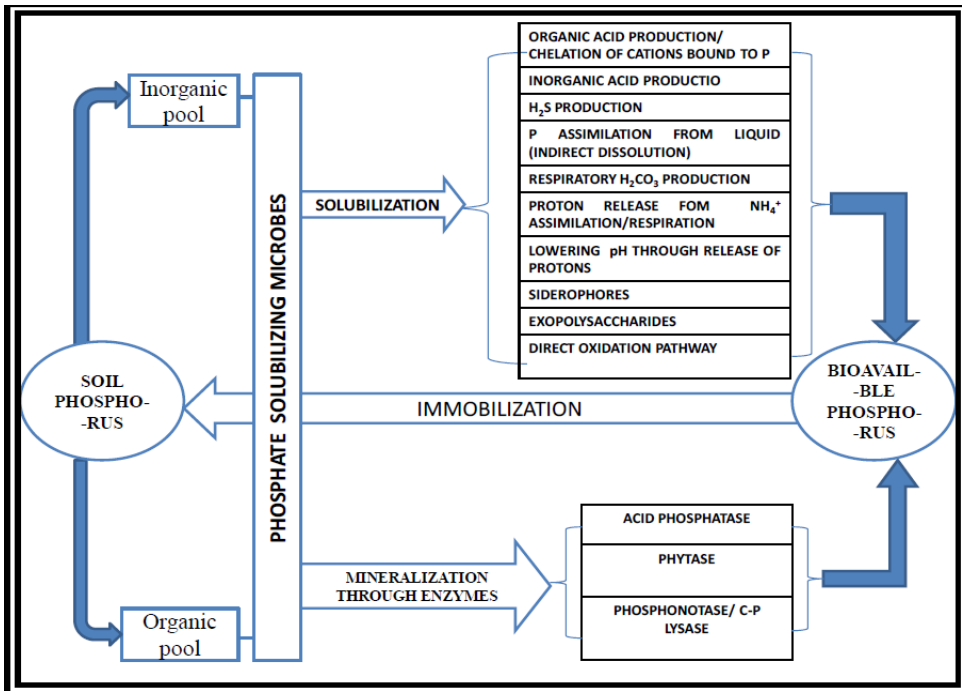


Fig. 2.2 Schematic representation of mechanism of soil P solubilization/mineralization and immobilization by PSM (Sharma et al., 2013a)

2.3.1.3 Mechanisms involved in phosphate solubilization

The process of phosphate solubilization by phosphate solubilizing microorganisms (PSM) involves the production of organic acids, which is accompanied by the acidification of the medium (Puente *et al.,* 2004). A decrease in the pH of the filtrate from the initial value of 7.0 to a final value of 2.0 was recorded by many workers (Gaur and Sachar 1980; Gaind and Gaur 1991; Illmer and Schinner 1992). The analysis of culture filtrates of PSMs has shown the presence of number of organic acids such as malic, glyoxalic, succinic, fumaric, tartaric, alpha keto butyric, oxalic, citric, 2-ketogluconic and gluconic acid Illmer and Schinner 1995; Fasim *et al*., 2002, White *et al*., 1997; Kim *et al*., 1997). Gluconic acid is the principal organic acid produced by *Pseudomonas* sp. (Illmer and Schinner, 1992), *Erwinia herbicola* (Liu *et al*., 1992) *Pseudomonas cepacia* (Goldstein 1994) and *Burkholderia cepacia* (Rodríguez and Fraga 1999). The production of gluconic acid by *Azospirillum* sp. (Rodriguez *et al*., 2004), *Citrobacter* sp. (Patel *et al*., 2008) and *P. fluorescens* (Di Simine *et al*., 1998; Park *et al*., 2009) has also been reported. Similarly, 2-ketogluconic acid production has been reported in *Rhizobium leguminosarum* (Halder *et al*., 1990), *Rhizobium meliloti* (Halder and Chakrabartty, 1993), *Acetobacter pasteurianus, Enterobacter intermedium, Pseudomonas fluorescens* and *Bacillus firmus* (Banik and Dey 1982).

The amount and type of the organic acid produced varies with the microorganism. The organic acids released in the culture filtrates react with the insoluble phosphate. The amount of soluble phosphate released depends on the strength and type of acid. Aliphatic acids are more effective in P solubilization than the phenolic acids and citric acid. Tribasic and dibasic acids are also more effective than mono basic acids (Gaur and Gaind 1999). Organic acid production by the microorganisms depends not only on the phosphate form but also on the type of soil and its buffering capacity, plant species and PGPR strain. Other mechanisms including H^+ excretion, production of carbon dioxide, H_2S and inorganic acids such as sulphidric, nitric, and carbonic acid have also been implicated in phosphate solubilization (Kucey 1983; Parks *et al*., 1990; Illmer and Schinner 1995; Ivanova *et al*., 2006). Proton-excretion accompanying ammonium ion assimilation has been reported as the most plausible explanation for microbial solubilization without

organic acid production (Illmer and Schinner, 1995). Phosphatase and phytase produced by the microorganisms cause mineralization of organic phosphates (Kremer, 1994; Kucharski *et al*., 1996; Idriss *et al*., 2002; Gulati *et al*., 2007; Gupta *et al.,* 2014).

2.3.2 Phytohormone production

The production of phytohormones such as auxins (IAA), cytokinins, gibberellins and abcissic acid by the natural soil microbial communities have been reported by various workers over the last 20 years (Poonguzhali *et al*.; 2008). Auxin is one of the important hormones produced by PGPR (Glick, 1995; Dobbelaere *et al.,* 2002; Richardson *et al.,* 2009). Indole-3-acetic acid, a main auxin in the plants is known to control many important physiological processes of the plants such as cell enlargement, cell division, root initiation, growth rate, phototropism, geotropism and apical dominance.

About 80% microorganisms isolated from the rhizosphere of various crops possess the ability to synthesize and release auxins as secondary metabolites (Patten and Glick 1996). Tryptophan exuding from the roots serves as the primary precursor of IAA biosynthesis by microorganisms (Fig. 2.3). The production of auxins in the presence of tryptophan have been reported for several bacteria, including *Acinetobacter*, *Acetobacter*, *Alcaligenes*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Bradyrhizobium*, *Pantoea*, *Pseudomonas*, *Rhizobium* and *Xanthomonas* (Patten and Glick, 1996; Bent *et al*., 2001; Kang *et al*., 2006; Boiler *et al*., 2007; Idriss *et al*., 2007; Sergeeva *et al*., 2007; Tsavkelova *et al*., 2007; Gulati *et al*., 2009; Park *et al*., 2009; Jha *et al*., 2009, Ahemad and Khan 2010 b). PGPR predominantly synthesize IAA from tryptophan mainly through the indole-3-pyruvic acid and indole-3-acetamide pathways (Patten and Glick 1996).

Bacterial IAA increases root surface area and length, and thereby provides the plant greater access to soil nutrients (Ahmed *et al.,* 2014). The supplementation of culture media with tryptophan increases the IAA production by most of the rhizobacteria (Spaepen and Vanderleyden, 2011). Auxins, particularly IAA have also been reported to be involved in host-parasite interactions (Gutierrez *et al*.,

2009).Various authors have reported the biocontrol action of IAA, either due to the inhibition of spore germination and mycelium growth of pathogenic fungi, or due to its involvement together with glutathione S-transferase in defense-related plant reactions (Droog 1997; Brown and Hamilton 1993; Martinez-Noel *et al*., 2001). Recent studies on the involvement of IAA in the *P. fluorescens* mediated control of *Fusarium* head blight (FHB) disease of barley, reduced both disease severity and yield loss caused by *F. culmorum* but no hormone affect in vitro was reported (Petti *et al*., 2012).

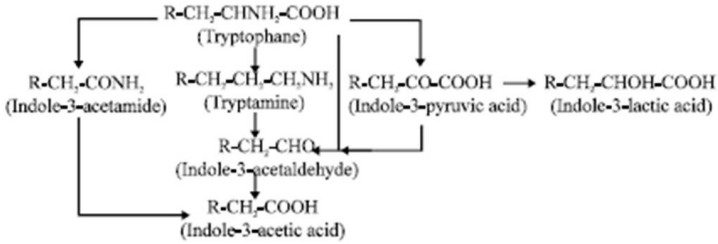


Fig.2.3. Biosynthetic pathway of IAA (Adapted from Ahemad and Khan, 2011)

The level of IAA produced by the PGPR may affect the host plants, either positively or negatively (Glick *et al.,* 1999). The optimum IAA concentration enhances root length and promotes better developed plant root systems. However, when the IAA concentration or inoculum is too high, or too low, plant root growth may be inhibited. Studies have shown that the concentration of IAA increases significantly with the addition of tryptophan in the growth medium (Patten and Glick, 2002; Khalid *et al*., 2004; Ahmad *et al*., 2005). Khalid *et al*., (2004) reported that plant roots also excreted tryptophan, which could be used as an IAA precursor source for PGPR. Studies have shown that the factors such as type of bacteria, levels of hormone produced by the bacteria, and plant response to these levels determine the actual effects of growth regulators on plant productivity (Glick, 1995; Cattelan *et al.,* 1999; Dobbelaere *et al.,* 2002). For example, the overproduction of IAA reduces plant growth (Glick 1995; Dobbelaere *et al.,* 2002; Richardson *et al.,* 2009). In addition to stimulating root growth, the role of IAA producing bacteria in increased growth of tubers has also been reported (Martinez-Viveros *et al.,* 2010).

Several reports have revealed that PGPR are able to produce ABA, or gibberellic acid, or cytokinins that stimulate plant cell division, proliferative induction of root hair, control root meristem differentiation, but inhibit lateral root formation and primary root elongation (Silverman *et al*., 1998; Riefler *et al*., 2006). Inoculation of the plants with bacteria producing cytokinin has been shown to stimulate shoot growth and reduce the root to shoot ratio (Arkhipova *et al.*, 2007). Cytokinin production (especially zeatin) has been documented in various PGPR such as *Arthrobacter giacomelloi, Azospirillum brasilense, Bradyrhizobium japonicum, Bacillus licheniformis, Pseudomonas fluorescens*, and *Paenibacillus polymyxa* (Cacciari *et al*., 1989; Timmusk *et al.*, 1999; de García Salamone *et al*., 2001; Boiero *et al*., 2007; Cassán *et al*., 2009; Hussain and Hasnain 2009). Bacterial genes involved in cytokinin biosynthetic pathways have been identified *in silico* but the role has not been validated through the functional analyses (Frébort *et al*., 2011). Gibberellins promote primary root elongation and lateral root extension (Yaxley *et al*., 2001). Production of gibberellins has been documented in several PGPR belonging to *Achromobacter xylosoxidans, Acinetobacter calcoaceticus, Azospirillum* sp., *Azotobacter* sp., *Bacillus* sp., *Herbaspirillum seropedicae, Gluconobacter diazotrophicus* and rhizobia (Gutiérrez-Mañero *et al*., 2001; Dodd *et al.*, 2010). The application of gibberellic acid on maize at a concentration similar to that produced by *Azospirillum*, promotes root growth (Fulchieri *et al.*, 1993). In addition to playing a role in plant root structural architecture, these two hormones are involved in plant defense mechanisms. Thus, PGPR producing these hormones may modulate the hormonal balance involved in plant defense, including the jasmonate and salicylic acid pathways (Pieterse *et al*., 2009). ABA is well-known for its involvement in drought stress. During the water stress, the increase in ABA levels causes closing of the stomata, thereby limiting water loss (Bauer *et al.*, 2013). ABA is also reported for significant role during the lateral root development (De Smet *et al*., 2006; Dodd *et al*., 2010). Inoculation with *Azospirillum brasilense* Sp245 led to an increase of ABA content in Arabidopsis, especially when grown under osmotic stress

2.3.3 1-aminocyclopropane-1-carboxylate deaminase activity

The plant hormone ethylene plays key role in root initiation and elongation, nodulation, senescence, abscission, ripening as well as in stress signaling (Arshad and Frankenberger 2002; Glick 2012). The high concentration of ethylene stimulates defoliation and other cellular processes that may lead to reduced crop performance (Bhattacharyya and Jha 2012; Glick 2012). Certain plant growth promoting rhizobacteria contain an enzyme, 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) that catalyses the cleavage of 1-aminocyclopropane-1-carboxylate (ACC), the immediate precursor of ethylene in the plants (Glick *et al*., 1995, 1998; Burd *et al*., 1998; Kaneko *et al*., 2000; Belimov *et al*., 2001; Babalola *et al*., 2003; Ma *et al*., 2003; Ghosh *et al*., 2003; Dey *et al*., 2004; Hontzeas *et al*., 2005; Dell'Amico *et al*., 2005; Shaharoona *et al*., 2006; Blaha *et al*., 2006; Saravanakumar and Samiyappan 2006; Glick 2012). Currently, bacterial strains exhibiting ACC deaminase activity have been identified in a wide range of genera such as *Ralstonia*, *Rhizobium*, *Rhodococcus*, *Sinorhizobium meliloti*, *Variovorax Acinetobacter*, *Achromobacter*, *Agrobacterium*, *Alcaligenes*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Pseudomonas*, *Ralstonia*, *Serratia* and *Rhizobium* (Shaharoona *et al*., 2007a, b; Nadeem *et al*., 2007; Zahir *et al*., 2008; Kang *et al*., 2010, Glick *et al*., 2012; Govindasamy *et al*., 2008). Several forms of stress are relieved by the ACC deaminase producers such as effects of phytopathogenic microorganisms (viruses, bacteria, and fungi etc.), and resistance to stress from the polyaromatic hydrocarbons, heavy metals, radiation, wounding, insect predation, high salt concentration, draft, extremes of temperature, high light intensity, and flooding (Glick, 2012; Lugtenberg and Kamilova 2009). The bacterium actually prevents ethylene caused inhibition of root elongation. The enzyme ACC is exuded together with the other components of the root, or seed exudates and taken up as a food source by the rhizosphere bacteria. ACC may be cleaved by the ACC deaminase to form ammonia and α-ketobutyrate compounds that are readily metabolized by the bacteria. IAA can also stimulate ACC synthase (Fig. 2. 4) to produce more ACC, which can be transformed into ethylene by ACC oxidase (Mayak *et al.*, 1999).

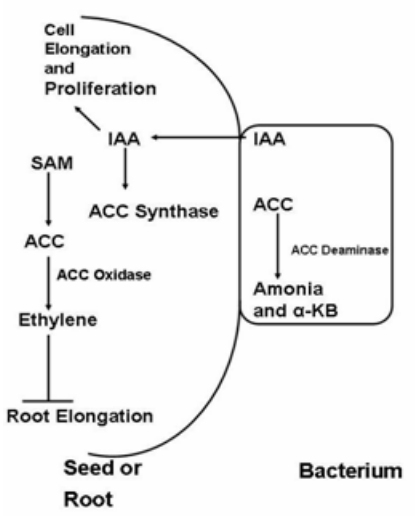


Fig. 2.4 Lowering of ethylene levels by ACC-deaminase activity (Glick *et al.,* 1998)

On the other hand, the simultaneously produced ACC deaminase can hydrolyze the ACC and inhibit ethylene production. As a consequence, the final effect on ethylene production, or root growth depends on the balance of the IAA and ACC. ACC that is exuded from the plant roots is taken up by the PGPR and hydrolyzed by the enzyme ACC deaminase to ammonia and α-ketobutyrate.

2.3.4 Associative biological nitrogen fixation

Nitrogen fixations by the symbiotic microorganisms or by free-living diazotrophs and transformations of nitrogen (N) between different pools have important implications for plant growth and for the loss of nitrogen from the soil systems (Jackson *et al*., 2008). The N has to be reduced to ammonia to be utilized by the plants to produce nucleic acids and proteins. However, symbiotic rhizobacteria, especially rhizobia, which are capable of fixing nitrogen in leguminous crops, are not usually considered as PGPR (Spaepen *et al*., 2009) and will not be discussed in this chapter while free-living diazotrophs, a group of bacteria, which are able to convert atmospheric nitrogen (N_2) into readily usable ammonia are considered as PGPR. Being a high energy-requiring process, it is biologically catalysed by the nitrogenase enzyme and regulated by nitrogen fixation genes (*nif*) and is called 'biological nitrogen fixation' (BNF) (Glick *et al*., 1999). One of the best studied diazotrophs for

nitrogen fixation is *Azospirillum* sp. isolated from nitrogen-poor soils by Beijernick in 1925 (Glick *et al*., 1999). Free-living diazotrophs have been identified in several genera of common rhizosphere-inhabiting microorganisms such as *Acetobacter, Azoarcus, Azospirillum, Azotobacter, Burkholeria, Enterobacter, Herbaspirillum, Gluconobacter Beijerinckia* sp., *Klebsiella pneumoniae, Pantoea agglomerans* and *Pseudomonas* (Baldani *et al*., 1997; Blaha *et al*., 2006; Vessey 2003; Antoun *et al.*, 1998; Riggs *et al*., 2001).

Evidence in favor of the participation of PGPR to the plant N budget has been reported for several plants, especially sugarcane (Boddey *et al*., 2003), radish (Antoun *et al*., 1998) and rice (Blaha *et al*., 2006), thereby contributing for the reduced dependence on N- based fertilizers. Nevertheless, studies in sorghum, maize and wheat inoculated with *Azospirillum* have revealed a contribution of only 5 kg N ha^{-1} yr^{-1} (Okon and Lanbandera- Gonzalez 1994). This quantity pales in importance when compared with the application of N fertilizers in a range of 150-200 kg N ha^{-1} yr^{-1}, which is commonly practiced in modern agriculture. This applies likely to other free living N fixers. However, the impact of N_2-fixation by PGPR has been debated and rarely credited for the stimulation of plant growth (Dobbelaere *et al*., 2003, Martínez-Viveros *et al*., 2010).

2.3.5 Siderophore`s production

Iron is an essential nutrient of the plants as it serves as a cofactor of many enzymes with redox activity. A large portion of iron in the soils is in the highly insoluble form of ferric hydroxide, thus acting as a limiting factor for plant growth even in iron-rich soils. Several PGPR produce siderophores, which are low molecular weight iron chelating compounds that bind Fe^{3+} with very high affinity and help in iron uptake. Plants can absorb bacterial Fe^{3+}- siderophore complexes, and this process is vital in the absorption of iron by plants (Wang *et al.*, 1993, Masalha *et al.*, 2000). According to the published results, several microorganisms, including *Alcaligenes, Azotobacter, Azospirillum, Bradyrhizobium, Bacillus, Enterobacter, Pseudomonas, Rhizobium* and *Serratia* are capable of producing siderophores, usually less than 1 kDa size under low-iron conditions (Yang *et al*., 1991; Raaijmakers *et al*., 1995; Khandelwal *et al*.,

2002; Rachid and Ahmed 2005; Sayyed *et al*., 2005; Silva *et al*., 2006; Storey *et al*., 2006; Sayyed and Chincholkar, 2006, 2009; Kuffner *et al.,* 2008)

Siderophores are usually classified by the ligands used to chelate the ferric iron. The major groups of siderophores include the catecholates (phenolates), hydroxamates and carboxylates (e.g., derivatives of citric acid). Based on their iron-coordinating functional groups, bacterial siderophores have been classified into carboxylate, hydroxamates, phenol catecholates and pyoverdines groups (Crowley 2006). Recent studies have demonstrated the suppression of soil-borne fungal pathogens through the release of iron chelating siderophores by fluorescent pseudomonads, rendering it unavailable to other organisms (Loper 1988; Paulitz and Loper, 1991; Dwivedi and Johri 2003). Siderophores also form stable complexes with other heavy metals that are of environmental concern, such as Al, Cd, Cu, Ga, In, Pb and Zn, as well as with radionuclides including U and Np (Neubauer *et al*., 2000; Kiss and Farkas, 1998). Studies have proven that the binding of the siderophore to a metal increases the soluble metal concentration (Rajkumar *et al*., 2010). Hence, bacterial siderophores help to alleviate the stresses imposed on the plants by high soil levels of heavy metals. Crowley and Kraemer (2007) revealed a siderophore mediated iron transport system in oat plants and inferred that siderophores produced by the rhizosphere microorganisms delivered iron to oat, which had mechanisms for using Fe-siderophore complexes under iron-limited conditions. Siderophore production confers competitive advantages to the PGPR that can colonize the roots and exclude other microorganisms from this ecological niche (Haas and Défago 2005). Under highly competitive conditions, the ability to acquire iron *via* siderophores may determine the outcome of competition for different carbon sources that are available as a result of root exudation, or rhizodeposition (Crowley, 2006). Plants such as sorghum, oats, peanut, cotton, cucumber and sunflower demonstrated the ability to use radio labelled microbial siderophores as a sole source of iron (Crowley *et al.,* 1988; Bar-Ness *et al.,* 1991; Cline *et al.,* 1984; Jurkevitch *et al.,* 1986; Wang *et al.,* 1993). Growth of cucumber in the presence of microbial siderophores resulted in increased plant biomass and chlorophyll content (Ismande 1998).

2.3.6 Production of anti-fungal metabolites by the PGPR

Many rhizobacteria have been reported to produce antifungal metabolites such as HCN, siderophores, butyrolactones, zwittermycin A, kanosamine, oligomycin A, oomycin A, phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin, viscosinamide, xanthobaccin, phenazines, pyrrolnitrin, 2, 4- diacetylphloroglucinol (2,4-DAPG), pyoluteorin, viscosinamide, tensin and cell-wall degrading enzymes such as chitinases, cellulases and proteases that reduce the growth, or activity of phytopathogens (Kremmer and Souissi, 2001; Raaijmakers *et al*., 2002; Nielson and Sørenson 2003; Raj *et al*., 2003; Kloepper *et al*., 2004; Compant *et al*., 2005; Domenech *et al*., 2006; Battu and Reddy 2009, Bhattacharyya and Jha 2012; Whipps 2001). 2, 4-DAPG has a wide spectrum of properties as it is antifungal (Loper and Gross, 2007; Rezzonico *et al*., 2007), antibacterial (Velusamy *et al*., 2006) and antihelmintic (Cronin *et al*., 1997). Many different bacterial genera have shown to be capable of producing HCN, including the species of *Alcaligenes, Aeromonas, Bacillus, Pseudomonas* and *Rhizobium* (Devi *et al*., 2007; Ahmad *et al*., 2008). Siderophore overproducing mutants were more effective at protecting the plants against fungal pathogens. Among the soil borne pathogens shown to be negatively affected by PGPR are *Aphanomyces* sp., *Fusarium oxysporum, Gaeumannomyces graminis, Phytophthora* sp., *Pythium* sp., *Rhizoctonia solani, Sclerotium rolfsii, Thielaviopsis basicola,* and *Verticillium* sp. (Kloepper *et al.,* 1999).Interaction of some rhizobacteria with the plant roots can result in plant resistance against some pathogenic bacteria, fungi, and viruses. This phenomenon is called 'induced systemic resistance' (ISR) (Lugtenberg and Kamilova, 2009). ISR does not target specific pathogens. Rather, it may be effective at controlling the diseases caused by different pathogens. ISR involves jasmonate and ethylene signaling within the plant and these hormones stimulate the host plant's defense responses to a range of pathogens.

2.4 Effect of PGPR on Plant Growth

The PGPR efficacy is dependent on establishing an effective population density of active cells in the plant rhizosphere. Interaction of the PGPR with the host plants is an intricate and interdependent relationship involving not only the two partners but

other biotic and abiotic factors of the rhizosphere region. Various factors that play a determining role on the survival and establishment in the rhizosphere include the composition of root exudates, properties of bacterial strain, soil status, and activities of other soil microbes. Treatments with the PGPR increase germination percentage, seedling vigor, emergence, plant stand, root and shoot growth, total biomass of the plants, seed weight, early flowering, grains, fodder and fruit yields (van Loon *et al*., 1998; Ramamoorthy *et al*., 2001). Though the exact mechanisms involved in growth promotion are still unclear, various mechanisms have been suggested to explain the phenomenon of plant growth promotion include increase in the nitrogen fixation, production of auxins, gibberellins, cytokinins, solubilization of phosphorous, oxidation of sulfur, increase in availability of nitrate, extra cellular production of antibiotics, lytic enzymes, hydrocyanic acid, increases in root permeability, strict competition for the available nutrients and root sites, suppression of deleterious rhizobacteria, and enhancement in the uptake of essential plant nutrients (Subba Rao 1982; Pal *et al*., 1999; Enebak and Carey 2000).

Dubey and Billore (1992) showed an increase in the yields of legumes after inoculation with rock phosphate and the phosphate-solubilizing bacteria, *Bacillus megaterium* and *Pseudomonas striata*, and the phosphate solubilizing fungus *Aspergillus awamori* and suggested using low-grade rock phosphate for both the neutral and alkaline soils with phosphate-solubilizing inoculants. The effect of inoculation with the phosphate-solubilizing bacterium *Bacillus firmus*, irrigation schedules and phosphorus levels on lentil (*Lens esculentus*) and black gram (*Vigna mungo*) resulted in significantly greater seed yield in field trials during the winter seasons (Tomar *et al*., 1993). A strain of *Burkholderia cepacia* displaying significant mineral phosphate solubilization and moderate phosphatase activity but no IAA production improved the yield of potato, tomato, banana, onion, citrics, and coffee in field tests, and was used as a commercial biofertilizer in Cuba (Martínez *et al*., 2001) Fraga 1999*).* Single and dual inoculation along with P fertilizer was 30-40% better than P fertilizer alone for improving the grain yield of wheat. The dual inoculation without P fertilizer improved the grain yield of wheat up to 20 % against sole P fertilization (Afzal and Bano 2008). Richardson *et al*., (2001) observed that the ability of pasture plants to acquire P from phytate was enhanced followed by

inoculation with the specified *Pseudomonas* sp. strains. Lifshitz *et al*., (1987) reported that a *P. putida* PGPR strain increased the uptake of 32P-labelled phosphate by canola seedlings and inoculation of the seeds with a *Pseudomonas* PGPR resulted in a significant increase of phosphate levels in the roots and in shoots.

Seed inoculation with wild-type *P. putida* GR12-2 induced the formation of roots in canola that were 35–50% longer than the roots from the seeds treated with the IAA-deficient mutant and the roots from uninoculated seeds (Glick 2012). Swain *et al.,* (2007) reported a positive effect of IAA producing strains of *Bacillus subtilis* on *Dioscorea rotundata* L when a suspension of *B. subtilis* was applied on the surface of the plant, which resulted in an increase in the root: stem ratio as well as the number of sprouts as compared with the non-inoculated plants. Similarly, shoot growths in maize and rice dwarf mutants were significantly promoted by gibberellins-like substances excreted by *Azospirillum* sp. (Boiero *et al*., 2007). Swain *et al*., (2007) reported a positive effect of *Bacillus subtilis* IAA producing strains on the edible tubercle, *Dioscorea rotundata* L in one of their studies. They applied a suspension of *B. subtilis* on the surface of the plants, which resulted in increased fresh weight of the stem and root, increased length of stem and root, increased root: stem ratio and increased numbers of sprouts as compared with the non-inoculated plants.

Ghosh *et al*., (2003) recorded ACC deaminase activity in three *Bacillus* species namely, *B. circulans* DUC1, *B. firmus* DUC2 and *B. globisporus* DUC3 that stimulated root elongation in *Brassica campestris*. Mayak *et al.,* (2004b) observed tomato plants inoculated with the bacterium *Achromobacter piechaudii* under water and saline stress conditions and reported a significant increase in fresh and dry weight of inoculated plants. *Pseudomonas fluorescens* TDK1 containing ACC deaminase has been reported to enhance the saline resistance and overall yield in groundnut as compared to those inoculated with *Pseudomonas* strains lacking ACC deaminase activity (Govindasamy *et al.,* 2008).

P. fluorescens strain WCS374 has been reported to suppress *Fusarium* wilt in radish leading to an average increase of 40% in yield (Bakker *et al.,* 2007). Studies revealed that Fe-pyoverdine complex synthesized by *P. fluorescens* C7 was taken up by *Arabidopsis thaliana* plants, which led to an increase of iron in the plant tissues

and improvement in plant growth (Vansuyt *et al.*, 2007). Sharma *et al*., (2003) studied the role of the siderophore-producing *Pseudomonas* strain GRP3 on iron nutrition of *Vigna radiate* and observed a significant decline in chlorotic symptoms accompanied with increased content of iron, chlorophyll a and chlorophyll b after 45 days of treatment compared to the control. Various studies have attributed a disease protective effect to HCN, e.g., in the suppression of "root-knot" and black rot in tomato and tobacco root caused by the nematodes *Meloidogyne javanica* and *Thielaviopsis basicota*, respectively (Voisard *et al*., 1989; Siddiqui *et al*., 2006). One of the important pest termite, *Odontotermes obesus* affecting the agricultural and forestry crops in India were also controlled by HCN (Devi *et al*., 2007).

PGPR strains with multiple plant growth-promoting activities have been reported to enhance the growth in many plants, e.g., *P. aeruginosa* strain PS1 through phosphate solubilization, IAA, siderophores, EPS, fungal antagonism and fungicide tolerance in green gram *Enterobacter asburiae* HPP16 through indole-3-acetic acid producer, synthesized siderophores and showed acid phosphatase activity in mung bean (Zhao *et al*., 2011), *Mesorhizobium* sp. through IAA production, HCN, siderophore and ammonia in chickpea (Wani *et al*., 2008), *Azospirillum amozonense* through the production of IAA and ammonia in rice (Elisete *et al*., 2008), etc. The selection of PGPR with multiple plant growth-promoting attributes has been advocated for the development of microbial inoculants with consistency in field performance.

2.5 Microbial consortium and application

A biotechnological approach to minimize the toxic effects of pollutants and maximize plant growth and nutrition is to use a combined inoculation of selected rhizosphere microorganisms. Selected combinations of microbial inocula can very well enhance the positive effect achieved by each microbial group, improving plant development. Syntrophic relationships between different organisms have been demonstrated in several microbial ecosystems. Therefore, mixed inoculants (combination of microorganisms) that interact synergistically are currently being devised, which yield better and quick results (Bashan 1998).

It has been suggested that the development of plant growth promoting consortium (PGPC) could be a feasible strategy for increased activity and better viability of PGPR. When these strains are made into consortium, each of the constituent strains of the consortium not only out competes with the others for rhizospheric establishments, but complement functionally for plant growth promotion (Shenoy and Kalagudi 2003). The benefits of mixture of PGPR include broad spectrum of actions such as improved efficacy, reliability and allowance of combinations of various traits without genetic engineering (Janisiewicz 1996). But, sometimes the incompatibility between the mixtures of inoculants may arise because of which inhibition of each other as well as target pathogens may occur. Hence, for the development of mixture inoculants, compatibility of microorganisms is a pre-requisite.

Afzal *et al.*, (2005) reported increased yield and P uptake of wheat plants due to inoculation of mixture of *Pseudomonas* and *Bacillus* sp. Combined inoculation of *A. brasilense* and the phosphate-solubilizing bacterium, *P. strica* or *B. polymyxa* on field grown sorghum significantly increased grain and dry matter yields and N and P uptake as compared with single inoculation of individual organisms (Alagawadi and Gaur 1992). Three unrelated bacteria- methylotrophic *Methylobacterium oryzae* along with *Azospirillum brazilenze* and *Burkholderia pyrrocinia* were reported to have positive effect on nutrient uptake, hence on the growth of tomato, red pepper and rice plants (Aronen *et al*., 2002; Madhaiyan *et al*., 2010). Similarly, studies have revealed that the presence of *S. meliloti* PP3, *R. leguminosarum* Pcc and *Bacillus* B1 did not have any detrimental effect on the viability of PGPR strain– *Burkholderia* sp. MSSP in wheat bran based multi species consortium (Pandey and Maheshwari 2007).

2.6 Stress tolerance of PGPR

Abiotic soil factors such as texture, pH, temperature, moisture content, and substrate availability largely determine the survival and activity of the introduced microorganisms (Gray 1975). Abiotic and biotic factors influenced the different mechanisms and limited the interactions between the plant and beneficial bacteria,

thereby resulting in less than acceptable performance in plant growth promotion and management of diseases (Egamberdiyeva and Hoflich 2002, 2003). Stress factors reduce the ability of the plants to absorb water, induce many metabolic changes causing rapid reduction in growth rate and the responses seems similar to those caused by the water stress. In such soils, inoculation of broad range of stress tolerating microorganisms could be of great importance. The response of the inoculant to the prevailing soil conditions depends on its genetic and physiological constitution. Thus, the effects of the aforementioned soil factors on the introduced microorganisms would differ in accordance with the ability of the inoculant to cope with adverse and fluctuating conditions, to survive, and to remain active. Hence, for the selection of an efficient PGPR, screening for abiotic stress tolerance would be an added advantage. Microbial adaptation to stress is a complex regulatory process in which a number of genes are involved (Tobor - Kapłon *et al*., 2006; Grover *et al*., 2010).

The occurrence of *Rhizobium, Bradyrhizobium, Azotobacter, Azospirillum, Pseudomonas* and *Bacillus* has been reported from desert ecosystems, acid soils, saline and alkaline areas and highly eroded hill slopes of India (Tilak *et al*., 2005, Selvakumar *et al*., 2008; Upadhyay *et al*., 2009). *Pseudomonas* sp. strain NBRI0987 caused thermo-tolerance in sorghum seedlings, which consequently synthesized high molecular weight proteins in the leaves, thus increasing the plant biomass. *Burkholderia phytofirmans* PSJN colonized grapevine residues and protected the plant against heat and frost by increasing the levels of starch, proline and phenols. Inoculation of wheat seeds with *Serratia marscescens* strain SRM and *Pantoea dispesa* strain 1A increased the biomass of seedlings and uptake of nutrients at low temperatures. PGPR mediated the increased growth of tomatoes, peppers, beans and lettuce grown in saline environments (Grover *et al*., 2010; Yildirium and Taylor, 2005). Crop inoculation with *B. amyloliquifaciens* led to the production of polysaccharides (EPS), which tended to improve the soil structure by facilitating the formation of macro-aggregates, and in turn increased the plant resistance to drought stress due to water shortage.

2.7 Rhizosphere competence

Rhizosphere competence of PGPR involves effective root colonization combined with the ability to survive and proliferate along growing plant roots over a large time period in the presence of the indigenous microflora (Weller, 1988; Lugtenberg *et al*., 1999). Root colonization by rhizobacteria is an important factor in plant growth promotion and biological control (De Weger *et al*., 1995; Knudsen *et al*., 1997; Roberts *et al*., 1999), which is affected by the characteristics of both PGPR, host plant and is also controlled by the combined effects of soil properties that include biotic and abiotic factors (Table 2.2).

Table 2.2 Major factors involved in root colonization (Adopted from Beniziri *et al*., 2010)

	Bacteria	Plant	Biotic actors	Abiotic factors
Attachment	Adhesion electrostatic charge Polarity Hydrophobicity Agglutination Pili, Fimbriae Polysaccharides Outer membrane proteins	Adhesion electrostatic charge Polarity Roughness of root Agglutination agglutinins	Number of sites	Soil type pH temperature texture structure density
Dispersion	Chemotaxis Flagella	Root elongation	Nematods Arthropods Fungi Annelids Protozoa	Water percolation Osmotic potential Soil Type Texture Density
Multiplication Competition Survival	Antibiotics Siderophores Generation time Catabolism Osmotolerance Catalases	Rhizodepostion Quantity Quality Stress Chemical treatment Growth stage	Bacterial biomass Nutrient quantity	Soil Temperature Soil water potential Moisture pH Climate

Indeed, the bacterial strain needs to possess particular traits such as chemotaxis towards root exudates, compounds mediating attachment (adhesions, fimbriae, pili, cell surface proteins and EPS) and a capacity to metabolize the root exudates compounds. In most cases, the population of many PGPR inoculants actually declines progressively in time after inoculation from 10^7-10^9 cells per gram dry soil to 10^5-10^6 cells per gram dry soil after 2-3 weeks Nevertheless, this population threshold is often sufficient to provide beneficial effects (Raaijmakers *et al*., 2002).

Different approaches and techniques have been used to quantify and identify the inoculated strains on the host plant (Lugtenberg *et al*., 2001; Gamalero *et al*., 2003; Haggag and Timmusk, 2008). Measurements of cell densities based on 16S rRNA gene copy numbers, or plating on agar and CFU enumeration are some of the commonly used methods for the quantification of inoculates. In one of the studies, the presence of "introduced" bacteria in the rhizosphere of various plant species of the Himalayan region has been confirmed through antibiotic markers. The use of genetic markers such as intrinsic resistance to various antibiotics is a simple and rapid method of strain identification and enumeration of the introduced bacterial isolates that exhibit resistance to selective antibiotics (Josey *et al*., 1979; Kluepfel 1993). Colonization studies have also used antibiotic-resistant mutants (e.g., rifampicin resistant) of the wild-type strain to study the root colonization by then PGPR (Lottmann *et al*., 2000; Moënne-Loccoz *et al*., 2001). Other approaches employ PCR methods to quantify the copy number of a particular functional gene, or expression of relevant mRNA for the genes encoding PGPR traits. Using such markers, the effective rhizosphere colonization of tea by *B. subtilis*, *B. megaterium* and *P. corrugate* was confirmed (Trivedi *et al.,* 2005b) indicating a close bacterial–plant association with beneficial effects on plant growth. These three bacterial species were also studied for colonization and survival in the rhizosphere of maize using field and pot experiments conducted for three consecutive years under rain-fed conditions of the Himalayan region (Kumar *et al.,* 2007a). The three bacterial inoculants showed good rhizosphere competence giving high inoculum numbers ($\log_{10}$ 11.13–11.34 cfu/ g). The rifampicin mutant of *Acinetobacter rhizosphaerae* and *Rahnella* sp. effectively colonized the pea rhizosphere without adversely affecting the resident microbial populations (Gulati *et al*., 2009; Vyas *et al*., 2010).

Rhizoplane population of fluorescent pseudomonads was reportedly maintained at a critical level (5.3 cfu) for up to 30 days of soybean growth, followed by a steep decline (Tripathi *et al*., 2005).

Immunological and molecular techniques have also been used to quantify and identify inoculated strains on plant roots (Benizri *et al*., 2001; Fould *et al*., 2006). *In situ* detection of bacteria based on specific staining methods, advanced microscopy using fluorescent antibodies and fluorescent *in situ* hybridization, and reporter gene technology have been stimulated to study their ecology on plant root surface, rhizosphere and bulk soil (Hong *et al*., 1991; Roberts *et al*., 1999; Lugtenberg *et al*., 2001; Timms-Wilson and Bailey, 2001; Bhatia *et al*., 2002; Gamalero *et al*., 2003; Mavrodi *et al*., 2006).

2.8 Identification and characterization of PGPR strains

Specific rhizobacterial strains from several hundreds of root-colonizing bacteria are isolated from excised roots of field grown plants. PGPRs are then initially selected for their ability to promote plant growth, or inhibit the growth of various phytopathogens, or miscellaneous rhizosphere bacteria and fungi *in vitro*. Pure cultures of potent rhizobacterial strains are screened in greenhouse trials. During the experiment, those PGPRs that consistently cause statistically significant increase in the root, or shoot development, or both are selected for further testing in agricultural field.

2.8.1 Polyphasic characterization of rhizobacteria

The current trend for the identification and characterization of microbes relies on polyphasic approach to taxonomy (Vandamme *et al.,* 1996). According to this systematics, several patterns of properties are taken into account for the identification, and not merely on one, or few characteristics. Identification initially begins at the domain level and descends to the level of phyla, classes, order, families, finally to genus and species. Polyphasic approach involves the collection of information from three basic features (phenotypic features, chemotaxonomic and genotypic features). Phenotypic features include morphological, physiological, biochemical and metabolical characteristics of bacteria. The bacterial morphology

includes both cellular (shape, endospore, flagella, inclusion bodies, Gram staining) and colonial (color, dimensions, form) characteristics. The physiological and biochemical features include data on the growth at different temperatures, pH values, salt concentrations, or atmospheric conditions, growth in the presence of various substances such as antimicrobial agents, and data on the presence, or activity of various enzymes, metabolization of the compounds, etc. The reproducibility of the results between labs and the conditional nature of gene expression in response to different environmental conditions are some of the limitations of phenotypic tests. Hence, phenotypic results must be compared with similar set of available data from type strain of closely related organisms.

Several miniaturized and automated commercial versions of traditional biochemical tests are available for the taxonomical studies enabling rapid identification of microorganisms. Addition of turbidometrically controlled aliquot of a pure bacterial suspension initiates the reaction of growth, production of enzymatic activity, utilization of carbohydrates, etc. The results are interpreted according to the recommendations of the manufacturer and are readily accessible with a minimal input of time. The phenotypic fingerprinting systems (API 50CH kit) have been used to identify *Bacillus* (Logan and Berkeley, 1984) and *Paenibacillus* strains (Seldin and Penido 1986); the API 20NE systems have been used for correct identification of *Pseudomona*s species (Barr et al. 1989). The differential utilization of 95 carbon sources using Biolog assay is considered a much less laborious system for bacterial identification (Miller and Rhoden 1991) and used for the identification of PGPR strains belonging to *Paenibacillus azotofixans* (Pires and Seldin 1997).

The chemotaxonomy includes the collection of information on different chemical constituents, or chemotaxonomic markers of bacterial cells to group, or organizes them into different taxonomic ranks (Rosello-Mora and Amann 2001; Vandamme *et al.,* 1996). PGPR identification using chemotaxonomic fingerprinting techniques include FAME profiling, PAGE analysis of whole-cell proteins, polar lipid analysis, quinone content, cell wall diamino acid content, pyrolysis mass spectrometry, Fourier transform infrared spectroscopy, Raman spectroscopy, and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. The analyses of fatty acid methyl ester composition (FAME analysis)

are increasingly used both in taxonomic studies and identification analysis (Welch, 1991; Park *et al.*, 2005; Logan *et al*., 2009). Cell wall/membrane components such as peptidoglycan, teichoic acids, polar lipids, fatty acids composition, lipopolysaccharide, isoprenoid quinones and polyamines are some of the commonly used chemical markers (Park *et al*., 1993). The data on the type, content, proportion and variation in the fatty acid profile are used to identify and characterize the genus and species by comparing it against the fatty acid profiles of known organisms. The limitations are also reported with the identical fatty acid profiles for different species (Welch, 1991; Vreeland *et al*., 2006; Logan *et al*., 2009).

Genotypic methods are based on the information obtained from the DNA, or RNA molecules and are thought to be more reliable than phenotypic identification. Some of the different techniques employed to subtype bacteria up to strain level are restriction fragment length polymorphism (RFLP), ribotyping, pulsed field gel electrophoresis (PFGE), plasmid profiling, amplified ribosomal DNA restriction analysis (ARDRA) and randomly amplified polymorphic DNA (RAPD). Numerous reports on the identification of PGPR using one, or more of these techniques are available (Oliveira *et al*., 2000; Depret and Laguerre 2008; von der Weid *et al*., 2000; Monteiro *et al*., 2009). One of the classical methods of bacterial systematics involves the analysis of mole percent of guanosine and cytosine. Within a species the variation of mole percent GC content is not more than 3% while within the genus the variation is not more than 10% (Stackebrandt and Goebel 1994). In DNA–DNA hybridization, or DNA–DNA reassociation technique, the percent DNA-DNA hybridization value and the decrease in thermal stability of the hybrid are used to delineate the species (Stackebrandt and Liesack 1993). The experimentally induced mis-pairing and data, which are obtained with short oligonucleotides for extrapolation of the entire genome could be still debatable. To overcome the experimental errors and inaccurate reproducibility another expensive method developed is DNA microarray, which involves the hybridization of fragmented DNA instead of the whole genome (Yoo *et al.,* 2010).

DNA based typing methods such as Restriction Fragment Length Polymorphism (RFLP), Amplified Ribosomal DNA Restriction Analysis (ARDRA), Plasmid profiling, Pulse Field Gel Electrophoresis (PFGE), Ribotyping and

Randomly Amplified Polymorphic DNA (RAPD) have been used for the bacterial taxonomic studies due to their reproducibility, simplicity, high discriminatory power to avoid strain duplications. Genotypic method based on gene, or DNA sequence analysis revolutionized the field of bacterial systematics. Sequences of the stable part of the genetic code throw light about the phylogenetic relationship of bacteria, or any other life forms. These stable genetic regions in bacteria include the genes that code for 5S, 16S, 23S rRNA and spaces between these genes. Currently, the most commonly used DNA, or house keeping genes for the taxonomical purpose in bacteria is 16SrRNA gene on the basis of its universal distribution, highly conserved nature, fundamental role of ribosome in protein synthesis, absence of horizontal transfer and evolution rate represent an appropriate level of variation between organisms .The universal primers are designed for the amplification of 16SrRNA, which are from the universal regions while variable region are used for comparative taxonomy (Figueiredo *et al.,* 2010).

Numerous PGPRs are being isolated, identified and characterized using polyphasic approach such as PGPR of *Bacillus* genus isolated from apple rhizosphere identified and divided into four groups of *B. subtilis, B. pumilis, B. megaterium and B. amyloliquefaciens* based on the morphological, biochemical, 16S rDNA partial sequencing and repetitive element sequence based (rep) PCR (Kumar *et al*., 2004), Salt tolerant and plant growth promoting *Alcaligenes, Bacillus* and *Ochrobater* isolated from the coastal saline soil of rice rhizosphere based on the morphological, biochemical, physiological, FAME analysis and 16S rDNA (Bal *et al.,* 2012), Nitrogen fixing and plant growth promoting six *Burkholderia* sp. isolated from the rice rhizosphere were identified based on the morphological, biochemical, physiological and 16S rDNA (Roy *et al.,* 2013).

2.9 Formulation of Microbial Inoculants

To facilitate the introduction of high cell numbers and increased survival of the microorganisms in soil, suitable carrier based preparation of microbial inoculants with right formulation is a prerequisite (Bashan 1998). The viability of such preparations under storage for some time is also important for the commercialization of this microbe-based technology. Several carrier materials used for bioformulation include talc, peat, lignite, charcoal, vermiculite, wheat bran, press mud, calcium

alginate, soil, farmyard manure, fly ash, burnt rice husk, coir dust and polymers (Hegde and Brahmaprakash, 1992; Saha *et al*., 2001; Gaind and Gaur, 2004; Trivedi *et al*., 2005a); Negi *et al*., (2005) mixed the bacterial suspension with talc powder containing 1% carboxymethyl cellulose to prepare the formulations of biocontrol PGPR. Formulations based on the polymers encapsulate containing the living cells protect the microorganisms against many environmental stresses, and release them into soil gradually but in large quantities during the degradation of polymers by soil microorganisms at the time of seed germination and seedling emergence (Trivedi and Pandey 2007a, 2008a, b). The use of each type of inoculant depends upon market availability, choice of farmers, cost, and the need of a particular crop under specific environmental conditions.

The optimization of growth conditions of the selected PGPR strains is worked on before the formulation, development of vehicles and appropriate technology for the application (Date 2001). The fermenter biomass was mixed with different carrier materials (talc/ peat/ kaolinite/lignite/vermiculite) and stickers (Vidhyasekaran and Muthamilan 1995). Formulation in dry powdered form is important for seed treatment and soil application. The survival of the PGPR in a dried formulation and the effectiveness of methyl cellulose in a powder formulation for coating sugar beet seed has been reported (Suslow 1980). Krishnamurthy and Gnanamanickam (1998) developed talc-based formulation of *P. fluorescens* for the management of rice blast caused by *Pyricularia grisea*, in which methyl cellulose and talc was mixed at 1: 4 ratio and blended with equal volume of bacterial suspension at a concentration of 10^{10} cfu/ml. Nandakumar *et al*., (2001) have also developed talc-based strain mixture formulation of fluorescent pseudomonads. The talc-based strain mixtures were effective against rice sheath blight and increased plant yield under field conditions than the application of individual strains. Storage of *P. fluorescens* isolate Pf1 survived up to 240 days in talc-based formulation. The initial population of Pf1 in talc-based formulation was 37.5 x 10^7 cfu/g and declined to 1.3 x 10^7 cfu/g after eight months of storage (Vidhyasekaran and Muthamilan 1995).

Chapter 3

MATERIALS AND METHODS

3.1 Collection of soil samples and isolation of native strains of the PGPR from Western *Ghats* regions of Kerala

3.1.1 Collection of soil samples

The soil samples were collected from the rhizosphere of plants grown in the Silent Valley region of Western *Ghats* [GPS coordinates for the sample site as 74º 52′ E, 8º 18′ N], located in the Nilgiri Hills, Palakkad district, Kerala. The soil samples were place in the polythene bags and brought to the laboratory and stored in cold room for further use.

3.1.2 Isolation of native strains of PGPR

One gram of thoroughly mixed soil sample was added to 9.0 ml of sterile saline in a 100 ml conical flask, which was placed on rotary shaker at 150 rpm for 30min. the resulting suspension was decimally diluted (100 ~ 10^{-6}) with sterilized saline. One ml of 10^{-5} and 10^{-6} dilution was plated separately on nutrient agar (NA) medium to isolate rhizobacteria (Aneja 2002). The plates were incubated at room temperature (28±2°C) for 48 h and colonies showing morphological difference were collected for further analysis.

3.2 Screening for Plant Growth Promoting Attributes

3.2.1 Phosphate solubilization

3.2.1.1 Qualitative screening

Qualitative screening to evaluate phosphate solubilization by the bacterial isolates was done on Pikovskaya agar (Appendix I) with tricalcium phosphate as the inorganic form of phosphate (Pikovskaya 1948). A loop full of each culture was placed on the agar plates and incubated at 28°C for five days. A clear zone around the colonies was scored as phosphate solubilization zone. The solubilization zone

was determined by subtracting the diameter of bacterial colony from the diameter of total zone.

3.2.1.2 Quantitative estimation

Quantitative estimation of inorganic phosphate solubilization was done by growing the bacterial isolates in National Botanical Research Institute's phosphate liquid growth medium (Appendix I) (Nautiyal 1999), containing 0.5% tricalcium phosphate (TCP). The flasks containing 50 ml medium inoculated with 1.0 ml bacterial culture (inoculum adjusted ~ 1 $\times 10^8$ CFU/ml) in triplicates were incubated at 28 °C at 200 rpm for five days. The uninoculated autoclaved medium, incubated under a similar set of conditions as the inoculated cultures, was employed as control. The cultures were harvested by centrifugation at 10,000 x g for 10 min.

The phosphorus in the culture supernatant was estimated by ammonium-phospho-molybdate-blue color method (Fiske and Subbarow 1925). To 0.1 ml aliquot of the supernatant, 500 µl of acid molybdate and 200 µl of 1, 2, 4-Aminonaphthol sulphonic acid reagent (Appendix II) was added and the volume was made to 5.0 ml with distilled water. The absorbance of the resultant color was read after 10 min at 660nm in UV/Visible spectrophotometer. The total soluble phosphorus was calculated from the regression equation of standard curve. The values of soluble phosphate liberated were expressed as µg ml^{-1} over control. The pH of culture supernatants was measured using a pH meter. For standard curve, a stock solution was prepared by dissolving 0.351 g KH_2PO_4 in 400 ml distilled water and 10 ml of 10N H_2SO_4 was added to it, then the volume was made up to one liter with distilled water. This stock solution was diluted by adding 50 ml distilled water to 50 ml of the solution to strength of 40 µg P ml^{-1}. After adding 500 µl of acid molybdate and 200 µl of 1,2,4-Aminonaphthol sulphonic acid reagent to 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0ml aliquots of the diluted stock solution, the volume made to 5.0 ml with distilled water. The absorbance of the resultant blue color was read at 660 nm after 10 min. Values of concentration versus optical density were plotted and regression equation was used to calculate the liberated phosphorus.

3.2.2 Production of IAA-like auxins

3.2.2.1. Growth conditions

Fifty milliliter NB containing 1.5mg/ml DL-tryptophan was inoculated with 500 µl of 24-h old bacterial cultures and incubated on a incubator shaker at 200rpm for 48 h. Then the cultures were centrifuged at 10,000 rpm at 4 °C for 10 min. Estimation of indole-3-acetic acid (IAA)-like auxins in the supernatants was done using colorimetric assay (Gordon and Weber 1951).

3.2.2.2 Colorimetric estimation

One milliliter supernatant was mixed with 4.0 ml Salkowski reagent (Appendix II) and the absorbance of the resultant pink color was read at 30 °C after 30 min at 535 nm in UV/Visible spectrophotometer. A standard curve was prepared by dissolving 1.0 mg IAA (Sigma Aldrich) in few drops of methanol and made to 10 ml volume with distilled water. After adding 4.0 ml Salkowski reagent to 50, 100, 150, 200, 300, 400 and 500 µl aliquots of the stock solution taken in 5 ml volumetric flasks, the volume made to 5.0 ml volume with distilled water. The absorbance of the resultant pink color was read at 535 nm after 30 min at 30 °C. Values of concentration versus optical density were plotted and regression equation used to calculate the indole-derivates. The values of IAA-like auxins were expressed as µg/ml over control.

3.2.3 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity

ACC-deaminase activity of rhizobacteria was determined qualitatively by using Dworkin and Foster (DF) salt minimal medium (Appendix I) containing ACC as sole nitrogen source (Dworkin and Foster 1958; Jacobson *et al*., 1994). A 500µl of 24-h old bacterial culture grown in nutrient broth was inoculated with 10ml of DF, DF with ammonium sulphate, and DF with 3mM ACC salts minimal medium. The inoculated tubes were incubated at 28°C and 150rpm and optical density (OD) was measured after 48 h at 600 nm using UV-VIS spectrophotometer. Isolates were categorized into three groups, as the strains with higher (> 0.7), medium (0.5-0.69)

and lower (< 0.5) ACC-metabolism rate, depending upon their OD value at 600 nm for ACC substrate as compared to ammonium sulphate.

3.2.4 *In vitro* phytopathogenic antagonistic activity

The well-diffusion assay was used for *in vitro* test of antagonistic activities of the bacterial isolates (Schillinger and Lucke 1989) against 8 common phytopathogens, *viz.*, against *Aspergillus flavus* KACC 42109, *Fusarium monoliformis* NII 08141, *Penicillium expansum* NII 08137, *Fusarium oxysporum* NII 08119**,** *Oidium* sp NII1201, *Aspergillus niger* NII 08117, *Geotrichum candidum* NII 08115, and *Penicillium chrysogenum* NII 08138.

Fungal phytopathogens were cultivated on PDB at 28°C for seven days. The fungal culture of 100µl was spread over PDA plates using sterile cotton swab. Five small well of 2mm diameter (one at the center and four wells at the periphery) was cut in the plate and 0.1 ml of 1X 10^8 CFU/ml of 48 h grown bacterial culture in nutrient broth was added. The autoclaved nutrient broth without bacterial inoculation served as control. The plates in triplicates were incubated at 28 °C for seven days and antagonistic activities were evaluated by measuring (in mm) the inhibition zones between the pathogens and tested bacteria.

3.2.5 Chitinase production

Preparation of colloidal chitin was done adopting the method of Berger and Reynolds (1958) (Appendix II). Chitinase activity was screened by spot inoculation on Nutrient agar plate with colloidal chitin using the method of Roberts and Selitrennikoff (1988).

3.2.6 Nitrogen fixation

Bacterial isolates were streaked on Jensen's solid N-free medium (Appendix 1) and their growth was observed at four days post inoculation. Growth on the N-free medium was used as an indication of isolates ability to fix free nitrogen. The plates were incubated at 28°C and observations on the growth were recorded at 24 h interval for two days; growth of the colonies indicated N fixation ability.

3.2.7 Phytase activity

Production of phytases by the bacterial isolates was determined following Richardson and Hadobas (1997). The bacterial isolates spot inoculated on phytase screening medium plates (Appendix I) were incubated at 28 °C for three days. The phytase activity was determined by subtracting the diameter of bacterial colony from the diameter of total zone.

3.2.8 Hydrogen cyanide (HCN) production

The assay for hydrogen cyanide (HCN) production by the isolates was done by adopting the method of Lorck (1948). Bacterial cultures were fully streaked on Nutrient Agar (NA) plates supplemented with 4.4 g/l glycine. A Whatman filter paper No. 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed inside the lid of a plate. Plates were sealed with parafilm and incubated at 28°C for four days. Development of orange to brown color indicated HCN production. Screening was done three times with two replications each.

3.2.9 Ammonia production

The bacterial isolates were tested for the production of ammonia as described by Cappuccino and Sherman (1992). Overnight grown bacterial cultures were inoculated in 10 ml of peptone broth and incubated at 28 °C for 48 h. Development of faint yellow to dark brown color on addition of 0.5 ml Nessler's reagent to the cultures indicated the production of ammonia.

3.2.10 Production of siderophores

The rhizospheric isolates were assayed for the production of siderophores on chrome azurole S (CAS) blue agar (CAS) plates as described by Schwyn and Neilands (1987). CAS plate (Appendix I) were streaked with a loopful of test isolates and incubated at 28 °C for five days. Growth of the colonies and development of halo clearance zone of blue color were considered positive for siderophore production.

3.3 Effect of PGPR isolates on plant growth promotion

3.3.1 Root initiation and root colonization bioassay

The inoculum of selected isolates having phosphate solubilizing traits and IAA–like auxins were prepared by inoculating the sterilized broth with individual isolates in the 250 ml conical flasks containing Nutrient broth (NB). The flasks were incubated at 28±1ºC for 48 h under shaking (100 rpm) in the orbital shaking incubator at 150 rev min^{-1}. An optical density of 0.5 at 600 nm of broth containing PGPR was achieved by dilution to maintain uniform cell density (10^8-10^9 CFU ml^{-1}). The suspension of selected rhizobacteria was used for seed inoculation.

The ability to initiate root formation and colonization was detected by a simple screening method developed by (Silva *et al*., 2003). Tomato seeds were surface sterilized by dipping in 95% ethanol solution for 5 min, 0.2% $HgCl_2$ solution for 3 min and washed thoroughly with distilled water. Thoroughly washed seeds were sown on sterilized filter paper sheets placed in Petri plates. The seeds were then left immersed in a suspension of the rhizobacteria (1X 10^8 cfu/ml) containing 1% Carboxymethyl Cellulose (CMC) for 24 h, and transferred onto sterile 0.8% water-agar in tubes. The tubes were incubated in dark chamber at 28°C with six replications for each treatment. The seedlings treated with uninoculated NB containing 1% CMC served as control. The seedlings were uprooted after seven days for biometric analysis. The root length and shoot length of seedlings was measured (in cm) after seven days of experiment conducted in tube.

3.3.1.1 Root colonization studies

For root colonization analysis by the selected strains, one centimeter of root (proximal region of root) from each treatment was cut off and placed in sterilized conical flask having sterile saline. The root suspension inoculated flasks were then incubated at 28°C at 150 rpm for 30 min. 0.1ml of serially diluted samples of each treatment was taken in sterilized NA Petri plate and spread onto it. The Petri plates were incubated at 28 ±1°C for four days. Bacterial colony forming units were counted in each Petri plate by using colony counter.

The root colonization pattern by the selected strain was qualitatively studied using Scanning Electron microscopy (SEM) for the direct visualization of colonization image patterns on tomato root surface. Tissue samples from the inoculated and non-inoculated seedling roots of cowpea were thoroughly washed in water to remove the soil particles and were fixed in 2% glutaraldehyde (made up in 0.1 M cacodylate buffer) in the refrigerator (8°C) for 1.5 h. Samples were washed two times in the same buffer for 10 min, post fixed in 1% OsO_4 for 4 h, and dehydrated as follows: 30, 50, 70, 85, and 95% ethanol for 15 min; 100% ethanol, two times for 15 min each. For SEM, sputter coating and a JEOL–JSM 5600LV model operating at 20 kV were used. Rhizobacteria colonization patterns were observed by SEM

Table 3.1 Treatments details of rhizobacterial isolates with tomato seeds

Treatment Label	Treatment details
T1	NII 0902
T2	NII 0905
T3	NII 0906
T4	NII 0907
T5	NII 0909
T6	NII 0912
T7	NII 0917
T8	NII 0918
T9	NII 0928
T10	NII 0929
T11	NII 0930
T12	NII 0931
T13	NII 0934
T14	NII 0943
T15	NII 1020
T16	Uninoculated control

3.3.2. Evaluation of plant growth promotion in short duration life cycle plant Cow pea (*Vigna sinensis) var.kairali*

The ability of rhizobacterial isolates to promote the growth of cow pea was detected by a simple screening method. Cowpea seeds were surface sterilized by dipping in 70% ethanol solution for 5 min, 2% sodium hypochlorite for 2 min and washed thoroughly with distilled water. Thoroughly washed seeds were sown on sterilized filter paper sheets placed in Petri plates. Optical density was measured at 600 nm by spectrophotometer and uniform cell density containing 10^8-10^9 CFU $ml^{-1}$was achieved. For all experiments, the inocula containing 10^8-10^9 CFU ml^{-1} was used; however, fresh inoculum was prepared for each experiment. Soil was autoclaved at 121°C for 1 h for two consecutive days to kill the existing microbes in the soil.

The seeds were then left immersed in a suspension of the rhizobacteria (1X 10^8 cfu/ml) containing 1% carboxymethyl cellulose (CMC) for 24 h, and transferred onto sterile soil in green net house. The seedlings treated with uninoculated NB containing 1% carboxymethyl cellulose (CMC) served as control. The cups were placed in green net house in completely randomized design (CRD) with six replications for each treatment containing three seeds per pot. After three weeks, the emerged seedlings were examined. After 21 days of germination, the seedlings were uprooted and the data regarding root and shoot growth were recorded.

Table 3.2 Treatment details of rhizobacterial cup experiments with cowpea seeds

Treatment Label	Treatment details
T1	Uninoculated control
T2	NII 0906
T3	NII 0909
T4	NII 0912
T5	NII 0917
T6	NII 0918
T7	NII 0928
T8	NII 0930
T9	NII 0943
T10	NII 1020

The colonization potential of rhizobacterial isolates towards the roots were studied using SEM. Tissue samples from the inoculated and non-inoculated seedling roots of cowpea were thoroughly washed in water to remove soil particles and were fixed in 2% glutaraldehyde (made up in 0.1 M cacodylate buffer) at 8^0C for 1.5 h. Samples were washed two times in the same buffer for 10 min, post-fixed in 1% OsO_4 for 4 h, and dehydrated as follows: 30, 50, 70, 85, and 95% ethanol for 15 min; 100% ethanol, two times for 15 min each. For SEM, sputter coating, and a JEOL–JSM 5600LV model operating at 20 kV were used. Rhizobacteria colonization patterns were observed by SEM.

3. 3. 3 Screening of effective single rhizobacteria isolates for growth promotion on black pepper (*Piper nigrum* L.)

For the bioassays, stem cuttings (ca. 15 cm in height) of the black pepper cultivar '*Karimunda*' were obtained from the runner shoots of the plants from a healthy black pepper orchard of Kerala Agricultural College, Vellayani, Trivandrum. To study the plant growth-promoting activities of the selected bacterial isolate, a natural soil was collected from a garden field in NIIST campus. This soil was mixed with sand and farm yard manure (FYM) in the ratio 1: 1: 1 (v/v) then transferred to plastic pots with 11cm diameter. For the bioassays, the disease-free cuttings (15 cm height) were surface-sterilized with ethanol (70%) for 5 min, followed by several washings with ample amounts of sterile water. Fresh cuts were made at the ends of the cuttings to get sterile end tissues. Pepper cuttings were thoroughly washed in tap water and then in sterile distilled water. Tapered cut ends were made at the lower part of the node. The cut end part was then dipped in water (control) and bacterial suspensions (10^8 cfu /ml) for 2 h prior to transplanting to the pots. After planting, 20 ml of culture filtrate (10^8 CFU) was given to the pots planted with PGPR treated stem cuttings and for the untreated control pots sterile nutrient broth was aseptically added. The potting mix used had pH 7.0, available N 286.04 kg/ha, available P 227.27kg/ha and available K 265.67 kg/ha.

The bioassays were conducted in the green net house without temperature and humidity control. Black pepper seedlings were assessed after 60 days of plant growth in the greenhouse. Growth promotion was observed in black pepper after 60 days. The number of roots per cutting and length of the roots were measured

initially. The experiment was conducted in two batches for practical convenience with separate untreated control plants. The First batch experiment consisted of seven treatments and six replications for each treatment. The second batch experiment consisted of three treatments and six replications for each treatment. The second batch of potting mix had pH 6.48, available N 301.06 kg/ha, P 256.6 kg/ha and K 320.77 kg/ha.

Table 3.3 Treatment details of rhizobacterial pot experiments with black pepper stem cuttings (first batch)

Treatment Label	Treatment details
T1	Uninoculated control
T2	NII 0906
T3	NII 0909
T4	NII 0912
T5	NII 0917
T6	NII 0918
T7	NII 0928

Table 3.4 Treatment details of rhizobacterial pot experiments with black pepper stem cuttings (Second batch)

Treatment Label	Treatment details
T1	Uninoculated control
T2	NII 0943
T3	NII 1020

3.3.4 Pot experiment of PGPR on Cassava (*Manihot esculenta* Cratz).

The experiment was conducted to evaluate the effect of rhizobacterial isolates on cassava root and shoot growth initiation. Stakes of cassava cv. *Jaya* were thoroughly washed in tap water and then washed thoroughly with sterile distilled water. Tapered cut ends were made at the lower part of the bottom node of the stakes. The cut end part was then dipped in uninoculated NB containing 1% Carboxymethyl Cellulose (CMC) for control treatment and bacterial suspensions (10^8 cfu /ml) for 2 h prior to transplanting to the pots. Cell concentrations in the whole culture were adjusted with sterile saline to $1x10^8$ cfu / ml, based on absorbance. In the experiments conducted under green net house condition, treated stakes were planted in the pots (30 cm diameter) containing soils from the NIIST campus. There were six replicate pots per treatment with one stake per pot. After 15 days, 2^{nd} dose of 150ml of PGPR (10^8 cfu /ml) was also given. The pots were watered daily with tap water and kept in a green net house. After 30 days, stakelings were harvested for biometric measurements of root and shoots.

Table 3.5 Treatment details of rhizobacterial pot experiments with Cassava stem cuttings

Treatment Label	Treatment details
T1	Uninoculated control
T2	NII 0928
T3	NII 0943
T4	NII 1020

3.4 Selection of suitable growth medium for high cell density culture of PGPR isolates

LB, Nutrient broth, TSB, TSB with 1% glycerol were tested for selecting the growth medium supporting maximum growth of three best PGPR on black pepper growth. Fifty milliliter medium in 250 ml Erlenmeyer flasks were inoculated with

250 µl of 12 h old NB culture and incubated at 28ºC at 180 rpm for 48h. The serial dilutions 10^{-2} to 10^{-7} of culture growth were spread plated on TSA plates at 24 h intervals up to 48 h of incubation. The plates were incubated at 28ºC for 48 h to determine the viable colony forming units per ml of the medium.

3.5 Compatibility test of the inoculants

PGPR isolates selected for preparation consortium in talc formulation were analyzed for their mutual compatible nature by cross streak assay (Anandaraj *et al*., 2010) in nutrient agar medium

To test the compatibility, each isolate was streaked as a strip at one end of the plate and incubated for 24 h to form a thick growth. The other test cultures were streaked perpendicular to previously streaked grown culture. The plates were incubated for 48h and observed for the growth of the perpendicularly streaked culture.

3.6 Talc based formulations of selected isolates

A loopful of PGPR isolate was inoculated into the TSB supplemented with 1% glycerol and incubated at 150rpm and 30^{o}C for 48h. Ten gram of CMC (used as adhesive) and 15g of $CaCO_3$ were mixed with 1.0kg of talc powder in a sterile tray and autoclaved for two consecutive days of 30 min each. 400ml of 48 h grown broth culture containing 9.0×10^{10} cfu/ml were added to the sterile talc- cellulose mixture under sterile conditions in a tray and left for 12h drying to reduce the moisture content to less than 20%. One gram of bioformulation was taken for initial colony count and then packed, sealed in sterile polyproplylene bag stored at 4ºC as well as at ambient temperature for viability count for six months. At the time of application, the population of the bacteria in talc formulation was plated

Culture broths containing 9.0×10^{10} cfu/ml of NII 0928, 9.0×10^{10} of NII 0943 and 9.0×10^{10}cfu/ml of NII 1020 were used for the preparation of single culture bioformulation as well as for the preparation of PGPR consortium formulation of these three best isolates on the basis of biocompatibility test done previously. Bioformulation consisting of PGPR mixture were made by adding equal volume (v/v) of 135 ml each bacterial suspension and finally mixed with talc – cellulose

mixture (Nandakumar *et al*., 2001). Each formulation of PGPR consortium comprised three isolates.

3.7 Shelf life evaluation of the talc based single inoculant PGPR formulation

The shelf-life of the talc-based formulations stored at 4^{o}C and ambient temperature (28±2°C) for six months were studied. The serial dilutions of one gram of bioformulation were spread plated on NA plates and incubated at 28 °C for 48 h to determine the viable colony count at one month interval till six months. The number of colony forming units of bacteria was counted two days after plating and expressed as the number of CFU/g of formulation. The shelf-life of talc was determined to recognize the viability and concentration of active cells present in the mixture. The plate counts were carried out in triplicates and the final CFU/ml were the average of three readings.

3.8 Pot experiment of PGPR individual and consortia based talc formulation in black pepper

A pot experiment was conducted using the most efficient three bacterial strains in consortium as well as in single culture under green net house conditions. The soil was collected from NIIST campus and mixed with sand and farmyard manure at 1:1:1 (v/v) proportion. The black pepper stem cuttings of variety Karimunda obtained from the Kerala Agricultural University, Vellayani, Trivandrum were used in the study. PGPR strains were inoculated following both stem treatment and soil application method. The pepper cuttings were thoroughly washed in tap water and then in sterile distilled water. Tapered cut ends were made at the lower part of the node. The cut end part was then dipped in 2% bioformulation prepared in sterile distilled water for 2 h. A slurry of bioformulation was coated at lower end of cutting and planted into the pots. Bioformulation mixed with soil in the ratio of 1:1 (20g BF and 20g of soil) were then applied around each planted cuttings. Two cuttings were planted in each pot. Potting mix used in the previous experiment of tapioca root initiation consortium black pepper experiment was also used for this PGPR consortium black pepper experiment. The plastic pots (15 cm diameter) containing 5.0 kg of potting mix had pH 7.2, available nitrogen 327.26kg/ha, P 242.44 kg/h and

K 217.82 kg/ha. For NPK treatment, each pot received 1.0, 0.5 and 2.0g of N, P and K the form of urea, single superphosphate (SSP) and muriate of potash (MOP), respectively at bimonthly interval as per the package of practices for bush pepper from Kerala Agricultural University (Kerala Agricultural University. 2011, Package of practices). The absolute control stem cuttings and soil were treated with sterile talc without any bacterial inoculation. A booster dose of 20g talc-based bioformulations in mixture with equal amount of soil was applied towards each cutting after 15 days of planting as well at two months interval also.

The experiment consisted of six treatments and six replications for each treatment. The biometric measurements were taken after eight months of planting for number of roots, root length, number of leaves, leaf surface area number of nodes, dry weight of root and shoots, NPK analysis of plant material (treated as well as control), and NPK analysis of soil.

Table 3.6 Treatments details of experiment using PGPR talc formulations with black pepper stem cuttings

Treatment Label	Treatment details
T1	NPK Control
T2	Uninoculated control
T3	NII 0928
T4	NII 0943
T5	NII 1020
T6	Consortium (NII 0928+ NII 0943 + NII 1020)

3.9 Chemical analysis

Soil samples were air-dried and sieved for determining the pH and available N, P, K. Plant samples were oven-dried and powdered for estimation of total N, P and K. Estimation of total nitrogen was done by the modified Micro Kjeldahl's method,

total phosphorus by vanado-molybdo phosphoric yellow colour method and total potassium by flame photometric method (Jackson, 1973). Available phosphorus was estimated by Bray colorimetric method (Jackson, 1973). Available N was estimated by alkaline permanganate method (Subbiah and Asija 1956) and available K was estimated by ammonium acetate method (Jackson 1973).

3.10 Characterization and identification of selected isolates

Identification using 16S rRNA gene sequencing and biochemical characterization were done for three most effective PGPR isolates, namely, NII 0928, NII 0943 and NII 1020. Genomic DNA was extracted according to the method of Sambrook and Russell (2011) with minor modifications. PGPR isolates were grown in nutrient broth and incubated at 28°C for overnight under aerobic conditions. About 1.5 ml of culture was spinned for seven minutes and supernatant was discarded. The pellet was re-suspended in 567µl of TE Buffer, 3.0 µl of 20 mg/ml proteinase-K, 30µl of 10% SDS and incubated at 37°C for 1h. To this, 100µl of 5 M NaCl and 80 µl of CTAB solution were added and left at 65°C for 10 min. Aqueous phase was transferred to a fresh microcentrifuge tube after extraction with equal volume of chloroform: isoamyl alcohol and again extraction was done with this equal volume of phenol: chloroform: isoamyl alcohol and subjected to centrifugation at 8,000 x g at 4°C for 5 min. A wash with chloroform:isoamyl alcohol was done for a clear supernatant and equal volume of chilled propanol was added, mixed gently for precipitation of DNA after overnight incubation at -20°C. The precipitate was washed with 70% ethanol and pellet was then air - dried. The pellet was then re-suspended in 100 µl TE buffer and quantified by electrophoresis on 0.8% agarose gel in tris-borate- ethylene diamine tetra acetic acid (TBE) buffer stained with ethidium bromide and visualised under ultraviolet light. The 16S rRNA gene fragment was amplified by using universal primers corresponding to positions A-8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and primer B-1492-1510r (5'-AAGGAGGTGATCCAGCCGCA-3'; *Escherichia coli* numbering system (Weisburg *et al*.,1991). 16S rRNA gene sequencing was performed using an automated ABI PRISM BigDye Terminator cycle sequencing kit (as recommended by the manufacturer). 16S rRNA gene sequences of the isolates were compared by the BLAST search with 16S rRNA sequences available from the GenBank (Altschul *et*

al., 1990). Nucleotide sequences were aligned using the Clustal-X program (Thompson *et al*., 1997). The method of Jukes and Cantor (1969) was used to calculate the evolutionary distances. Phylogenetic and molecular evolutionary analyses were conducted using the MEGA software (Kumar *et al*., 2004). The phylogenetic trees were constructed by the neighbour-joining method (Saitou and Nei 1987) using the distance matrix from the alignment and tree topologies were evaluated by performing bootstrap analysis of 1,000 datasets. Distances were calculated using the method by Kimura (1980). The sequenced data were then deposited in GenBank nucleotide sequence database under the specific accession number for each isolates.

For the phenotypic characterization of bacteria, purified bacterial strains were observed for their shape, size as well as colony morphology on NA medium. Gram reaction of the isolates was determined by using 3% potassium hydroxide test (Suslow *et al*., 1982b). Biochemical characterization were performed using a miniaturized identification Profiles Index, API 20 ZYM and API 50CHB kits (bio-Mérieux, Marcy, I'tole, France) for Gram-negatives and Gram-positive, respectively following the manufacturer's instructions.

3.11 Quantitative estimation of nitrogen by N_2 fixers

The selected isolates were incubated in 10 ml of Jensen's broth at 150 rpm at 28°C for five days and subjected to quantification using the Kjeldahl N digestion and distillation system (Kelplus system, Classic Dx [VA]). The amount of N fixed in the microbial tissues contained in the broth was determined by the method described by Kizilkaya (2009).

3.12 Detection of organic acids produced by best isolates during phosphate solubilization

The bacterial isolates were grown in 10 ml NBRIP broth supplemented with 0.5% TCP at 28°C. and 180 rpm for five days. The cultures centrifuged at 10,000 x g for 10 min were filtered through a 0.22 µm nylon filter. Detection and quantification of organic acids produced during phosphate solubilization was done on Shimadzu Prominance High Performance Liquid Chromatogram (HPLC) equipped with PDA detector SPD-M20A, SIL-20 A HT auto sampler, CBM-20A controller, LC-20AD pump, online degasser DGU-20A3, and Rezex ROA column 300 × 7.8 mm and 5 µm particle size (Phenomenex) and Rezex ROA organic acid H+ 5x7.80 mm guard column. The mobile phase consisted of 5% Acetonitrile (SRL-HPLC grade) in de-ionized water. The flow rate was adjusted to 0.6 ml/ min. The sample injection volume was 20 µl. Eluates were detected at λ 210 nm and identified by retention time and co-chromatography by spiking the sample with authentic organic compounds. The organic acids were quantified by reference to the peak areas obtained for authentic standards for acetic acid, citric acid, formic acid, lactic acid, succinic acid, propionic acid, tartaric acid, levulinic acid, gluconic acid, and oxalic acid (Sigma-Aldrich). The samples were analyzed in triplicate.

Table 3.7 Regression equations for authentic organic acid standards y is the peak area and x is the concentration; R^2 = regression co-efficient

Standard	Regression equation	R^2 value
Acetic acid	y=548980x	0.998
Citric acid	y=86194x+2E+07	0.998
Formic acid	Y=94142x	0.998
Lactic acid	y=69485x	0.999
Succinic acid	y=55140x	0.998
Propionic acid	y=87994x	0.999
Tartaric acid	y=20238x	0.994
Gluconic acid	y=51058	0.996

Table 3.8 Retention times of authentic organic acids

Component	Retention time in minutes
Acetic acid	18.58
Citric acid	9.4
Formic acid	16.1
Lactic acid	15.9
Succinic acid	15.09
Propionic acid	20.1
Tartaric acid	10.8
Gluconic acid	10.1

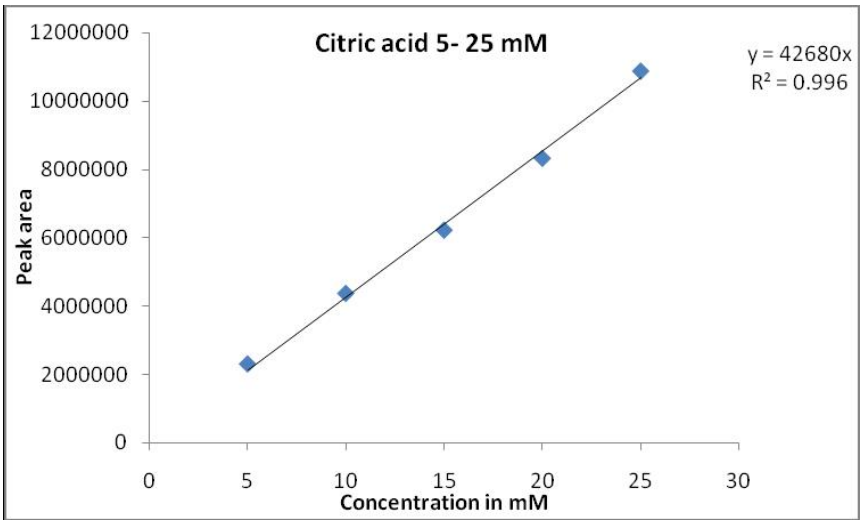


Fig. 3.1: Standard curve for citric acid

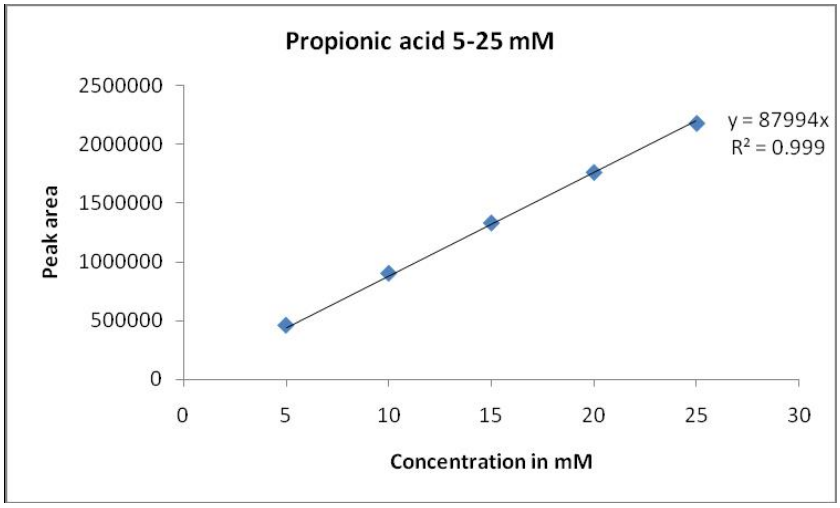


Fig. 1.2 Standard curve for propionic acid

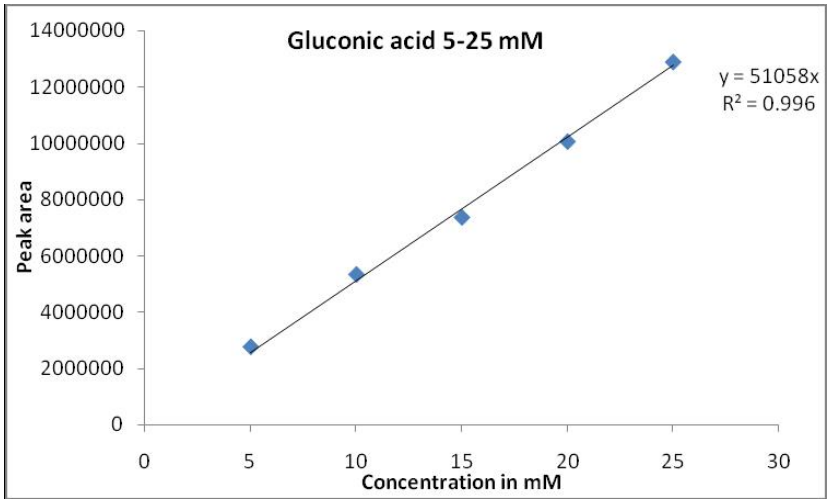


Fig. 3.3 Standard curve for gluconic acid

3.13 Experimental design and data analysis

Completely Randomized design (CRD) was adopted for conducting the plant based net house experiments. The data were subjected to the analysis of variance (ANOVA) using the GenStat Discovery edition 4. All values are means of six replicates and experiments repeated thrice unless stated otherwise. The differences among treatment means were determined using the Least Significant Difference (LSD) and Duncan's Multiple Range Test (DMRT) comparison method (if applicable) at 5% level of significance.

Chapter 4

RESULTS

4.1 Isolation of phosphate-solubilizing rhizospheric bacteria

A total of 200 colonies were isolated and were purified from the rhizospheric soil samples by plating serial dilutions on nutrient agar (NA). Representative colonies on the NA plates were picked up, purified by repeated sub-culturing and preserved in 20% glycerol at -80°C. All the isolates were assigned the code numbers. Bacterial isolates selected on the basis of distinct colony size, shape, color and elevation of colonies. These were subjected for the screening of various PGPR attributes.

4.2 Screening for Plant Growth Promoting Attributes

4.2.1 Solubilization of inorganic phosphates

Potential isolates were screened and selected on the basis of halo zone produced in Pikovskaya agar. Eighty out of 200 isolates showed the development of phosphate solubilization zones, ranging from 3 to 24 mm on modified PVK agar (Fig. 4.1). Forty seven isolates, which showed larger than 5 mm solubilization zone were selected for further quantification of phosphate solubilization studies.

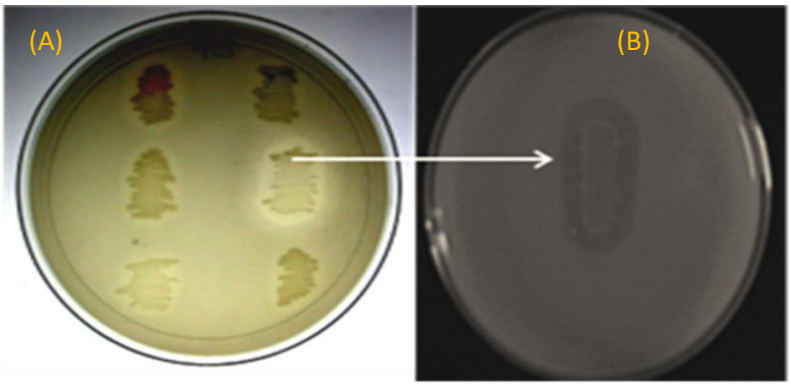


Fig. 4.1 (A) Zone of tricalcium phosphate solubilization by rhizospheric isolates on Pikovskaya agar at 28 °C after 48hrs incubation, (B) NII 0943 after 48hrs incubation

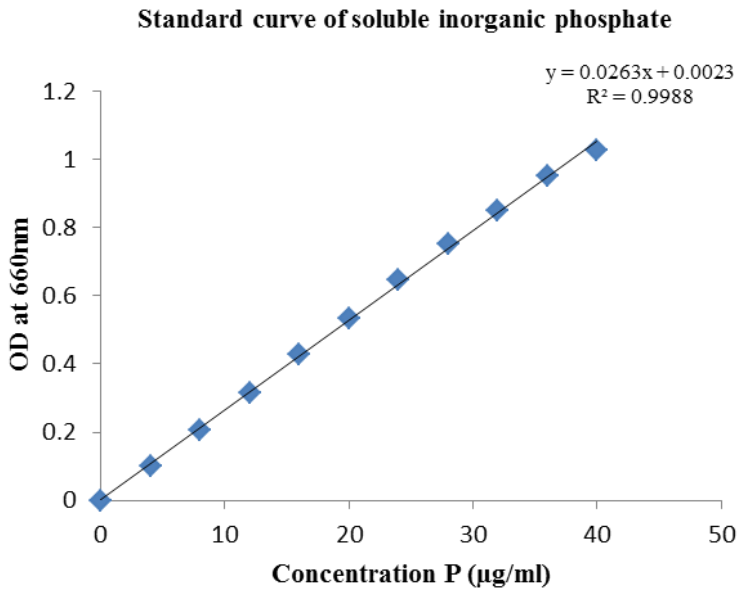


Fig. 4.2 Standard curve for soluble phosphate

Maximum TCP (Tricalcium phosphate) solubilization in liquid medium was observed in NII 1020 (529.07µg/ml), followed by NII 0906 (519.77 µg/ml), NII 0928 (518.35) and NII 0943 (518.20µg/ml) (Table 3.2). Isolate NII 0916 showed the least solubilization of 117.02 µg/ml (Table 4.1)

The pH of the medium also showed a decrease from 7.0 in majority of the isolates. In case of isolates NII 1020, NII 0906, NII 0928 and NII 0943, the pH fell

from initial 7.0 to a minimum of 3.5, 4.78, 4.82, 5.05 and 4.78, respectively on the day of maximum solubilization (Table 4.1).

Table 4.1 Amount of phosphorus solubilized (µg/mL) on solid and liquid medium and pH change by PSB isolates from Silent Valley region of Western *Ghats*.

Isolate	Solubilization zone (mm)	P- Solubilization (µg/ml)	pH change from neutral
NII 0901	10	218.60 ± 2.1	5.40
NII 0902	14	390.91 ± 1.2	3.94
NII 0903	13	220.07 ± 2.5	4.91
NII 0904	12	235.61 ± 2.6	5.32
NII 0905	18	415.43 ± 0.8	4.46
NII 0906	24**	519.77 ± 2.6	4.78
NII 0907	12	364.85 ± 2.3	5.08
NII 0908	5*	122.37 ± 2.2	5.79
NII 0909	22	515.82 ± 2.0	4.06
NII 0910	11	233.46 ± 3.9	4.08
NII 0911	5	125.54 ± 2.5	6.48
NII 0912	13	370.30 ± 1.8	6.52*
NII 0913	5*	126.28 ± 2.1	5.91
NII 0914	6	194.76 ± 1.7	5.37
NII 0915	8	215.86 ± 2.6	5.13
NII 0916	5*	117.02* ± 1.5	6.29
NII 0917	9	326.90 ± 1.1	5.63
NII 0918	11	319.83 ± 1.6	4.64
NII 0919	12	239.81 ± 1.1	4.61
NII 0920	14	244.74 ± 3.6	5.37
NII 0921	8	216.99± 3.8	4.82
NII 0922	6	130.20 ± 2.2	6.48
NII 0923	12	228.40 ± 0.9	4.70
NII 0924	12	238.03 ± 2.4	4.91
NII 0925	6	140.66 ± 1.9	6.42
NII 0926	12	248.33 ± 2.8	3.95

NII 0927	8	164.22 ± 1.1	6.10
NII 0928	24**	518.35 ± 1.6	4.82
NII 0929	22	506.57 ± 4.9	4.95
NII 0930	24**	517.54 ± 3.3	5.05
NII 0931	19	487.11 ± 3.5	4.81
NII 0932	12	243.76 ± 3.9	4.92
NII 0933	5*	124.86 ± 2.1	5.80
NII 0934	10	341.44 ± 3.1	4.08
NII 0935	12	242.02 ± 2.2	4.43
NII 0936	6	149.00 ± 3.9	5.74
NII 0937	13	240.08 ± 2.8	4.78
NII 0938	8	182.09 ± 2.2	5.74
NII 0939	14	248.64 ± 1.0	5.60
NII 0940	8	187.19 ± 2.3	6.20
NII 0941	13	231.91 ± 2.8	4.42
NII 0942	7	198.70 ± 1.8	5.73
NII 0943	22	518.20 ± 3.2	4.78
NII 0944	11	216.86 ± 2.0	4.94
NII 0945	10	216.92 ± 4.3	5.84
NII 0946	11	211.9 ± 1.8	5.91
NII 1020	21	529.07** ± 3.0	3.50**

Values are the means of six replicates with ± SD (Means ± SD, n=6).

**= Highest value; *= Lowest value and in case of pH it is maximum decrease and minimum decrease respectively.

4.2.2 Production of IAA-like auxins

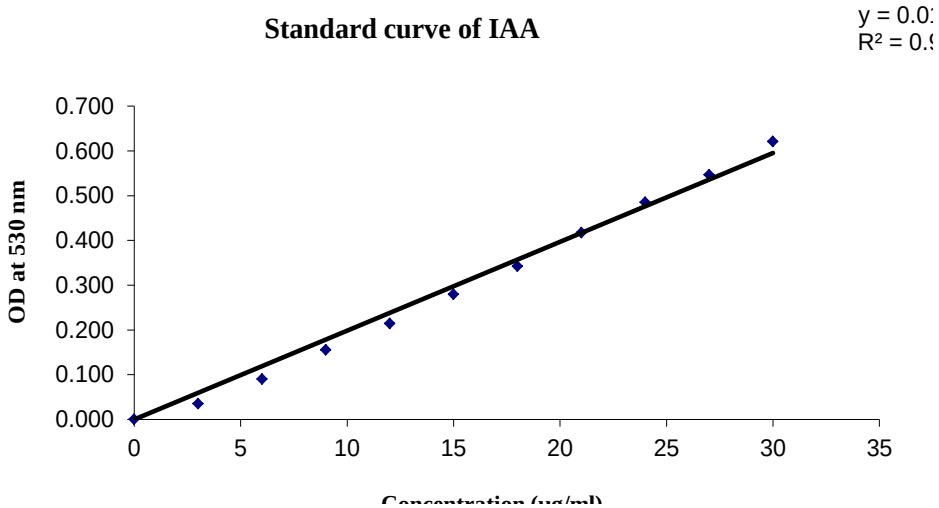


Fig. 4.3. Standard curve of IAA

All the 15 phosphate-solubilizing rhizobacterial isolates showed the production of IAA-like auxins ranging from 16.8-129.1 µg ml^{-1} in tryptophan-supplemented medium (Table 4.2). All the rhizobacterial isolates varied quantitatively in the production indole-derivatives. The highest production was exhibited by NII 0917 (129.1µg/ml), followed by isolates NII 0943, NII 0909, NII 0932, NII 0901, and NII 0916, which showed 127.6,109.0, 104.9, 103.4 and 103.1 µg/ml, respectively. Isolates NII 0944 and NII 0937 showed the least production of 18.8 and 16.82 µg/ml, respectively (Table 4.2).

Table 4.2 Production of IAA-like auxins by phosphate solubilizing PGPR isolates in tryptophan supplemented medium after 48 h of incubation at 28±0.1°C

Isolate	IAA conc. (µg/ml)
NII 0902	20.48 ± 1.4
NII 0905	24.87 ± 0.2
NII 0906	38.03 ± 1.6
NII 0907	22.24 ± 2.9
NII 0909	105.10 ± 2.5
NII 0912	31.97 ± 2.5
NII 0917	129.09** ± 3.3
NII 0918	22.24 ± 0.1
NII 0928	50.90 ± 1.0
NII 0929	57.72 ± 5.3
NII 0930	20.37 ± 1.9
NII 0931	40.82 ± 1.7
NII 0934	27.21 ± 1.9
NII 0943	127.55 ± 1.2
NII 1020	42.51 ± 2.7

Values are the means of three replicates with ± SD (Means ± SD, n=3).

**= Highest value, *= Lowest value

4.2.3 ACC-deaminase activity

The presence of ACC deaminase enzyme in rhizobacterial isolates were analysed qualitatively on the basis of bacterial growth in the presence of ACC as sole source of nitrogen. On the basis of growth, measured in terms of OD_{600}, the 12 strains out of 15 were divided into three groups (Table 4.3) consisting of high (OD_{600}>0.7), medium (OD_{600}:0.5- 0.69) and low (OD_{600} <0.5). Rhizobacterial strains NII 0906, NII 0934 and NII 0943 were the most efficient strains in utilizing ACC while NII 0902, 0909, NII0912,0917, 0928, 0929, 0931and 1020 were placed in medium group. The rest of all the isolates were low utilizers of ACC.

Table 4.3 Growth of rhizobacterial isolates (measured as OD) in minimal media with ACC as sole nitrogen source.

Isolates	Grouping of isolates based on growth OD using ACC as sole nitrogen source		
	Group H (OD>0.75)	Group M (OD=0.75-0.5)	Group L (OD<0.5)
NII 0902		√ (0.546)	
NII 0905			√(0.510)
NII 0906	√(0.757)		
NII 0907			√ (0.084)
NII 0909		√(0.661)	
NII 0912		√(0.672)	
NII 0917		√(0.527)	
NII 0918			√(0.506)
NII 0928		√(0.655)	
NII 0929		√(0.531)	
NII 0930			√(0.080)
NII 0931		√(0.751)	
NII 0934	√(0.763)		
NII 0943	√(0.782)		
NII 1020		√(0.637)	

OD values are the means of three replicates

4.2.4 *In vitro* antagonistic activity

The antagonistic activity was only observed for NII 0906 among the 15 strains tested against *Aspergillus flavus* KACC 42109, *Fusarium monoliformis* NII 08141, *Penicillium expansum* NII 08137, *Fusarium oxysporum* NII 08119**,** *Oidium* sp NII1201, *Aspergillus niger* NII 08117, *Geotrichum candidum* NII 08115, and *Penicillium chrysogenum* NII 08138.The zone of growth inhibition varied to different test fungal isolates ranging from 8 – 22mm (Table 4.4; Fig 4.9).

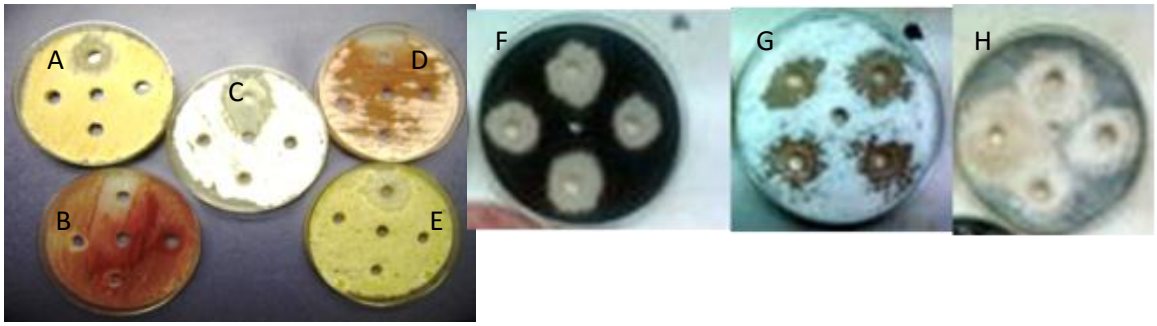


Fig. 4.4 Growth inhibition of test fungi by NII 0906 culture supernatant by agar well diffusion method (A) *A. flavus* (B) *F. monoliformis* (C) *P. expansum* (D) *F. oxysporum* (E) *Oidium* sp (F*) A. niger* (G) *G.candidum* (H) *P. chrysogenum*

Table 4.4: The zone of inhibition of NII 0906 for selected phytopathogenic fungi

Test Fungi	Zone of inhibition (mm)
Aspergillus flavus KACC 42109	8 ± 0.3
Fusarium monoliformis NII 08141	17 ± 0.6
Penicillium expansum NII 08137	22 ± 0.3
Fusarium oxysporum NII 08119	10 ± 0.5
Oidium sp. NII1201	20 ± 0.2
Aspergillus niger NII 08117	18 ± 0.3
Geotrichum candidum NII 08115	22 ± 0.5
Penicillium chrysogenum NII 08138	24 ± 0.2

Values are the means of triplicate readings (mean ± SD, n=3)

4.2.5 Chitinase activity:

Chitinase activity was exhibited by one isolate out of the 15 isolates screened with halo zone of chitin degradation around the colonies (Fig.4.5 and Table 4.5). Only isolate NII 0928 showed chitinase activity in plates out of the 15 rhizospheric isolates screened.

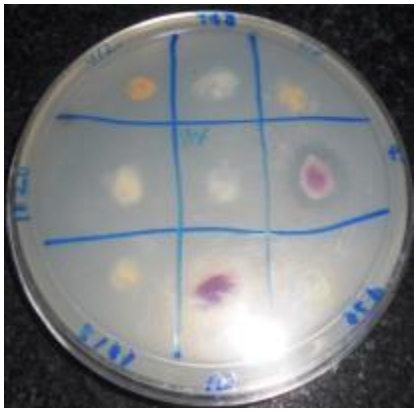


Fig. 4.5 Zone of chitin solubilization on colloidal chitin agar plates by NII 0928

4.2.6 Isolation of nitrogen fixing bacteria

A total of seven colonies showed positive growth out of the 15 isolates streaked on Jensen's N free agar plates (Jensen, 1942). NII 0902, 0905, 0906, 0907, 0909, 0928, 0938 and 1020 showed positive test for nitrogen fixation ability (Fig. 4.6 and Table 4.5).

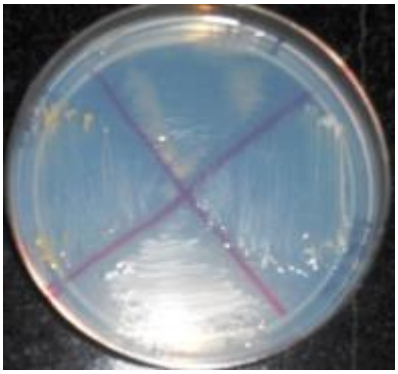


Fig. 4.6 Growth of PGPR isolates on Jensen's N free medium

4.2.7 Phytase activity:

Phytase activity was exhibited by five isolates out of the 15 isolates screened with zone size ranging from 1.2-8.4 mm on agar medium supplemented with calcium-phytate (Fig.4.7 and Table 4.5). The highest phytate solubilization zone size was shown by NII 0906 (8.4mm), followed by NII 0928 (7.1mm) and NII 1020 (6.8mm).

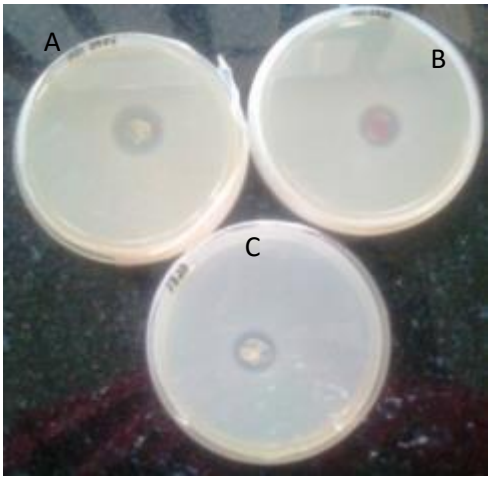


Fig. 4.7 Zone of phytate solubilization around the colonies of (A) NII 0906 (B) NII 0928 (C) NII 1020

4.2.8 Hydrogen cyanide production

Among the 15 isolates, 12 isolates were positive for hydrogen cyanide production. All these 12 rhizobacterial strains were moderate hydrogen cyanide producers due to the orange color change of filter paper. (Fig. 4.8 and Table 4.5). The absence of colour change in filter paper discs indicated the lack of HCN production by other strains.

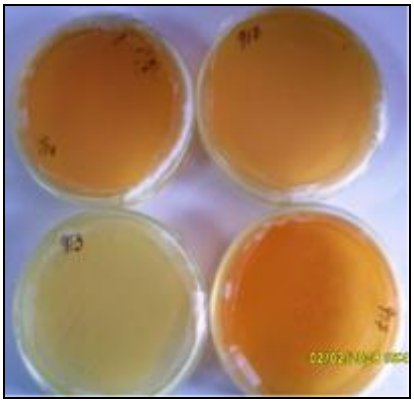


Fig. 4.8 HCN production by rhizobacterial isolates indicated as colour change of filter paper to orange; no colour change in control plate

4.2.9 Ammonia production:

Ammonia production was shown by all the isolates with variation in the intensity of yellow-brown colour on the addition of Nessler's reagent to 48 h old cultures grown in peptone broth (Table 4.5). NII 0906, NII 0928, and NII 0930 produced dark brown colour indicating strong ammonia production while other isolates showed moderate and weak ammonia production by change of the colour to dark yellow and light yellow, respectively (Fig. 4.9).

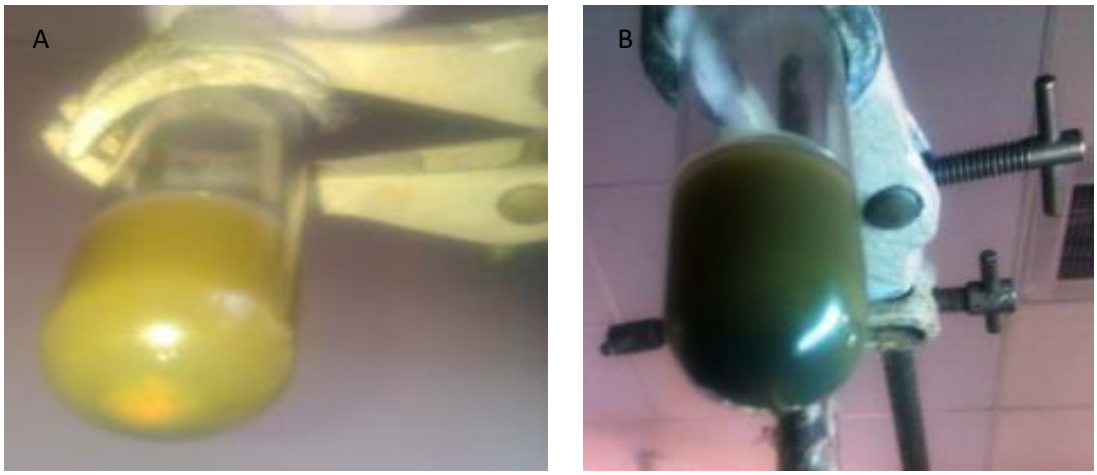


Fig. 4.9 Ammonia production by rhizospheric isolates (a) orange colour indicates medium ammonia producer (b) brown color indicates strong ammonia producer

4.2.10 Siderophore production

Production of siderophores by the rhizobacterial isolates was analysed by spot inoculation on Chrome Azurole S (CAS) agar medium. Of the 15 isolates, 11 were able to grow and showed a blue colour clearing halo zone around their respective colonies after five days of incubation at 28°C (Fig.4.10; Table 4.5)

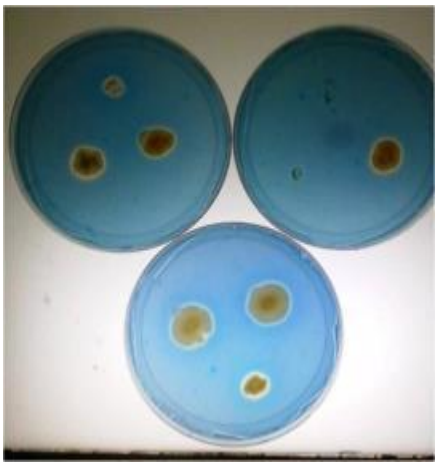


Fig. 4.10 Growth of PGPR isolates on CAS blue agar medium and halo zone around the colonies indicating the production of siderophores

Table 4.5 Characterization of rhizospheric isolates for phytase activity, chitinase activity, ammonia production, cyanide production, siderophore, N- fixation by rhizobacterial isolates.

Rhizospheric isolates	Phytase	Chitinase	Siderophore	Ammonia	HCN	N fixation
NII 0902	-	-	-	++	-	+
NII 0905	-	-	+	+	-	-
NII 0906	+	-	+	+++	++	+
NII 0907	-	-	-	+	-	+
NII 0909	+	-	+	++	++	+
NII 0912	-	-	+	++	-	-
NII 0917	-	-	-	+	++	-
NII 0918	-	-	+	+	++	-
NII 0928	+	+	+	+++	++	+
NII 0929	-	-	-	+	-	+
NII 0930	-	-	+	+++	++	-
NII 0931	-	-	+	+	++	-
NII 0934	-	-	-	+	-	-
NII 0943	+	-	+	++	++	-
NII 1020	+	-	+	++	++	+

+ = weak activity, ++ = moderate activity, +++ = strong activity, - = negative activity, values are the mean of three replicates,

4.3 Root initiation and root colonization bioassay on tomato (*Lycopersicon esculentum*)

4.3.1 Growth parameters

Significant difference was observed for the growth parameters among 14 rhizobacterial treatments and uninoculated control treatments in tomato seeds (Table 4.6 and Fig. 4.11). The root length was significantly higher with 14 treatments over uninoculated control treatments. The strains induced an increase in root length ranging from 15.9 -278% over uninoculated controls (Fig. 4.11a). The root length was significantly higher over control with 14 rhizobacterial isolates (Table 4.6). The root length was statistically at par with the control on treatment with NII 0905. The highest root length was obtained with NII 1020 (6.2cm), followed by NII 0906 (5.83cm), NII 0928 (5.5 cm), NII 0943 (5.47 cm), NII 0912 (4.94 cm) and NII 0909 (4.83 cm). The rhizobacterial colonization was visually observed as a milky turbid zone around the roots of inoculated tomato seedlings and this region was absent in uninoculated control seedlings.

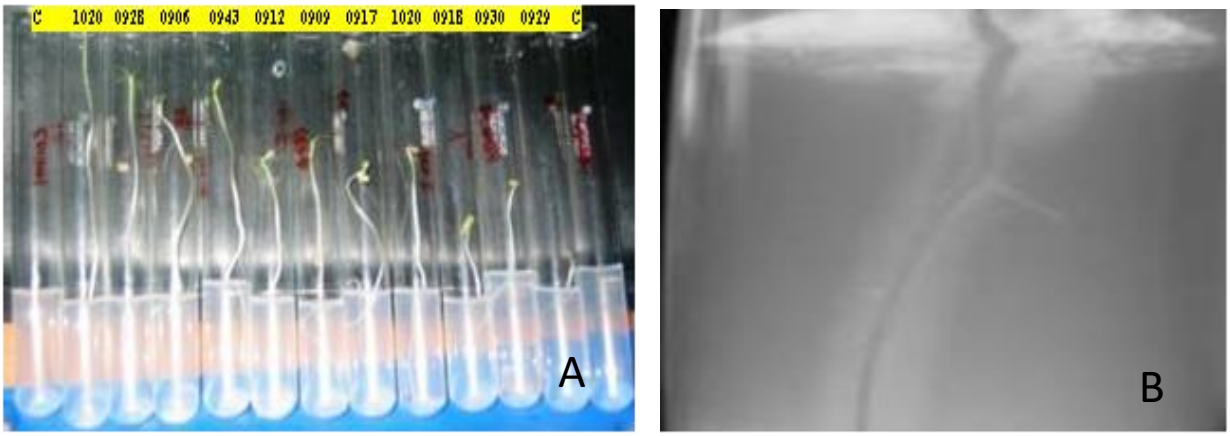


Fig. 4.11 A) Effect of rhizobacterial isolates on root initiation of tomato seeds. B) Root colonization over root surface of inoculated plants seen as turbid zones around rhizospheric region

Inoculation with rhizobacteria increased the shoot length of tomato seedlings with 14 rhizobacterial strains from 51% to 428.6% over uninoculated control (Table 4.6). The shoot length was statistically at par with control on treatment with NII 0905. The highest shoot length was obtained with NII 1020 (8.3cm), followed by NII 0928 (7.63cm), NII 0906 (6.97 cm), NII 0943 (6.59 cm), NII 0909 (6.43 cm) and NII 0912 (6.32 cm).

Table 4.6 Effect of rhizospheric isolates on root initiation of tomato seeds

Isolates (Treatments)	Root length (cm)	Shoot length (cm)
902	3.31bc	3.53cd
905	1.83a	1.58a
906	5.83ef	6.97fg
907	2.88b	3.52cd
909	4.83d	6.43f
912	4.94de	6.32f
917	3.83c	4.74e
918	3.63bc	4.21de
928	5.50def	7.63gh
929	3.82c	4.06cde
930	3.80c	4.33de
931	3.03bc	3.31c
934	2.94bc	2.37b
943	5.47def	6.59f
1020	6.20f	8.30h
c	1.64a	1.57a
LSD	0.82	0.75

Mean values sharing similar letter (s) in a column are non-significant at $P < 0.05$, according to Duncan's multiple range test

4.3.2. Root colonization assay

Root colonization abilities of the rhizobacteria were measured by quantification of colony forming units (cfu) per cm of root length when plated on agar media as well as through the microscopic visualization of the adherence of the inoculated PGPR. The highest root colonizing ability was found in rhizobacterial isolate NII 1020 that had 9.1×10^6 cfu / cm of root length, followed by NII 0906 that produced 8.4×10^6 cfu / cm of root length. The rest of the rhizobacterial isolates NII 0928, 0943, NII 0909, and NII 0912 produced 7.6×10^6, 7.4×10^6, 1.2×10^6 and

8.2×10^5 cfu/cm of root length in descending order (Table 4.7). As expected in control non-inoculated plants, there was no presence of bacteria in the roots.

Table 4.7 Root colonization by rhizospheric isolates on tomato roots

Treatments	cfu/cm of root
NII 0902	$3.1 \text{ X } 10^2 \pm 1.5 \text{ X } 10^1$
NII 0905	26 ± 2.3
NII 0906	$8.4 \text{ X } 10^6 \pm 0.8 \text{ X } 10^6$
NII 0907	$5.5 \text{ X } 10^3 \pm 0.4 \text{ X } 10^3$
NII 0909	$8.2 \text{ X } 10^5 \pm 1.8 \text{ X } 10^5$
NII 0912	$1.2 \text{ X } 10^6 \pm 2.7 \text{ X } 10^5$
NII 0917	$6.4 \text{ X } 10^4 \pm 1.5 \text{ X } 10^3$
NII 0918	$9.4 \text{ X } 10^3 \pm 3.4 \text{ X } 10^2$
NII 0928	$7.6 \text{ X } 10^6 \pm 1.1 \text{ X } 10^6$
NII 0929	$5.1 \text{ X } 10^4 \pm 0.7 \text{ X } 10^4$
NII 0930	$5.8 \text{ X } 10^4 \pm 2.4 \text{ X } 10^3$
NII 0931	$2.8 \text{ X } 10^2 \pm 3.1 \text{ X } 10^1$
NII 0934	$1.3 \text{ X } 10^2 \pm 0.8 \text{ X } 10^1$
NII 0943	$7.4 \text{ X } 10^6 \pm 0.04 \text{ X } 10^6$
NII 1020	$9.1 \text{ X } 10^6 \pm 1.0 \text{ X } 10^6$
control	0

Each cfu value is the average of three replicates ± standard deviation

SEM results clearly marked the direct visualization of the colonization of bacteria on the root surface of PGPR inoculated plants (Fig. 4.12B and 4.12C) while the root surface of uninoculated control plants showed a smooth and undisturbed entire plane surface (Fig 4.12A). A stable and strong PGPR- root association was found by the formation of micro colonies and consistent matrix like biofilm formation over the entire root surface.

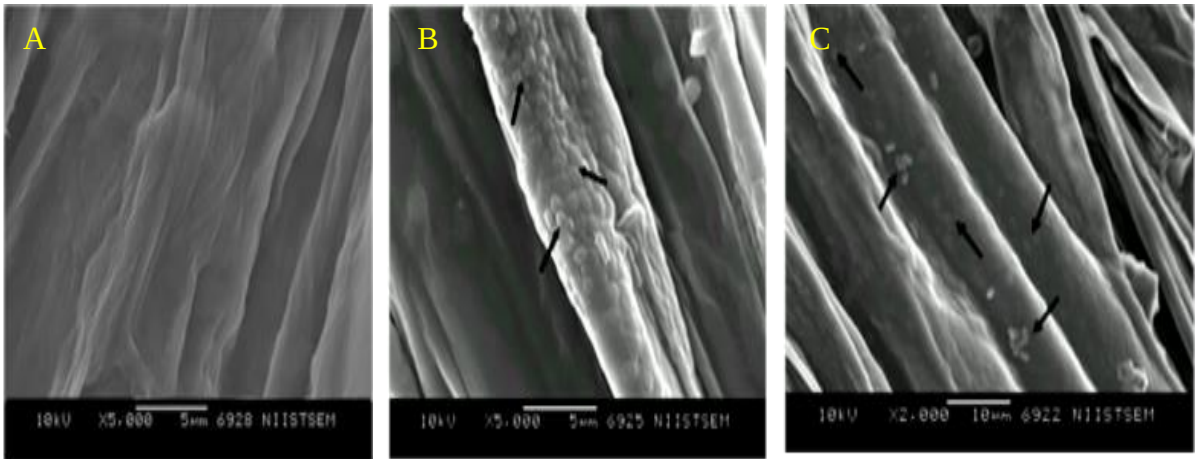


Figure 4.12 Scanning electron micrographs of tomato root segments seven days after treatment with NII 0906 A) control roots treated with sterile nutrient broth B) tomato roots treated with NII 0906 (C) NII 0906 treated roots with high magnification

4.4 Evaluation of plant growth promotion in short duration life cycle plant cowpea (*Vigna sinensis) var.kairali*

4.4.1 Growth parameters

Significant increase in the growth of cowpea was shown by all the nine best strains selected from the previous tomato based experiments with different efficacy in sterile soil conditions (Fig. 4.13 and Table 4.8). The incremental influence on root length, shoot length, number of roots and dry matter over control was to the extent of 15.7-162.7, 16.5-55.5, 24.1-116.7 and 11-90.3% in cowpea var. *Kairali*, respectively.

Fig.4.13 Effect of rhizobacterial isolates on growth of cowpea under cup conditions

A) treatment with NII 0928 B) treatment with NII 0943 C) uprooted cow pea seedlings of treatment NII 0943, NII 0928, NII 1020, NII 0906, NII 0909 and control.

Table 4.8 Effect of rhizospheric isolates on cowpea seeds after 21days of treatment in sterile soil under cup conditions

Treatments	Root numbers	Root length (cm)	Shoot length (cm)	Dry weight (mg/plant)
C	18.00a	6.57a	14.02a	0.27a
906	35.67cd	14.74de	20.83ef	0.45cde
909	32.00c	13.33d	19.45cde	0.40bcd
912	32.33c	10.33c	17.50bc	0.38bc
917	25.33b	7.60ab	18.21bcd	0.36bc
918	24.67b	8.93bc	18.37bcd	0.37abc
928	36.67d	15.72ef	20.07def	0.49de
930	22.33b	9.02bc	16.33b	0.30ab
943	39.00d	17.26f	21.80f	0.52e
1020	38.00d	16.63f	20.77ef	0.46cde
l.s.d.	3.68	1.79	1.94	0.09

Mean values sharing similar letter (s) in a column are non-significant at $P < 0.05$, according to Duncan's multiple range test

In cowpea, all the treatments showed significantly higher shoot length over the control (Table 4.8).The maximum increase of 55.5 % in shoot length was produced by the isolate NII 0943 and the minimum of 16.5 % by the isolate NII 0930 with respect to un-inoculated control (Table 4.8). The root length was significantly higher over the control with eight rhizobacterial treatments. The maximum increase of 162.7% in root length was produced by the isolate NII 0943 and the minimum of 15.7 % by the isolate NII 0917 with respect to un-inoculated control. NII 0917 showed root length statistically at par with control. All the treatments showed significantly higher number of roots over the control. The maximum increase of 116.7% in root numbers was produced by the isolate NII 0943 and the minimum of 24.1% by the isolate NII 0930 with respect to un-inoculated control. The dry weight was significantly higher over the control with seven rhizobacterial treatments

(Table 4.8). The maximum increase of 90.3% in dry weight was produced by the isolate NII 0943 and the minimum of 11% by the isolate NII 0930 with respect to un-inoculated control. NII 0918 and NII 0930 showed dry weight statistically at par with the control.

4.4.2 Root colonization of inoculants

The ability of inoculated PGPR to colonize the cowpea root surface soil was assessed after one month of inoculation using scanning electron microscope (SEM) (Fig. 4.14). The same pattern of adherence as shown in tomato roots were followed in cowpea also. SEM results clearly marked the direct visualization of the colonization of bacteria on the root surface of PGPR inoculated plants (Fig. 4.14 B) while the root surface of uninoculated control plants (Fig. 4.14 A) showed a smooth and undisturbed entire plane surface. A stable and strong PGPR-root association was found by the formation of micro colonies and consistent matrix-like biofilm formation over the entire root surface.

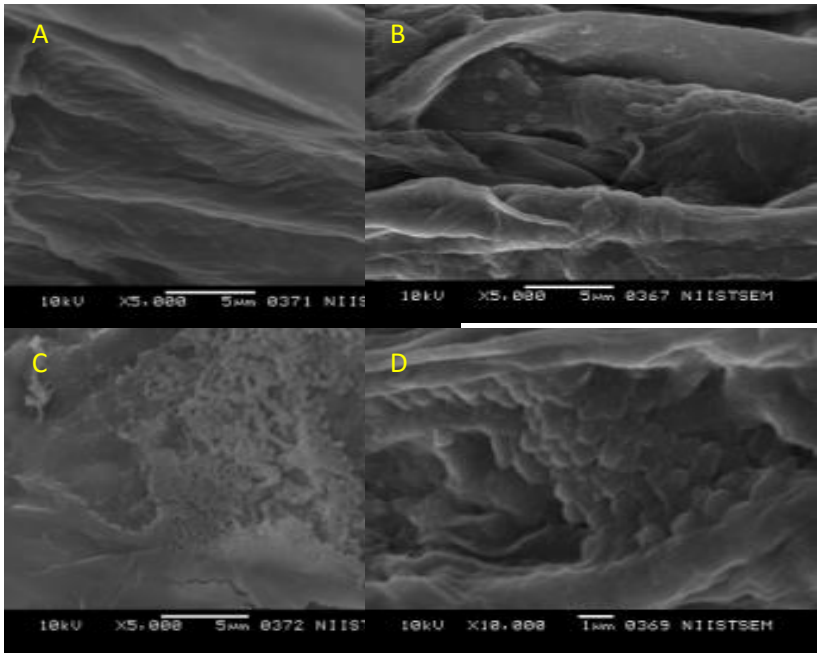


Fig. 4.14 Scanning electron micrographs of cow pea root segments 21 days after treatment A) control roots treated with sterile nutrient broth B) tomato roots treated with NII 1020 C) NII 0943 treated roots D) NII 0943 treatment with high magnification

4.5 Evaluation of plant growth promotion in Black pepper (*Piper nigrum* L.) *var. karimunda*

4.5.1 Growth parameters of First Batch of experiment

In the first batch of experiments conducted with seven isolates in black pepper var. Karimunda under green net house conditions with non-sterile soil, only two isolates, NII 0906 (Fig. 4.15)and NII 0928 showed highly significant increase in growth promotion compared to un-inoculated control (Fig. 4.16). Other treatments were more or less on par with the control (Table 4.9).

Fig. 4.15: Effect of rhizobacterial isolates on black pepper under pot conditions a) treatment with NII 0906 and uninoculated control.

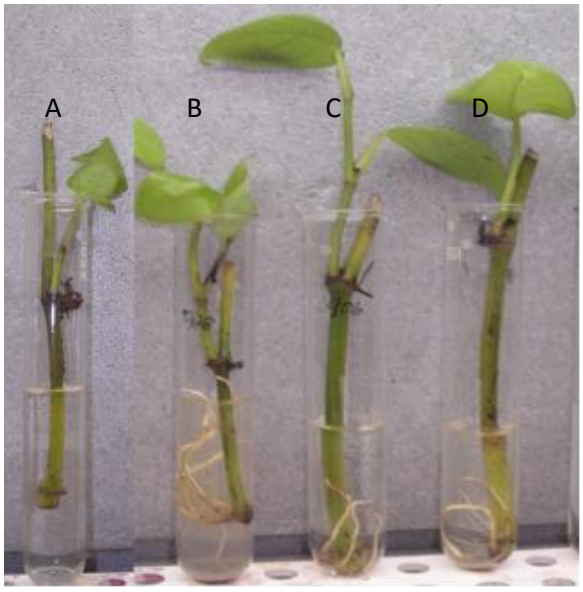


Fig. 4.16: Uprooted view of black pepper cuttings after 60 days of rhizobacterial treatments A) and D) uninoculated control B)treated with NII 0928 C)treated with NII 0906 (First Batch)

Table 4.9: Effect of rhizobacterial isolates on growth of black pepper after 60 days of planting

Treatments	Root length (cm)	Root Numbers	LAI	Total dry wt. (mg/plant)
Control	3.07a	1.67a	1.07a	1.53a
NII 906	7.70c	7.33c	8.40c	3.97bc
NII 909	5.37b	4.67b	3.37ab	3.07b
NII 0912	5.43b	5.00b	4.67b	4.10bc
NII 0917	4.70b	5.33b	2.80ab	3.00b
NII 0918	4.13ab	4.67b	4.07b	3.10b
NII 0928	9.10c	7.67c	7.60c	4.80c
lsd	1.27	1.73	2.59	1.20

Mean values sharing similar letter (s) in a column are non-significant at $P < 0.05$, according to Duncan's multiple range test

The incremental influence on root length, number of roots, LAI and dry matter over the control was to the extent of 34-196.7, 179.6-359.3, 161.7- 685.1, and 96.1-213.7% in black pepper var. Karimunda, respectively (Table 4.9). All the treatments showed significantly higher root length over the control. The maximum increase of 196.7 % in root length was produced by the isolate NII 0928 and the minimum of 34% by the isolate NII 0918 with respect to un-inoculated control. Out of the six, four treatments showed significantly higher number of roots over the control. The maximum increase of 359.3 % in root numbers was produced by the isolate NII 0928 and the minimum of 179.6% by the isolate NII 0918 and 0909 with respect to un-inoculated control.

The LAI was significantly higher over the control with four rhizobacterial treatments. The maximum increase of 685% in LAI was produced by the isolate NII 0906 and the minimum of 161.7% by the isolate NII 0917 with respect to un-inoculated control. The dry weight was significantly higher over the control with six rhizobacterial treatments. The maximum increase of 213.7% in dry weight was produced by the isolate NII 0928 and the minimum of 96.1 % by the isolate NII 0917 with respect to un-inoculated control.

4.5.1.1 Plant NPK content

The total NPK content of PGPR treatments were increased for the PGPR treatments compared to uninoculated control (Table 4.10). An increase of 147.1 - 433.7% in total N uptake occurred with PGPR treatments compared to the uninoculated control. The statistically higher total N content was shown by NII 0928, followed by NII 0906, which was at par with NII 0912 and NII 0918. An increase of 120 - 686% in total P uptake occurred with the PGPR treatments compared to uninoculated control. The statistically higher value for the total P content was again shown by NII 0928, which was followed by NII 0906, which was at par with NII 0912. The other four treatments were on par with uninoculated control. An increase of 95 - 272.3% in total K uptake occurred with the PGPR treatments compared to uninoculated control. Again, the statistically higher value for total K content was shown by NII 0928 at par with NII 0912.

Table 4.10: Effect of rhizobacterial isolates on NPK content of black pepper after 60 days of planting (First Batch)

Treatments	Total N uptake (mg/plant)	Total P uptake (mg/plant)	Total K uptake (mg/plant)
Control	0.026a	0.001a	0.022a
NII 906	0.099c	0.006b	0.060bc
NII 909	0.067b	0.002a	0.046b
NII 0912	0.086bc	0.004b	0.067cd
NII 0917	0.063b	0.002a	0.043b
NII 0918	0.078bc	0.002a	0.045b
NII 0928	0.136d	0.008c	0.082d
lsd	0.028	0.002	0.019

Mean values sharing similar letter (s) in a column are non-significant at $P < 0.05$, according to Duncan's multiple range test

4.5.2 Second batch of experiments conducted with the next set of PGPR isolates

4.5.2.1 Growth parameters

In the second batch of experiments conducted with last two isolates in black pepper var. *Karimunda* under green net house conditions with non-sterile soil, both the isolates NII 0943 and NII 1020 showed highly significant increase in all the growth parameters analysed compared to un-inoculated control (Fig. 4.17; Fig. 4.18 and Table 4.11).

Fig. 4.17 Effect of rhizobacterial isolates on black pepper under pot conditions a) treatment with NII 1020 and uninoculated control (Second batch)

Fig. 4.18 Uprooted view of black pepper cuttings after 60 days of rhizobacterial treatments A) NII 0943 B) control C) NII 1020

Table 4.11 Effect of rhizobacterial isolates on growth of black pepper after 60 days of planting (Second batch)

Treatments	Root length (cm)	Root Numbers	LAI	Total dry wt. (mg/plant)
Control	8.80a	5.00a	2.60a	2.93a
NII 0943	16.30b	17.67b	7.87b	5.85c
NII 1020	19.70b	16.33b	6.67b	4.36b
lsd	6.84	4.14	1.53	1.36

Mean values sharing similar letter (s) in a column are non-significant at $P < 0.05$, according to Duncan's multiple range test.

The incremental influence on root length, number of roots, LAI and dry matter, over control for NII 0943 and 1020 was to the extent of 85.2 -123.9, 253.4-226.6, 67.8-171.2, and 48.8- 99.7 in blackpepper var. Karimunda, respectively (Table 4.11). Both the treatments were statistically on par for root length and number of roots, but significantly higher over the control. NII 0943 was highly significant over NII 1020 for LAI and total dry weight.

4.5.2.2 Plant NPK

Both the PGPR treatments showed highly significant increase in total NPK content compared to uninoculated control (Table 4.12). The statistically higher total N content was shown by NII1020, which was at par with NII 0943while statistically higher value for total P content was shown by NII 0943 than NII 1020. The total K content was at par for both, NII 0943 and NII 1020 treatments.

Table 4.12: Effect of rhizobacterial isolates on NPK content of black pepper after 60 days of planting

Treatments	Total N uptake (mg/plant)	Total P uptake (mg/plant)	Total K uptake (mg/plant)
Control	0.064a	0.003a	0.043a
NII 0943	0.142b	0.019c	0.089b
NII 1020	0.185b	0.007b	0.106b
lsd	0.049	0.003	0.023

Mean values sharing similar letter (s) in a column are non-significant at $P < 0.05$, according to Duncan's multiple range test.

4.6 Evaluation of plant growth promotion in Cassava (*Manihot esculenta* Cratz), var *Jaya*

4.6.1 Growth parameters

The best three isolates from the previous plant experiments were selected for the evaluation of growth promotion of cassava var. Jaya under green net house conditions with non-sterile soil. All the three isolates, NII 0928, NII 0943 and NII 1020 showed highly significant increase in growth promotion compared to uninoculated control (Fig.4.19, Fig. 4.20 and Table 4.13).

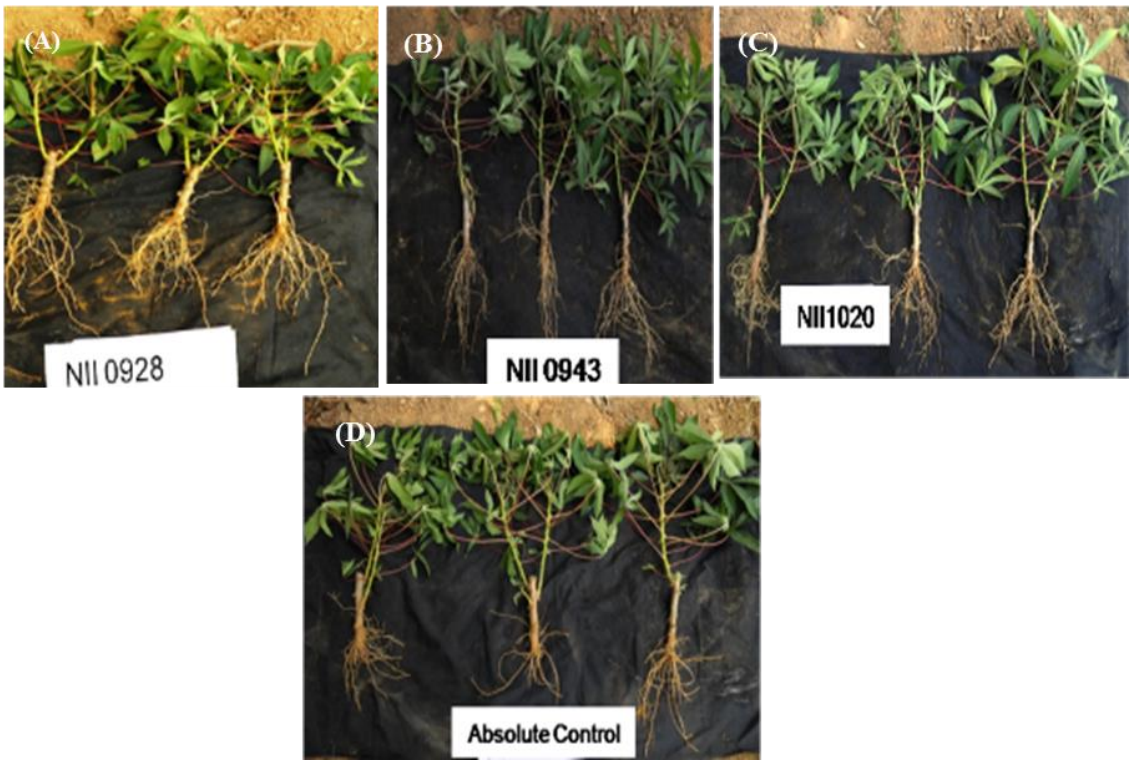


Fig. 4.19 Effect of rhizobacterial isolates on Cassava under pot conditions

(A) treatment with NII 0928 (B) treatment with NII 0943 (C) treatment with NII 1020(D) uninoculated control.

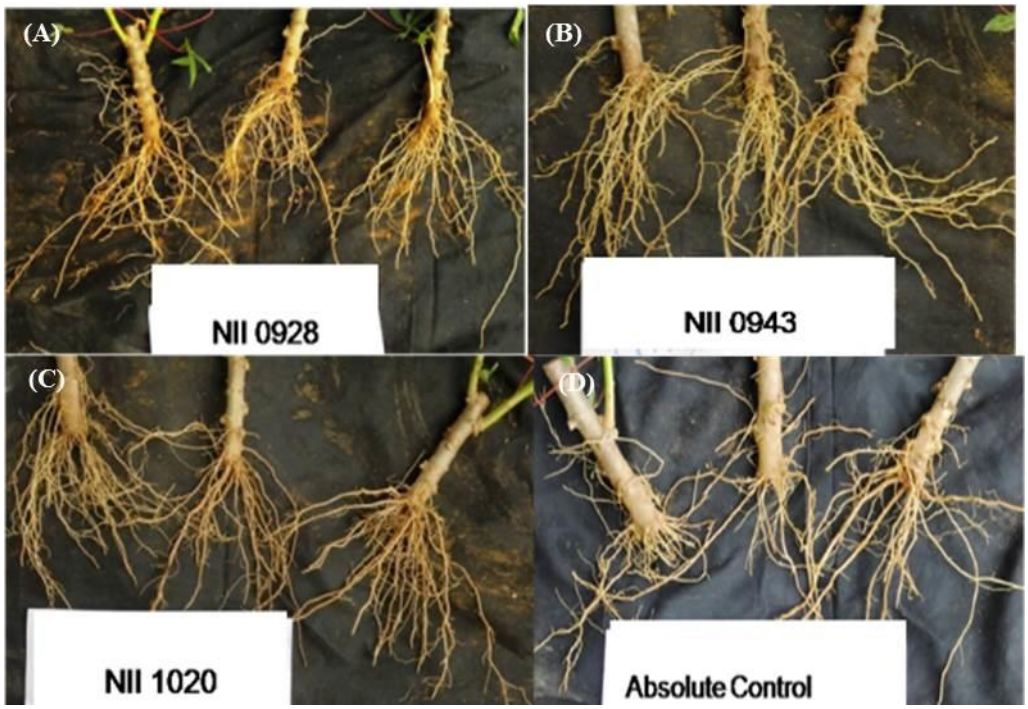


Fig. 4.20 Uprooted view of Cassava plants after 30 days of rhizobacterial treatments A) NII 0928 B) NII 0943 C) NII 1020 and D) Control

Table 4.13: Effect of rhizobacterial isolates on growth of Cassava under pot conditions

Treatments	Root length (cm)	Root number	Plant height (cm)	Dry stem weight (g/plant)	Dry root weight (g/plant)	Dry Leaf weight (g/plant)	LAI
Absolute control	15.13a	21.33a	19.53a	1.38a	0.50a	8.91a	1.17a
NII 0928	19.50bc	33.33bc	23.67b	2.68bc	0.91b	13.48bc	3.67bc
NII 0943	19.13b	30.33bc	22.63b	2.49b	1.02bc	13.10b	3.53b
NII 1020	22.90c	35.00c	26.00c	2.70bc	1.12c	14.09bc	3.91bc
lsd	3.57	6.74	1.94	0.46	0.20	1.06	0.50

Mean values sharing similar letter (s) in a column are non-significant at $P < 0.05$, according to Duncan's multiple range test

The incremental influence on root length, number of roots, plant height, LAI , dry weight of stem, leaf and root over uninoculated control was 26.4-51.4, 56.3-64.1, 21.2-33.1, 213.4-234.4, 93.6-122.7, 51.3-62.4 and 82.1-123.4% in cassava var. Jaya, respectively (Table 4.13) . All the three treatments showed significantly higher root length over the control. The maximum increase in root length was produced by the isolate NII 1020, which was statistically on par with NII 0928. The number of roots was maximum for NII 1020, which was statistically on par with NII 0928 and NII0943. Dry root weight maximum was shown by NII 1020 on par with NII 0928.

4.7 Selection of suitable growth medium for high cell density bioformulation

Three bacterial isolates, NII 0928, 0943 and 1020 were identified as potential soil inoculant for plant growth based on the bioassay studies on tomato, cowpea, black pepper and cassava. For mass production of these PGPR, three different culture media, *viz.*, nutrient broth (NB), tryptic soya broth (TSB) and tryptic soya broth supplemented with 1% glycerol (TSBG) were tested for their growth. TSBG supported maximum growth of all the three isolates after 48 h (Table 4.14; Fig.4.21). Therefore, TSBG was selected as the growth medium for bioformulation.

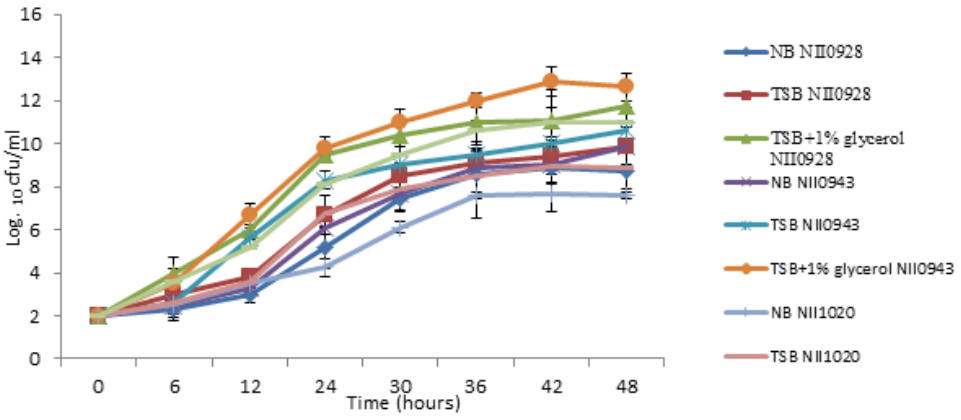


Fig. 4.21: Growth of NII 0928, NII 0943 and NII 1020 in different media at 28°C. NB = Nutrient broth, TSB = trypticase soya broth and TSBG = trypticase soya broth with 1% glycerol

Table 4.14 Growth of PGPR isolates in different media with pH 7.0 at 28 ± 0.1°C

PGPR Isolates	Biomass – gm/l/hr		
	NB	TSB	TSB+ 1% Glycerol
NII 0928	0.131	0.195	0.312
NII 0943	0.171	0.265	0.325
NII 1020	0.094	0.198	0.256

4.8 Survivability of PGPR isolates on talc based bioformulation on storage

In general, the initial high cell population were not retained throughout the six months of storage period for both 4ºC and at ambient temperature (Fig.4.22 and Table 4.15).The viability count at 4ºC exhibited a slow and gradual reduction for all the three formulations while a rapid reduction in viable population were observed after three months at ambient temperature storage.

The population of NII 0928 showed a decline from an initial count of 7.6×10^9 to 3.8×10^8 at 4ºC and 2.1×10^7 at ambient temperature while for NII0943, a drop from initial count of 8.2×10^9 to 5.6×10^8 and 8.2×10^7 at 4 ºC and ambient temperature, respectively. In the case of NII 1020, a different pattern from the other two isolates was shown by a viability loss from 4.9×10^9 to 9.7×10^7 and 6.4×10^6 at 4 ºC and ambient temperature, respectively. However, the talc bioformulation of NII 0928 and NII 0943 exhibited adequate population density while the viability of NII 1020 was found to be below the recommended viability according to the BIS standard (10^7 cfu/ g). The maximum survivability of 10^7 was shown by NII 0943 while the least survivability was exhibited by NII 1020.

Fig. 4.22 Talc based bioformulation of selected PGPR isolates (A) NII 0928), (B) NII 0943 and (C) Talc based bioformulation in sterile tray kept for shade dry

Table 4.15 Population dynamics of talc based bioformulation of PGPR isolates on storage at 4°C and at room temperature

cfu/g		NII 0928	NII 0943	NII 1020
Initial		7.6×10^9	8.2×10^9	4.9×10^9
30 days	4°C	7.4×10^9	7.9×10^9	4.1×10^9
	RT	6.9×10^9	7.3×10^9	3.2×10^9
60 days	4°C	6.3×10^9	6.6×10^9	3.8×10^9
	RT	4.4×10^9	5.6×10^9	2.7×10^9
90 days	4°C	1.2×10^9	3.2×10^9	9.8×10^8
	RT	9.6×10^8	2.8×10^9	6.1×10^8
120 days	4°C	8.7×10^8	9.5×10^8	7.6×10^8
	RT	1.4×10^8	9.6×10^8	8.5×10^7
150 days	4°C	5.3×10^8	8.0×10^8	3.8×10^8
	RT	9.2×10^7	6.3×10^8	2.0×10^7
180 days	4°C	3.8×10^8	5.6×10^8	9.7×10^7
	RT	2.1×10^7	8.5×10^7	6.4×10^6

4.9 Mutual Compatibility between PGPR isolates for consortium bioformulation

The cross streak assay for the *in vitro* compatibility among the PGPR strains NII 0928, 0943 and 1020 in the plates showed that none of these were antagonistic, or growth inhibitory to each other (Figure 4.23). Hence, the talc-based bioformulation

consisting of consortium of three PGPR isolates were prepared as described in Materials and Methods chapter.

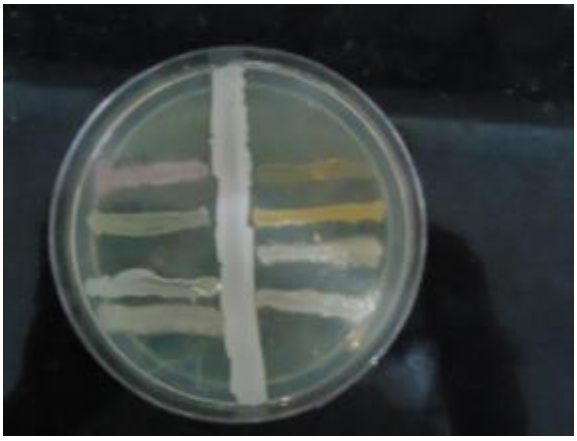


Fig. 4.23 Biocompatibility between the rhizospheric isolates shown by cross streak assay plate

4.10 Effect of Talc based formulation of PGPR Consortia on Black pepper

4.10.1 Growth parameters

A pot culture experiment conducted to study the inoculation effect of single and consortium of PGPR isolates, *viz,* NII 0928, NII 0943 and NII 1020 on the growth parameters and NPK content of black pepper, which showed a significant increase with the consortium treatment compared to the uninoculated talc-based control as well single inoculant treatments. Compared to the uninoculated control treatment, all the bacterial treatments significantly increased the growth of black pepper vine, except for root length, for which the treatments had a non-significant effect (Fig. 4.24 and Table 4.16).

Table 4.16 Effect of PGPR talc based bioformulation of single and consortium inoculants on growth of black pepper after 8 months of planting

Treatments	Root length (cm)	Root Nos.	No. of nodes.	LAI	Shoot length (cm)	Dry weight root (mg/ plant)	Dry weight shoot (mg/ plant)
T1(NPK)	22.33	27.33bc	52.33c	2.79d	277.60b	2.38b	27.88d
T2(control)	21.48	12.33a	19.33a	0.91a	107.10a	1.30a	10.21a
T3(NII 0928)	23.18	29.33bcd	34.00b	1.63bc	203.40b	3.53c	16.71b
T4(NII 0943)	29.17	30.67cd	31.33ab	2.02c	216.70b	3.57c	19.48bc
T5(NII 1020)	24.61	24.33b	30.67ab	1.51b	202.90b	2.67b	16.31b
T6(Consortium)	22.00	34.67d	48.67c	2.47d	240.20b	4.07d	22.92c
lsd	NS	5.61	12.73	0.42	78.50	0.47	4.16

Mean values sharing similar letter (s) in a row are non-significant at $P < 0.05$, according to Duncan's multiple range test

The increase in the number of roots was significantly higher with PGPR consortium treatment, which was statistically at par with NII 0943 and NII 0928. The numbers of nodes were maximum for NPK treatment and statistically at par with PGPR consortium treatment, followed by NII 0928 statistically at par with NII 0943 and NII 1020 (Table 4.16). The maximum value for shoot dry weight and number of nodes, LAI was shown by NPK treatment followed by the consortium and single PGPR inoculants. The shoot dry weight was significantly higher for NPK treatment, followed by PGPR consortium treatment at par with NII 0943. The root dry weight was significantly higher for PGPR consortium treatment, followed by NII 0943 and NII 0928. The dry root weight of NPK treatment and NII 1020 were at par statistically (Table 4.16). The increase in shoot length was higher with NPK treatment and at par with the consortium treatment and single PGPR inoculants. The consortium showed an increase of root number by 26.9% over NPK control and 13% over the single inoculant NII 0943. The consortium also showed an increase of dry root weight of 70.9% over NPK control, 14.1% over the single inoculant NII 0943 and 15.5% increase over the NII 0928. There was an increase of dry shoot weight of 17.7% (in consortium) over the maximum value attained by the single inoculant NII 0943 and an increase of number of nodes by 43% over the maximum value attained by the single inoculant NII 0928.

4.10.2 Plant NPK content

PGPR consortium showed a significant increase in the root and shoot NP content of eight month planted black pepper vine over the single PGPR as well as uninoculated control treatment, while there was a significant decrease in macro-nutrient content over the recommended NPK control treatment (Table 4.17). All the PGPR treatments showed a non-significant effect on K content of the root and shoot. Among the PGPR treatments, the consortium showed the significantly highest root N content of 5.5% increase over NII 0928, which was statistically at par with the consortium and NII 0943. The shoot N uptake for the consortium was increased up to 7% over NII 0928, which was at par with other PGPR treatments. The P uptake of the roots of consortium was statistically significant over NPK treatment and single PGPR inoculants. The root P content was increased up to 68.8% over NPK treatment and 50% increase over the highest value attained by the single inoculant NII 0943. The shoot P uptake of consortium was statistically significant over the single inoculant and uninoculated control (Table 4.17). The shoot P uptake of the consortium was increased up to 12.5% over NII 0928, which was at par with other PGPR treatments. The root and shoot K uptake of consortium showed an increase of 15.6 and 5.0 %, respectively over the single inoculant even though all the PGPR treatments were statistically at par with each other.

Table 4.17 Effect of NPK uptake on black pepper by single inoculants and consortium based PGPR treatments

	N uptake (mg/plant)		P uptake (mg/plant)		K uptake (mg/plant)	
Treatments	Root	Shoot	Root	shoot	Root	Shoot
T1	0.079e	0.585d	0.016c	0.075d	0.131c	0.962c
T2	0.019a	0.222a	0.004a	0.030a	0.032a	0.277a
T3	0.068c	0.399b	0.018c	0.054b	0.067ab	0.518b
T4	0.067bc	0.398b	0.018c	0.056bc	0.065ab	0.644b
T5	0.057b	0.384b	0.011b	0.053b	0.069ab	0.606b
T6	0.077de	0.487c	0.027d	0.063c	0.089b	0.676b
lsd	0.010	0.050	0.004	0.010	0.041	0.198

Mean values sharing similar letter (s) in a column are non-significant at $P < 0.05$, according to Duncan's multiple range test

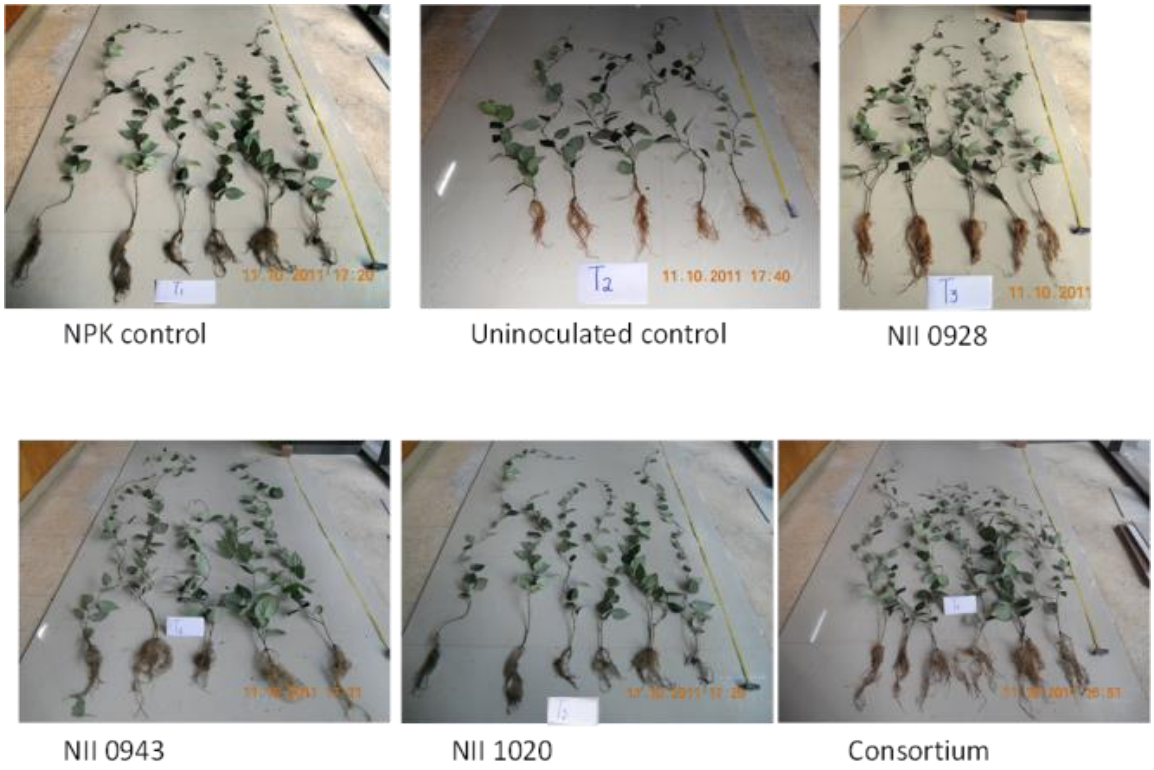


Fig. 4.24 Effect of single and consortium based PGPR inoculants on growth of black pepper

4.10.3 Soil properties

Table 4.18: Effect of single and consortium based PGPR treatments on pH and NPK content of soil

Treatments	T1	T2	T3	T4	T5	T6	LSD
pH	6.80d	7.00e	6.03b	5.99b	6.16c	5.91a	0.07
Available N (kg/ha)	614.30f	148.3a	522.80d	514.80c	414.00b	547.80e	1.73
Available P (kg/ha)	329.20b	98.40a	579.20d	607.00 e	523.10c	615.8f	0.70
Available K (kg/ha)	330.90f	105.60a	327.50e	317.80c	311.80b	324.6d	0.77

Mean values sharing similar letter (s) in a row are non-significant at $P < 0.05$, according to Duncan's multiple range test

The results of post planted soil analysis showed a significant effect of PGPR treatments on the pH, and available N, P, K (Table 4.18). A significant reduction of pH from an initial value of 7.2 to 5.91 was found with various treatments. The macronutrient concentration status of soil also statistically increased with the application of single as well as consortium PGPR. The maximum increase in available nitrogen was shown by NPK treatment, followed by the PGPR consortium, NII 0928, NII0943 and NII 1020. The maximum increase in available phosphorus was shown by PGPR consortium, followed by NII 0928, NII0943, NII 1020 and NPK treatment while maximum value for available K was in NPK, followed by NII 0928, PGPR consortium, NII0943 and NII 1020. The available NPK in uninoculated absolute control treatment was reduced from the initial values.

4.11 Identification and Characterization of best PGPR isolates

On the basis of the previous plant based experiments conducted in the pots, three best isolates, namely, NII 0928, NII 0943 and NII 1020 were selected for molecular identification (Table 4.19) as well as for the morphological, physiological and biochemical characterization (Table4.20 and Table 4.21).

Molecular analysis based on 16S rRNA gene sequencing revealed that the isolate NII-0928 showed highest similarity to *Serratia nematodiphila* KCTC 22130 (Fig. 4.25), NII-0943 showed maximum similarity to *Bacillus tequilensis* NRRL B-41771 (Fig 4.26) and NII 1020 showed maximum similarity to *Kocuria* sp. BVB03 (Fig. 4.27) available in the public domain. The sequenced data were deposited in the GenBank nucleotide sequence database under the accession numbers FJ897465 for NII 0928, FJ897473 for NII 0943 and KC470544 for NII 1020.

Table 4.19: 16S rRNA gene sequences of PGPR isolates submitted with NCBI GenBank

Isolate	Size of sequenced rRNA bases (bp)	Nearest Neighbour	Maximum identity	Accession number
NII 0928	1105	*Serratia nematodiphila*	99.40%	FJ897465
NII 0943	1111	*Bacillus tequilensis*	99.50%	FJ897473
NII 1020	1428	*Kocuria* sp	99%	KC470544

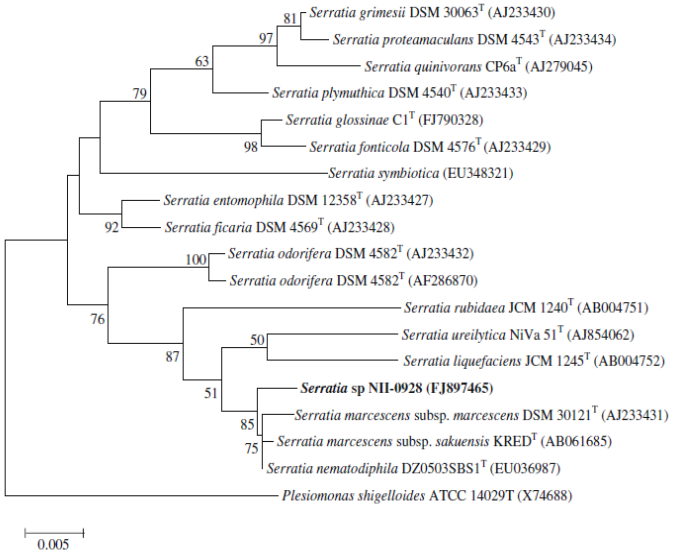


Fig. 4.25 Neighbour – joining phylogenetic dendrogram based on 16S rRNA sequences showing relationships between isolate NII 0928 and their related taxa. Only the bootstrap percentages higher than 50% are shown at branching points. Bar 0.005 substitutions per nucleotide position

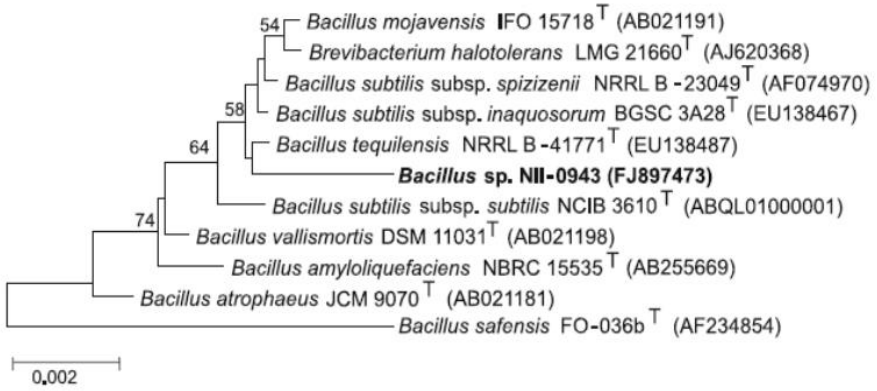


Fig. 4.26 Neighbour – joining phylogenetic dendrogram based on 16S rRNA sequences showing relationships between isolate NII 0943 and their related taxa. Only the bootstrap percentages higher than 50% are shown at branching points. Bar 0.002 substitutions per nucleotide position

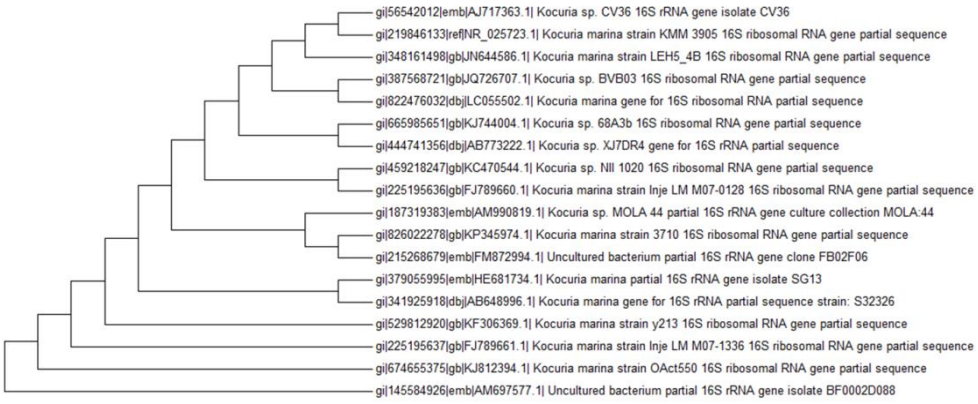


Fig.4.17 Neighbour – joining phylogenetic dendrogram based on 16S rRNA sequences showing relationships between isolate NII 1020 and their related taxa. Only the bootstrap percentages higher than 50% are shown at branching points. Bar 0.002 substitutions per nucleotide position

All the three isolates were catalase positive. NII 0928 was Gram negative while NII 0943 and NII 1020 were Gram positive bacteria (Table 4.21). The

preliminary results obtained using API ZYM and API 50CH revealed the enzyme activity as well as the carbohydrate utilization profile of the potent isolates. All the three isolates were positive for esterase, esterase lipase, leucine arylamidase, acid phosphatase, naphthol- AS-BI-phosphohydrolase and negative for the cysteine arylamidase, trypsin, α-chymotrypsin, α-galactosidase, β-galctosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N acetyl- β-glucosaminidase, α-mannosidase, and α-fucosidase. All the isolates were positive for the utilization of glycerol, D-ribose, D-adonitol, D-galactose, D-glucose, D-fructose, D- Mannose, Inositol, D-mannitol, D-sorbitol, N-acetyl glucosamine, arbutin, esculin ferric citrate, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, glycogen,xylitol, gentiobiose, D-lyxose, D-fucose, L-fucose, L-arabitol, potassium 2- ketogluconate, potassium 5-ketogluconate, while all the three isolates were negative for inulin, D-meltizitose, D-raffinose, D-turanose, D-tagatose, D-arabitol, methyl- α D-mannopyranoside, dulcitol, methyl-β, D-xylopyranoside, L-xylose (Table 4.20).

Fig. 4.28 API 50CH kit for characterization of carbon source utilization pattern for rhizospheric isolates

Table 4.20 Carbohydrate utilization profiles of the three best PGPR isolates selected using API-50 CH strips

Carbon source	NII 0928	NII 0943	NII 1020
Glycerol	+	+	+
Erythritol	+	-	+
D-arabinose	-	+	+
L-arabinose	-	+	+
D-ribose	+	+	+
D-Xylose	-	+	+
L-xylose	-	-	-
D-adonitol	+	+	+
Methyl-βD-Xylopyranoside	-	-	-
D-Galactose	+	+	+
D-glucose	+	+	+
D-fructose	+	+	+
D-Mannose	+	+	+
L-Sorbose	-	+	-
L-rhamnose	-	+	-
Dulcitol	-	-	-
Inositol	+	+	+
D-mannitol	+	+	+
D-sorbitol	+	+	+
Methyl-αD-Mannopyranoside	-	-	-
Methyl-α D-glucoyranoside	+	-	-
N-Acetylglucosamine	+	+	+
Amygdalin	+	-	-
Arbutin	+	+	+
Esculin ferric citrate	+	+	+
Salicin	+	-	+
D-Cellobiose		+	+
D-maltose	+	+	+
D-lactose	+	+	+
D-melibiose	+	+	+

D-saccharose	+	+	+
D-trehalose	+	+	+
Inulin	-	-	-
D-melizitose	-	-	-
D-raffinose	-	-	-
Amidon	-	+	+
Glycogen	+	+	+
Xylitol	+	+	+
Gentiobiose	+	+	+
D-turanose	-	-	-
D-lyxose	+	+	+
D-tagatose	-	-	-
D-fucose	+	+	+
L-fucose	+	+	+
D-arabitol	-	-	-
L-arabitol	+	+	+
Potassium gluconate	+	+	-
Potassium 2-ketogluconate	+	+	+
Potassium 5-ketogluconate	+	+	+

Table 4. 21 Morphological, physiological and biochemical characteristics of the best three PGPR isolates selected

Test	NII 0928	NII 0943	NII 1020
Colony colour	pink	whitish	lemon yellow
Colony morphology	circular , entire, elevated, smooth, opaque	circular , entire, elevated, smooth, opaque	circular , entire, elevated, smooth, opaque
Gram's test	-	+	+
Catalase	+	+	+
Range of Growth temperature			
4	+++	++	++

20	+++	+++	+++
30	+++	+++	+++
40	++	+++	+++
pH range for growth			
4	+++	+++	-
5	+++	+++	++
6	+++	+++	+++
7	+++	+++	+++
8	+++	+++	+++
9	+++	+++	+++
10	+++	+++	+++
11	++	+++	+
12	-	-	-
Salinity range for growth			
2.5	+++	+++	+++
5	+++	+++	+++
7.5	+++	+++	+++
10	-	+++	+++
API ZYM test			
Alkaline phosphatase	+	-	-
Esterase	+	+	+
Esterase lipase	+	+	+
Lipase	+	-	-
Leucine arylamidase	+	+	+
Valinearylamidase	-	+	-
Cystinearylamidase	-	-	-
Trypsin	-	-	-

α-chymotrypsin	-	-	-
Acid phosphatse	+	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	+
α-galactosidase	-	-	-
β- galactosidase	-	-	-
β- glucuronidase	-	-	-
α-glucosidase	-	-	-
β- glucosidase	-	-	-
N- acetyl-β-glucoseaminidase	-	-	-
α-mannosidase	-	-	-
α-fucosidase	-	-	-

All the three isolates possessed a fairly good growth tolerance towards the extreme pH, temperature and salinity (Table 4.21).The three isolates shared common growth characteristics in the pH range of 5.0 -11.0, temperature range of 20°C – 40°C and salinity range of 2.5 -7.5%. NII 0928 and NII 0943 were able to grow at 4^{o}C and at pH of 4.0, while NII 0943 and NII 1020 showed growth at 10% NaCl.

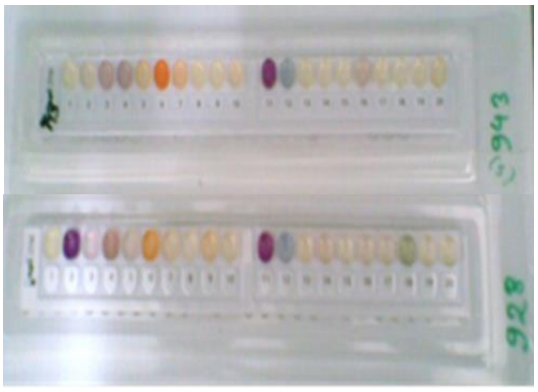


Fig. 4.29 API 20ZYM kit for characterization of the isolated rhizobacterial enzyme activity

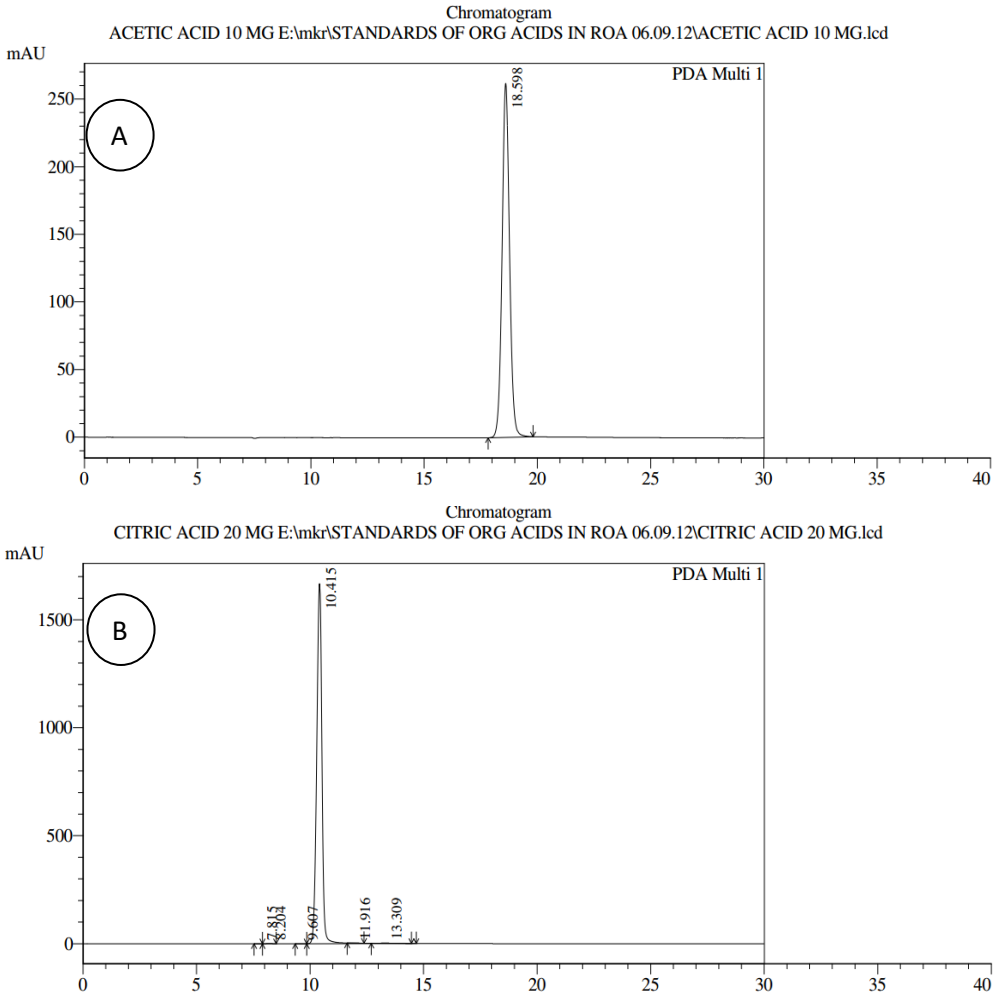
4.12 Determination of Fixed Nitrogen by the best three selected isolates

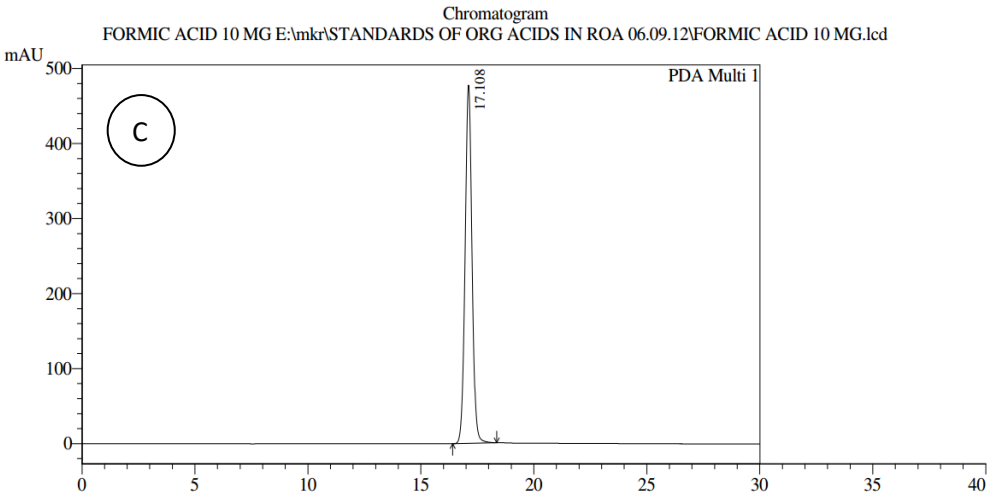
The Quantification of the fixed nitrogen results showed that the maximum N fixing capacity was shown by NII 0928 (25.64 µg/ml) and by NII 1020 (22.2 µg/ml).

4.13 Production of organic acids during phosphate solubilization

The organic acids from the culture filtrate of rhizobacterial isolates were quantified using the retention times and peak areas of chromatograms with pure standards of acetic, citric, formic, gluconic, propionic, lactic acid, tartaric acid and succinic acid (Fig. 4.30).

During TCP solubilization, all the strains showed the production of gluconic, citric, formic, succinic and tartaric acids (Table 4.22). Acetic and propionic acid production was detected in NII 0928 and 1020. The production of lactic acid was restricted to NII 0928 and 0943.




Chromatogram
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mAU
PDA Multi 1
17.108
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500
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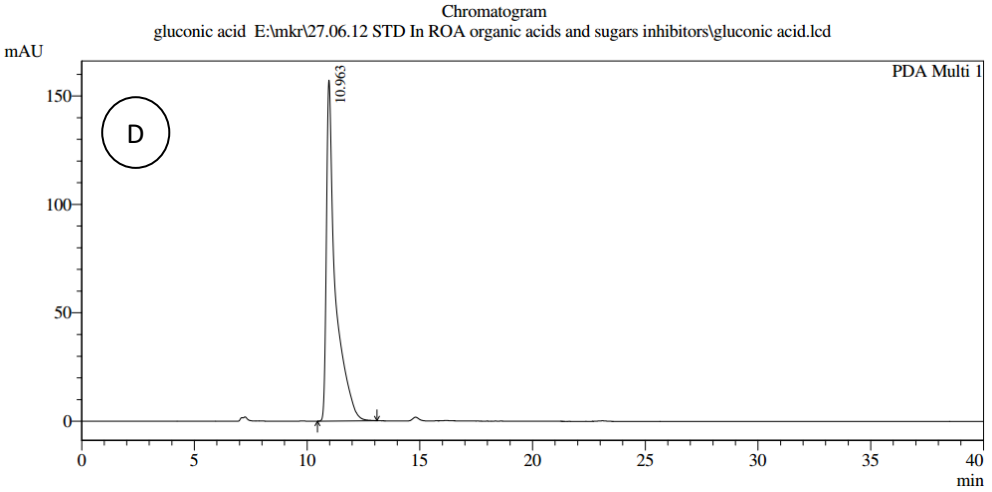
40


Chromatogram
gluconic acid E:\mkr\27.06.12 STD In ROA organic acids and sugars inhibitors\gluconic acid.lcd
mAU
PDA Multi 1
10.963
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10
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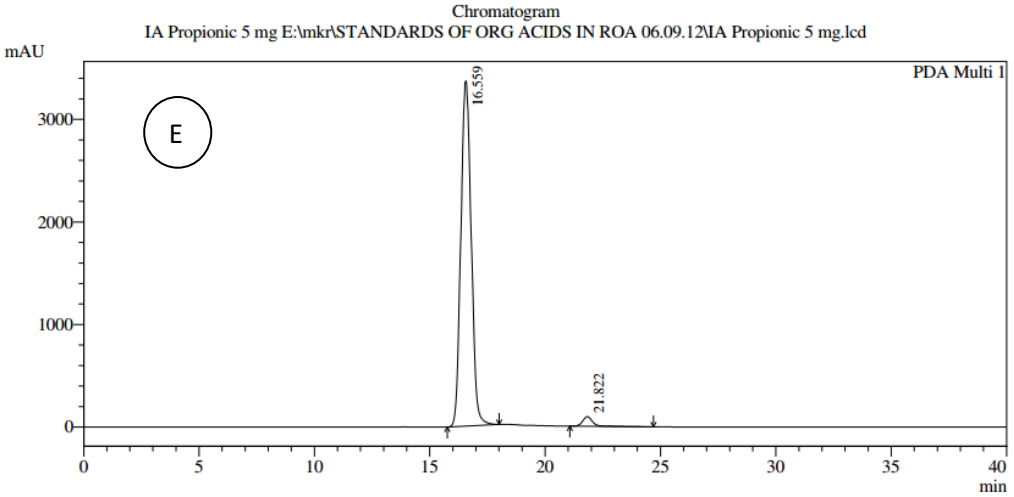
Chromatogram
IA Propionic 5 mg E:\mkr\STANDARDS OF ORG ACIDS IN ROA 06.09.12\IA Propionic 5 mg.lcd
mAU
PDA Multi 1
16.559
21.822
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3000
2000
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min

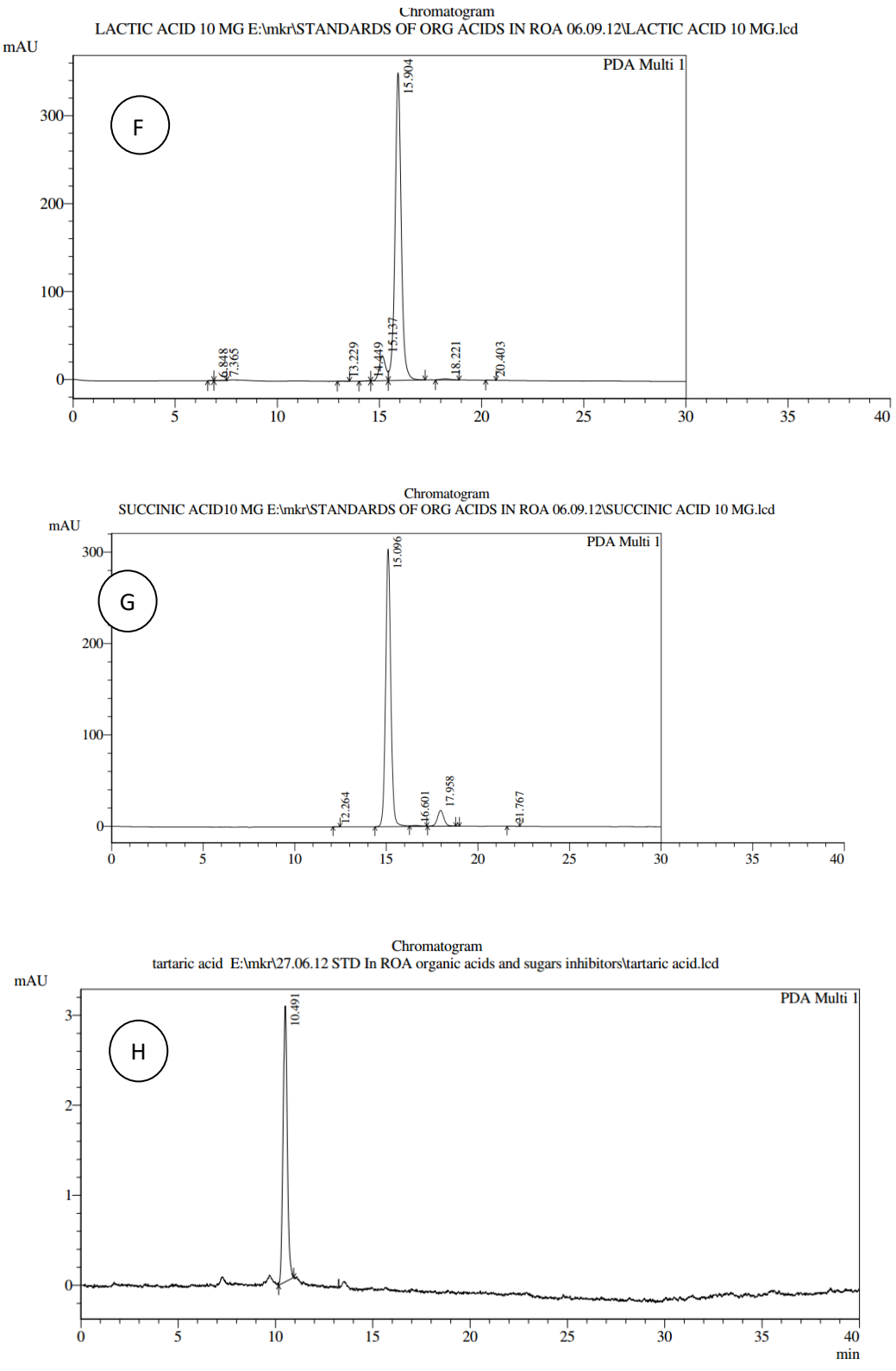

Fig. 4.30 HPLC chromatograms of standard organic acids (A) acetic acid (B) citric acid (C) formic acid (D) gluconic acid (E) propionic acid (F) lactic acid (G) succinic acid (H) tartaric acid

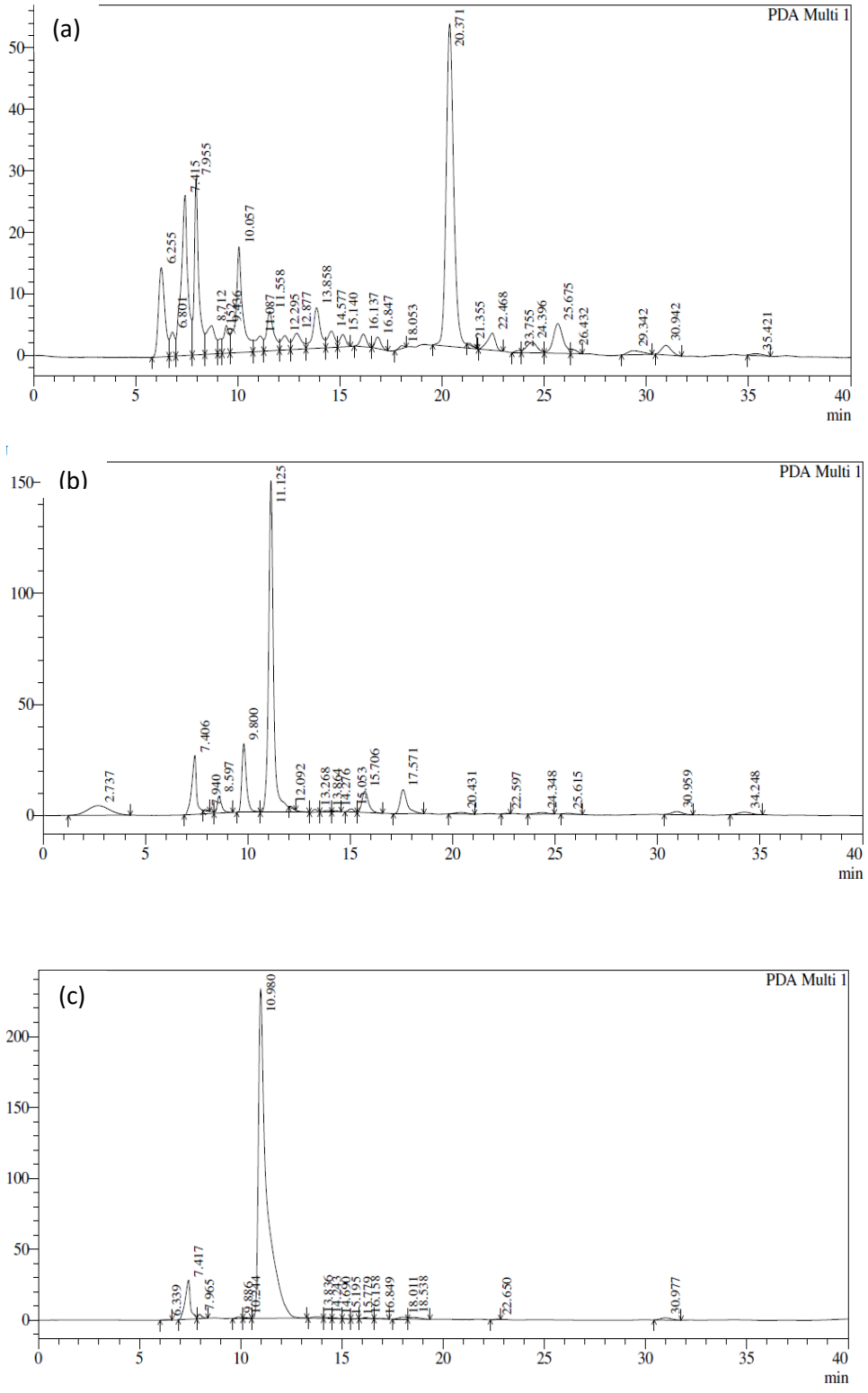

Fig. 4.31 HPLC chromatograms of culture supernatant of PGPR NII isolates grown for 5 days at 28 °C in NBRIP broth with tricalcium phosphate (a) culture supernatant of NII 0928 (b) culture supernatant of NII 0943 (c) culture supernatant of NII 1020

Quantitative difference in the production of organic acids was observed during the solubilization of TCP by the rhizobacterial strains (Tables 4.22). Gluconic acid production was highest in NII 1020 (22766.2 µg ml^{-1}), followed by NII 0943 (8730.1 µg ml^{-1}) and NII 0928 (228.9 µg ml^{-1}) (Table 4.22).The highest concentrations of citric acid, formic acid, and tartaric acid was shown by NII 0943.

Table 4.22: Organic acid production by rhizospheric isolates during tricalcium phosphate solubilization after 5 days incubation at 28±0.1 ºC

	Organic acids (µg/ml)								
Rhizospheric isolates	GA (10.96)*	AA (18.5)	CA (10.415)	FA (17.12)	PA (10.6559)	LA (15.904)	SA (15.096)	TA (10.491)	Total organic acids
NII 0928	228.9	13	308.4	36.41	11124.3	55.2	67.2	543.3	12376.7
NII 0943	8730.1	ND	375.7	258.3	ND	285.03	39.7	3502	13190.8
NII1020	22766.2	80	7.8	10.04	3066.8	ND	3	13.8	25947.6

*Retention time is given in brackets

Chapter 5

DISCUSSION

The ability of introduced bacterial strains to colonize the roots and survive in the soil is one the major scientific challenges, reducing the expected Plant Growth promoting effect. Hence, there is a desirable need for the search and selection of PGPR from the natural soil devoid of any human disturbances, such as forest areas, specifically, which thrives and sustains well under the prevailing soil conditions and promote the growth of plants adapted to the same soil environment.

The Western *Ghats* designated as one of the 25 global "mega-diversity" hotspots that support unique sets of the plant, animal and microbial species are a valuable resource for the search of natural potent PGPR around rhizosphere region of wild forest plants, which is devoid of human intervention. In light of this, in this study efforts were made to isolate the bacteria from the rhizospheric region of Western *Ghats* dense forest and screen them for various plant growth promoting attributes such as for phosphate solubilization, biocontrol ability, hormone production, in *vivo* pot experiments for plant growth promotion and characterization of selective bacterial isolates were studied. The impacts of PGPR consortium on plant growth promotion were also studied; these findings have been discussed in this chapter.

Microbial phosphate solubilization is one of the important parameters related to plant growth promotion. There are several reports regarding the plant growth promotion due to inoculation of phosphate solubilizing microorganisms under greenhouse as well as in field conditions (Wahid and Mehana 2000; Reyes *et al*., 2002). Due to the rapid P fixation and its insolubility and in the soils, only 0.1% of the total P exists in a soluble form available for plant uptake (Zhou *et al*., 1992). The

overall phosphate use efficiency following phosphate fertilizer application is generally below 30% because of the formation of insoluble complexes with Ca^{2+} in neutral and high pH soils, or with Fe^{3+} or Al^{3+} in low pH soils (Goldstein 1986, Harris *et al.,* 2006).The modern resource for P fertilizer, rock phosphate are very limited in India and about two million tons of rock phosphate are imported annually from other countries (Sharma *et al*. 2013a). Moreover, the depletion of rock phosphate within this century has been alarmed by geologists of IFDC (Sharma *et al*., 2013a).Hence, in this current scenario, an attempt to isolate potent phosphate solubilizers from western *ghats* forest soil was done.

5.1 isolation and characterization of rhizospheric bacterial isolates for PGPR attributes

5.1.1 Phosphate solubilization

A total of 200 bacterial isolates were screened for qualitative assay for phosphate solubilization in PVK agar supplemented with insoluble tricalcium phosphate. Forty seven isolates were selected having halo solubilization zone of 5-24mm in PVK agar plates (Fig. 4.1; Table 4.1). A direct relation between solubilization zone of qualitative assay and solubilized P in liquid NBRIP broth could not be established since the solubilization zone of 22mm was shown by NII 0909, NII 0929 and NII 0943 but their respective solubilization varied for each having 515.82, 506.57 and 518.2µg/ml. In plate assay, the maximum solubilization zone was 24mm shown by three isolates, *viz,* NII 0906, NII 0928 and NII 0930 but the maximum quantitative solubilization in liquid medium was shown by NII 1020 (529.07µg/ml), NII0906 (519.77µg/ml)) and NII 0928 (518.35µg/ml).Similar studies have been reported by many researchers (Gupta *et al*., 1994; Nautiyal 1999, Nautiyal *et al*., 2000). Quantitative solubilization of P was determined in NBRIP broth containing 0.5% TCP, by UV – Vis- spectrophotometric method at 660nm using KH_2PO_4 as standard. All the isolates were able to solubilize phosphate varying from 117.02 to 529.07µg of $Ca_3 (PO_4)_2$ ml^{-1} day^{-1} at 28±2°C after 5^{th} day of incubation. The phosphate solubilization was also accompanied with a drop of pH of the of the culture medium from an initial value of 7.0 to 6.52 – 3.5 (Table 4.1).The pH drop of growth medium also differed among each isolates and could be related with the fact of production of organic acids and secretion by P-solubilizing bacteria. Similarly, the

decrease in the pH of culture filtrates were well correlated with the increased level of orthophosphate in culture filtrate as reported by several early researchers (Hwangbo *et al*., 2003; Rashid *et al*., 2004, Alikhani *et al*., 2006). It has been suggested that microorganisms that decrease the medium pH during the growth are efficient phosphate solubilizers (Khan *et al*., 2006). Secretion of the organic acids by PGPR directly solubilize the mineral phosphate through anion exchange or indirectly chelate Ca, Fe and Al ions associated with phosphate (Walpola and Yoon 2013).

A sharp relation between the solubilization of P and pH drop could not be established in this study because a similar pH for different isolates showed variation in the amount of P solubilized. The pH drop of 5.37 was shown by NII 0914 and NII 0920 but the soluble P varied with 194.76 and 244.4, respectively. These results very well corroborated with similar findings of many researchers who could not draw any correlation between the quantity of P solubilized and pH decline (Gulati *et al*., 2008; Sujatha *et al*., 2004; Dave and Patel 1999). Hence, pH or organic acid production did not seem the sole factor for P solubilization (Gaur 1990). Numerous researchers also studied the involvement of H^+ translocation ATPase or the release of H^+ to outer surface in exchange for cation uptake (Ehrlich 1990; Asea *et al*., 1988) and proposed their involvement in phosphate solubilization. Many researchers also supported the production of chelating substances (Altomare *et al*., 1999; Illmer and Schinner 1992) and inorganic acids such as sulphidric, nitric and carbonic acid (Sperberger 1958). Occurrence of phosphate solubilizing microorganisms has been reported from many different environmental niches (Banik and Dey 1982; Illmer and Schinner 1995; Johri *et al*., 1999; Nautiyal *et al*., 2000; Vazquez *et al*., 2000; Thakuria *et al*., 2004). Use of these PSB as bioinoculants could increase the available phosphorous in the soil and help to minimize the P-fertilizer application, reduce the environmental pollution and promote sustainable agriculture.

Phosphate-solubilizing bacteria serve as an environmental-friendly supplier of soluble forms of phosphorus compared to the ecological damages resulted by phosphatic chemical fertilizer. Production of organic acids (OA) by PGPR is one of the principal mechanisms of phosphate solubilisation accompanied with acidification of growth medium (Marra *et al*., 2012; Khan 2014). The solubilisation involves

direct anion exchange, or indirectly through chelation of Ca, Fe, and Al ions associated with phosphate (Omar 1998; Mahdi *et al*., 2011).Some of the more prominent acids released by phosphate solubilizing microorganisms involved in solubilisation are gluconic acid (Bar-Yosef *et al*., 1999), oxalic and citric acid (Kim *et al*,. 1997), lactic acid, tartaric acid, aspartic acid (Venkateswarlu *et al*., 1984). A study conducted using HCl and gluconic acid revealed the chelation of Al^{3+} by gluconicacid and researchers claimed that GA might have been a major factor in the solubilization of colloidal Al phosphate (Whitelaw *et al*., 1999). Simultaneous production of different organic acids as well as quality of organic acids based on the strength and type of acids count for solubilization of insoluble inorganic phosphates more than total amount of acids produced (Marra *et al*., 2012; Scervino *et al*., 2010).

HPLC analysis of highly efficient phosphate solubilizing strains in terms of *in vitro* and *in vivo* experiments indicated the involvement of organic acids in the solubilisation process. All the three PGPR isolates showed multiple organic acid productions into the growth medium varying in terms of the quantity and the types of acids (Table 4.22; Figure 4.30; Figure4.31). All the three strains produced gluconic, citric, formic, succinic and tartaric acids with varying concentrations. Acetic acid and propionic acid was detected in NII 0928 and NII 1020, while lactic acid was produced by NII 0928 and NII 0943.NII 1020 showed an increase in total no of acids, gluconic acid production, maximum decline in pH and higher levels of phosphate solubilisation in liquid NBRIP medium supplemented with TCP. Similar observations have been reported by Vyas *et al*., (2009) who found that the *Pseudomonas* sp. BIHB 751, which had a lower TCP solubilisation compared to other *Pseudomonas* isolates showed lower gluconic acid production proving the involvement of gluconic acid in solubilization of calcium-bound phosphates. Reports on propionic acid (PA) production by PGPR are very few, e.g., by *Bacillus megaterium, B. atrophaeus, Bacillus* sp., *Enterobacter sakazakii* M2PFe (Vazquez *et al*., 2000; Chen *et al*., 2006; Puente *et al*., 2009a, b; Lopez *et al*., 2011).In the present study, two isolates, NII 0928 and NII 1020 produced PA in high concentration indicating their involvement in phosphate solubilisation. Succinic acid production was in low quantity in high phosphate solubilizer NII 1020 compared to other two isolates. This result was in contrast to the report of Vyas *et al*., (2009) who pointed

the role of succinic acid in TCP solubilisation as it was in high concentration in their highly efficient *Pseudomonas* strains.

The aluminium and iron bound phosphate share a similar structure (Lopez Hernandez *et al*., 1979; Cline *et al*., 1983; Prijambada *et al*., 2009), hence, the most effective organic acids to chelate Al and Fe bound phosphates are citric, oxalic, tartaric and malic acids (Cline *et al*., 1983, Prijambada *et al*., 2009, Johnson *et al*., 2006; Fankem *et al*., 2008; Hue *et al*., 1986; Panhwar 2014). In the present study, three isolates also produced citric and tartaric acid in high concentrations, except NII 1020, which showed a comparable lower concentration of citric acid and tartaric acid production proving the innate ability to solubilize the aluminium and iron bound phosphate in addition to the solubilisation of Ca bound phosphate.

The microbial mineralization of organic phosphorus also plays an important role in providing nutrient phosphorus element to agricultural farm lands. A major portion of organic phosphorus is in the form of phytate (Borie *et al*., 1989; Turner *et al*., 2003; Martinez-Viveros *et al*., 2010) and the exploration of phytase producing PGPR enables for the application for the degradation of soil phytate to lower phosphate esters which are finally available to plant nutritional requirements. Hence, the selected isolates were analyzed for their phytase activity, which hydrolyses the soil phytate. Among the fifteen rhizobacterial isolates, five isolates were positive for the presence of phytase activity and especially the among the finally selected best isolates, NII 0928 and NII 1020 showed translucent phytate clearing region around colonies on phytase screening medium (Fig. 4.7; Table 4.5). Several Gram-positive and Gram-negative soil bacteria such as *Bacillus subtilis*, *Acetobactor* sp., *B. laevolacticus, Klebsiella terrigena*, *Pseudomonas* sp., *Enterobacter* sp., *Advenella* sp. and *Cellulosimicrobium* sp. have been reported for phytase activity (Hussin *et al*., 2007; Idris *et al*., 2002; Mukeshkumar *et al*., 2011; Singh *et al*., 2014; Pandey et al., 2001). Increased plant growth on treatment with phytase positive PGPR have been reported by many researchers (Idris *et al*., 2002; Richardson 2001).

5.1 .2 Production of IAA-like auxins

IAA producing rhizobacteria play a very important role in the growth of associated plants by primarily altering root architecture through involvement in lateral and adventitious root formation and root elongation (Spaepen and Vanderleyden, 2011).The production of IAA in the presence of a suitable precursor such as tryptophan has been reported for several PGPR belonging to the genera *Pseudomonas, Enterobacter, Azospirillum, Azotobacter, Serratia, Bacillus, Burkholderia, Erwinia* and *Pantoea*. The root exudates of various plants contain rich supplies of tryptophan, which are used by the microorganisms for synthesis and release of auxins as secondary metabolites in the rhizosphere. IAA alters root patterns and enhances nutrient absorption.

All the 15 isolates of the present study were able to produce IAA but the amount of IAA produced varied widely between the isolates ranging from16.8-129.1µg ml^{-1} in tryptophan supplemented (Table 4.2). The earlier reports of 10.4-28.3 µg ml^{-1} (Farah *et al*., 2008), 4.97 to 46.66 µg ml^{-1} (Yasmin *et al*., 2009), 107.6 – 213.2 µg ml^{-1} (Kumar *et al*.,2012) and 24.3 – 126.4 µg ml^{-1} (Ashraf *et al*., 2011) were very well comparable with the results obtained in the present study and also agreeable with the statement that the isolates from the rhizosphere were more efficient auxin producers than the isolates from the bulk soil (Sarwar and Kremer, 1992). The increased rooting due to the bacterial IAA production enhances plant mineral uptake and root exudation, which in turn stimulates bacterial colonization.

5.1.3 ACC deaminase activity

PGPR is known to promote the root and plant growth and protect plants from the abiotic and biotic stresses through the activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase involved in the reduction of plant ethylene levels(Glick 2012; Minaxi *et al*., 2012; Hameeda *et al*., 2006). ACC deaminase activity has been reported in many soil organisms, including *Azospirillum, Rhizobium, Azotobacter, Achromobacter, Burkolhderia, Ralstonia, Bacillus, Pseudomonas* and *Enterobacter* (Blaha *et al*., 2006; Gosh *et al*., 2003; Arshad *et al*., 2008; Nukui *et al*., 2000).A preliminary screening for ACC deaminase of 15 isolates

selected on the basis of previous *in vitro* experiments of phosphate and IAA attributes was done by evaluating the growth of isolates in minimal medium containing ACC as sole N source. Though all the selected rhizobacterial isolates were capable of utilizing ACC, they differed in their ACC utilization, or metabolization rate as shown through the variable cell densities measured ranging from 0.08 to 0.78 (Table 4.3).On the basis of growth OD, four isolates were grouped into high, seven into medium and four into low utilizers of ACC. Similar works have been reported by many researchers (Husen *et al*., 2009; Kaur and Sharma 2013). NII 0943 (0.782) showed highest growth in DF medium with ACC (OD), followed by NII 0934 (0.763) and NII 0906 (0.757).

The variations in ACC utilization could be due to the variation in the genetic, physiological and biochemical nature of ACC deaminase from each isolate. Growth promotional effect of root, shoot and nutrient uptake in various crops on inoculation with ACC deaminase- producing rhizobacteria on the seed and root treatment have been reported by many researchers (Nadeem *et al*., 2007; Glick, 2012).

IAA is well known for the activation of ACC synthetase transcription (Kende 1993; Kim *et al*., 1992) and ethylene inhibits IAA transport and signal transduction (Pratiyon *et al*., 2006). Ethylene concentration is lowered in plant roots under the presence of ACC deaminase containing PGPR, and thereby relieve ethylene repression of auxin response factor synthesis, and indirectly promotes plant growth.Thus, PGPR having ACC deaminase activity plus IAA production facilitates plant growth by decreasing ethylene inhibition and permitting IAA stimulation without the negative effects of increasing ACC synthetase and plant ethylene levels. (Glick 2012; Carmen and Roberto 2011).

5.1.4 Siderophore production, HCN production, ammonia production, chitinase production and *in vitro* antagonism towards fungal pathogens

The indirect aspect of plant growth promoting activity of PGPR essentially involves the suppression of phytopathogens through the production of various antifungal metabolites, sidrophores, lytic enzymes (e.g., chitinase, protease, cellulose and β-1, 3- glucanase), and hydrogen cyanide (El Sayed *et al*., 2014). Among the 15 isolates, nine isolates were siderophore producers, especially the finally selected best

three isolates (Table 4.5). The ability of siderophore production by the PGPR has been shown to antagonize many pathogenic fungi through the deprivation of nutrient iron and ultimate limiting the growth of phytpothogens. (Kloepper *et al*., 1980; Bagnasco *et al*., 1998; Deshwal, 2012a; Bholay *et al*., 2012; EI Sayed *et al*., 2014). A pseudobactin siderophore mediated suppression of *Fusarium oxysporum* in iron deficient soil by *P. putida* B10 strain was reported and the suppression was lost after the replenishment of the soil with iron (Kloepper *et al*., 1980; Beneduzi *et al*., 2012). Chitinase was produced by *Serratia nematodiphil*a sp. NII 0928 among the 14 isolates screened on cholloidal chitin containing plate (Fig. 4.5; Table 4.5). The innate chitinase activity of NII 0928 potentially can result in the degradation of the structural matrix of fungal cell wall (Dunne *et al*., 1998). Several strains from the genera *Serratia* have been reported by many authors to be the most competent chitin degrading bacteria (Frankowskii *et al.*, 2001; Someya *et al*., 2011; Brurberg *et al*., 1996; Mehmood *et al*., 2009; Fahdil *et al*., 2014). The purified 60KDa chitinase enzyme from *S. proteamaculans* 18A1 showed antifungal activity against *Fusarium oxysporum* and *Aspergillus niger* (Mehmood *et al*., 2009), while two chitinolytic enzymes, CHIT60 and CHIT100 from *S. plymuthica* HRO-148 inhibited spore germination and germ tube elongation of fungus *Botrytis cinera* (Frankowskii *et al*., 2001) All the 15 isolates were ammonia producers, while 10 isolates where HCN producers with varying intensity of production (Table4.5). Growth suppression of certain fungi and inhibition of germination of spores have been reported due to the accumulation of ammonia in the soil. The ammonia produced by the PGPR's has been reported to act as a signaling molecule when bacteria and plant interact with each other benefit plant as a source of nitrogen for growth of plants and also increase the glutamate synthetase activity, which is major assimilatory enzyme for ammonia to incorporate ammonia efficiently into several organic configurations (Chitra *et al*., 2002; Wani *et al*., 2014). Microbial production of HCN has been reported as an important antifungal trait to control root infecting fungi (Ramette *et al*., 2003).The major mechanism of biocontrol activity for protection of black pepper and ginger has been reported due to the production of HCN along with siderophore (Diby 2006)

Among the 15 isolates screened for the antifungal activity in plate assay, only NII 0906 showed the growth inhibition of various test phytopathogens with varying degrees of efficacy represented as growth inhibition zones for each test fungi used

(Fig. 4.4; Table 4.4).The growth inhibition of phytopathogenic fungi by the culture filtrate of PGPR are mainly attributed to the antibiotic production and secretion to the culture medium by the PGPR. These types of antibiotic producing bacteria are common constituents of natural microflora in the soil and plant associated environments (Raajimakers *et al.*, 2008).The antibiotic produced by the PGPR are known to possess antiviral, antimicrobial, insect and mammalian anti-feedant, anti-helminthic, phytotoxic, antioxidant, cytotoxic, antitumour and plant growth promoting activities (Fernando *et al*., 2006) and the major nonvolatile antibiotic involved in the suppression of fungal phytopathogens are butyrolactones, zwittermycin A,kanosamine, oligomycin A, oomycin A,phenazine-1-carboxylic acid, pyoluteorin, pyrrolnitrin, viscosinamide, xanthobaccin,and 2,4-diacetyl phloroglucinol (2,4-DAPG) (Whipps, 2001; Martinez-Viveros *et al.*, 2010).

5.1.5 Growth of isolates on Jensen's nitrogen free medium

On the basis of growth on Jensen's N free agar plate, eight isolates were grouped into nitrogen fixers among the 15 rhizospheric isolates (Fig. 4.6; Table 4.5) . Out of the three best isolates finally selected on the basis of plant experiments, two isolates positive for the qualitative nitrogen fixation assay were assayed quantitatively using Kjeldhal's method. NII 0928 showed the maximum value for fixed nitrogen of 25.64µg/ml, while NII 1020 showed 22.2 µg /ml of fixed nitrogen.Similar works nitrogen fixers using Kjeldhal's method have been quantified have been reported earlier by many authors (Kizilkaya 2009; Anjanadevi *et al*., 2013) and the level of fixation were well correlated.

5.2 Effect of PGPR on plant growth promotion

5.2.1 Effect of PGPR on plant growth promotion of tomato

On the basis of high *in vitro* soulbilization of TCP accompanied with other PGPR attributes such as IAA production, ACC deaminase, siderophore, ammonia and HCN, N-fixation, phytase production, a total of 15 isolates were selected for root initiation experiment on tomato seeds. Except NII 0905, all the other isolates significantly increased the root length and shoot length of tomato seedlings compared

to uninoculated control (Fig.4.11). The isolate NII 1020 showed significantly highest root length and at par with NII 1020, NII 0943, NII 0928 and NII 0912. The significant increase in shoot length was shown by NII 0928 at par with NII 1020, NII 0906, followed by NII 0943, NII 0912 and NII 0909 (Table 4.6).Similar reports on increase of root length and shoot length of various crops on PGPR treatment of seeds have been reported by several workers (Mishra *et al*., 2010; Glick *et al*., 1998; Bhattacharyya and Jha, 2012).The inherent ability of multiple PGP attributes with varying degrees of efficacy as well as the strong attraction and adherence of isolates towards the tomato roots might be the factors responsible for increased root and shoot growth. Though all the isolates tested in this experiment were phosphate solubilizers the involvement of phosphate solubilisation mechanism for growth promotion might be ruled out due to the substrate used for the growth of seeds are inert agar gel devoid of any insoluble phosphates.IAA production was the common PGPR attributes shared by all the isolates selected for this experiment and might be the major effect for increased root and shoot growth of germinates tomato seeds.

Many researchers have related PGPR production of IAA enhances root proliferation, root length (Tsavkelova *et al*., 2007; Arshad and Frankenberger, 1993). Even though the isolates possessed ACC deaminase activity of varying efficacy, the operational effect of this enzyme also could not be ruled out since this enzyme have a very important role in the reduction of ethylene levels of plants, which causes inhibition of seed germination and root formation at increased concentration level (Matilla, 2000; Armstrong and Drew, 2002; Norastehnia *et al*., 2007). ACC deaminase positive PGPR when attached to the seed coat, or plant root modulates the ethylene level within the plant's tissues so as to ensure the formation of longer roots and shoots, especially during the first few days after the seeds are planted (Saraf *et al*., 2010).The phenomenon of cross-talk between ethylene and IAA have been documented by many researchers. In this present study also the IAA produced by the isolates caused the transcriptional activation of ACC synthase and ultimate production of ethylene through ACC synthesis. The isolates used in this study possessed ACC deaminase activity also, the exuded ACC from roots or seeds might have hydrolyzed by the PGPR isolates to ammonia and α-ketobutyrate (Glick *et al*., 1998). The dual trait of IAA production and ACC deaminase benefits the test plant by the IAA induced stimulation of cell proliferation and elongation without the effect

of increasing the endogenous ethylene level of plants. Similar observations have been reported in other PGPR isolates too (Huesen *et al*., 2009). Huesen *et al*., 2009 reported that the IAA producing plus ACC deaminase activity of *Pseudomonas* strains increased soya bean seedling root length and weight compared to other *Pseudomonas* with any of the single trait. The different efficacy of growth promotion by isolates might be due to the varied intensity of attraction for the root exudates, attachment, multiplication or in general variation of rhizospheric competency over the rhizospheric region of test plants, variation in the level of production of PGPR attributes in vitro as well as in vivo and production of other growth promoting or inhibiting products produced by the same isolates.

5.2.2 Effect of PGPR on plant growth promotion on cowpea

The best nine isolates, which showed highly significant growth promotion on tomato were selected for growth promotion experiment in cowpea. All the PGPR showed increase in growth promotion over the uninoculated control The isolate NII 0943 showed highly significant increase in all the biometric measurements taken and at par with NII 0928 and NII 1020, followed by NII 0906 (Fig. 4.13; Table 4.8).All the best isolates of previous tomato based experiments repeated their growth promoting effect in cowpea also but with varying degrees of efficacy. The growth promoting effect of many PGPR on various cowpea varieties have been reported by many researchers (de Lima *et al*., 2011). In contrast to the host plant specificity towards PGPR, the results of this study showed growth promotion for all the four types of test crops. The application, or inoculation of single PGPR to more than one crops and their increased growth promoting effect has also been reported by many researchers (Kloepper., 1996; Ma *et al*., 2011; Sharma *et al*., 2011). PGPR strains can be crop specific, cultivar specific, or non-specific for root colonization and PGPR with narrow to broad host plant specific have been reported earlier (Kloepper 1996; Vacheron *et al*., 2013). Experiments were conducted with*Azospirillum brasilense* Sp7for growth promoting effect on great variety of plant species (Lucy *et al*., 2004; Okon and Labandera-Gonzalez, 1994) and subsequently showed positive increased growth response in majority of the trials. Reports of poor results were also obtained with other crops on inoculation with *Azospirillum brasilense* Sp7 in greenhouse and field trials (Baldani *et al*., 1987; Boddey *et al*., 1995). Experiments

conducted with *Pseudomonas* sp. showing IAA and ACC deaminase production as well as P solubilization enhanced wheat growth and yield under greenhouse and field conditions (Zabihi *et al*., 2010).Over all, the growth promoting effect in terms of number of roots, root and shoot length as well as the dry biomass can be attributed to IAA production, ACC deaminase activity and phosphate solubilisation activity and siderophore production, which all the selected PGPR possess. The role of IAA production (Kaymak *et al*., 2008; Egamberdieva and Kucharova 2009), phosphate solubilisation (Rotaru, 2010; Singh and Sale 2000), ACC deaminase (Shaharoona *et al*., 2006; Belimove *et al*., 2002) on root formation and the effect of siderophore production (Rajkumar *et al*., 2010; Sharma *et al*., 2003), ammonia production (Jha *et al*., 2012; Goswami *et al*., 2014), and HCN (Wani and Khan 2010) and their subsequent impact on overall growth of plant have been reported.. The increased biomass may be well related to the increased root system and nutrient absorptive surface, which explored the bulk of soil for water and other mineral nutrients uptake which comes in contact with the roots.

5.2.3 Effect of PGPR on plant growth promotion on black pepper

In the first batch of black pepper experiments, the isolate NII 0928 was highly significant compared to other PGPR treatments for all the biometric measurements taken, followed by NII 0906 and NII 0912 (Fig. 4.15; Fig. 4.16 and Table 4.9). The growth promoting effect of NII 0928 and NII 0906 could be attributed towards the better root colonization capacity on black pepper roots where the PGPR could exert the high phosphate solubilisation and IAA production and ACC deaminase activity. In the second batch of black pepper experiment, both the two isolates NII 0943 and NII 1020 were highly significant compared to the uninoculated control, whereas NII 0943 showed high significance over NII 1020 for LAI, total dry weight and total P uptake(Fig. 4.17; Fig. 4.18; Table 4.11 and Table 4.12). The growth promoting effect of NII 0943 and NII 1020 could also be attributed towards the better root colonization capacity on black pepper roots where the PGPR exert the high phosphate solubilisation and IAA production and ACC deaminase activity. Though the *invivo* and *invitro* PGPR attributes might not always be exactly similar, the high IAA production of NII 0943 could be the increased rooting of stem cuttings of black

pepper compared to NII 1020. Similar observations have been documented by other workers (Khalid *et al*., 2004; Ramirez and Mellado, 2005).

In general, the growth promoting effect in terms of number of roots, root and shoot length, LAI, dry biomasses well as the nutrient uptake could be attributed to multiple attributes such as IAA production, ACC deaminase activity, phosphate solubilisation activity and siderophore production, which all the selected PGPR possessed in addition to the rhizospheric competency towards the roots of black pepper. The increased nutrient absorption by increased root formed due to the PGPR IAA and ACC deaminase activity as well as the availability of more solubilized phosphorus probably contributed to higher plant growth, development and mineral content.

5.2.4 Effect of PGPR on plant growth promotion on Cassava:

All of the three best isolates selected from the previous plant experiments showed significant growth increase over the uninoculated control. NII 1020 was highly significant over NII 0928 and NII 0943 (Fig. 4.19; Fig. 4.20 and Table 4.13). As discussed in the previous plant experiments, the degree of efficiency varied, although all the PGPR strains increased the growth of experimental plants conducted. In black pepper, the highly significant isolate was NII 0943, while in cassava, the best isolate was NII 1020. In cowpea and tomato based experiments, both the two isolates NII 0943 and NII 1020, including NII 0928 and NII 0906 were mostly at par with each-other. These observations have been discussed earlier. High rooting percentages were observed in rose hip and sour cherry cuttings after the inoculation with PGPR for rooting in rose hip and sour cherry cuttings and found that PGPR were effective to obtain high rooting percentages (Ercisli *et al*., 2003; Esitken *et al,* 2003.) .

5.3 Shelf life evaluation of PGPR isolates on Talc based bio formulation

For the successful commercial application of PGPR, the bio-inoculants should be formulated in suitable carrier, which supports the survival of bacteria for a prolonged period of time. The results from the shelf-life evaluation clearly showed the survivability of all the three potent isolates selected in talc-based formulation after six months of storage, which was is exact agreement with the standards set by

Bureau of Indian Standard (2000). However, the survivability differed for each isolates due to the varied genera (Table 4.15).Numerous workers have attributed talc as the suitable carrier for long term viability of bio inoculants because of the very low moisture equilibrium, relative hydrophobicity, chemical inertness, reduced moisture absorption and the prevention of formation of hydrate bridges (Bora *et al*., 2004; Bakthavatchalu *et al*., 2012).

5.4 Effect of PGPR consortium on plant growth promotion on black pepper

PGPR consortium proved to be significant over single PGPR inoculants for all the biometric measurements taken, except for shoot length in which all the PGPR treatments and NPK treatment were at par (Fig. 4.24; Table 4.16). The consortium was highly significant in root P uptake over NPK treatment and single PGPR inoculants, while in shoot P uptake the significance was shown over single incoculants NII 0928 and NII 1020 and at par with NII 0943 (Table 4.17). The shoot N uptake and K uptake of root and shoot of consortium and single PGPR treatment were at par. The increased growth promoting effect of PGPR consortium was in conformity with the earlier reports of workers on several other crops (Megala and Elango., 2014; de Lima *et al*., 2011; Ghallab and Saleem 2001; Sivamurugan *et al*., 2000). Observations on significant increase in seedling growth on inoculation with mixed culture of *Pseudomonas* and *Bacillus* on wheat under field experiment has been reported (Van Elsas 1986). Co-inoculation of two PGPR, *Enterobacter* with *Pseudomonas* (Neyra *et al*., 1995) and *Azospirillum* with *Azotobacte*r (Elshanshoury 1995) resulted in increased better survival of these strains as compared to the individual strains.

Earlier studies performed on the microbes without the involvement of plants indicated that some PGPR combinations allowed the bacteria to interact with each other synergistically thereby providing nutrients and stimulate each other through their gene transfer, metabolic cross- feeding, physical and biochemical activities that might induce some beneficial aspects of their physiology (Bashan 1998). The PGPR consortium employs the principles of natural ecosystem, sustained by their constituents and specific ecological parameters that means the greater the diversity and number of inhabitants, the higher the order of their interaction and more stable the ecosystem. The combinational effect of PGPR causes an effort to shift the

microbiological equilibrium in favour of increased plant growth production, nutrient uptake and protection (Higa 1991; Parr *et al*., 1994).

Hence, from the results obtained in this consortium based experiments, it was obvious that all the three bacteria employed in the consortium interacted synergistically for the increase growth of all the biometric measurements and nutrient uptake, except the shoot length and K uptake of root and shoot compared to single inoculants and uninoculated control. The role of ACC deaminase in the development of a better root system due to hydrolysis the ACC synthesized in the roots, which subsequently affected shoot growth also have been reported for various crops (Glick *et al*., 1998; Belimov *et al*., 2002; Zahir *et al*., 2003). PGPR with IAA and ACC deaminase production promoted root, shoot and other growth indices of rice to a greater extent due to the coordinated action of both IAA and ACC deaminase for growth stimulation (Glick *et al*., 2007; Bal *et al*., 2012). The PGPR production of IAA causes the increased production of ACC through the stimulation of ACC synthetase and increases the root exudation of ACC from plant roots as a consequence of cell wall loosening effect of bacterial IAA. The exuded ACC serves as a unique nitrogen source for the selective proliferation and survival of ACC deaminase positive PGPRs over other soil microbes (Hontzeas *et al*., 2006).Thus, the dual attributes of IAA and ACC deaminase inhabiting PGPR serves as a sink for the ACC and lowers the ethylene production of the plant formed in response to the biotic and abiotic stresses (Saraf *et al*., 2010). Though the N content of plant was increased, there was no statistical significance of N content between the N fixing isolates (NII 0928 and NII 1020) and non-N–fixer (NII 0943). This results was in contrast to the reports of increased N content on inoculation with N-fixing PGPR on various plants (Bhattacharjee *et al*., 2008; Antoun *et al*., 1998; Blaha *et al*., 2006).The N fixation of non-symbiotic -free living organisms are highly limited due to high energy requirement and competition for root exudate outside the nodule environment (Martinez-Viveros *et al*., 2010; Glick 2012). Hence, eventhough the nitrogen fixing capacity of PGPR *in vitro* conditions are shown easily, the green house and field studies results are highly variable and complex (Bottomley and Myrold 2007; Peoples *et al*., 2002; Martinez-Viveros *et al*.,2010).

5.5 Identification and characterization:

The selected PGPR were all catalase positive indicating the high resistance nature towards the environmental, mechanical and chemical stress prevalent in natural soil ecosystem (Kumar *et al*., 2012; Varma and Shahi., 2015).The utilization of 27 types of carbohydrate out of 49 by all the three isolates indirectly revealed the successful survivability of these isolates in rhizospheric region consisting of various types of sugar exudates. The wide range of metabolic activity and variety of C utilization as energy source of bacteria are well suited for the survival in a competitive region such as rhizosphre (Dunfield and Germida 2001).

Some of The extremities prevalent in the soil are pH, temperature and salinity, which influences the microbial activity and their survival.The threes isolated selected shared a wide range of tolerance towards the above stated extremities. These results enabled to utilize these potent strains in acidic soils, mainly prevalent in Kerala state as well as the alkaline soil of up to pH 11.0. The rich forests of the Western Ghats harbor a large portion of India's biological diversity and include most of the endemic species. Western Ghats, coupled with the region's complex geography, produces a great variety of vegetation types. These include scrub forests in the lowlying rain shadow areas and the plains, deciduous and tropical rainforests up to about 1500 meters. Such temporal and climatic selection processes are bound to help in the evolution of a variety of living organisms that are highly adapted to changing extremities of weather, and are known to be a rich source of microbial diversity (Greenland and Losleben 2001). All the three isolates were tolerant to the extreme high temperature of 40^{o}C, while only NII 0928 and NII 0943 were tolerant towards the extreme low of 4^{o}C, while NII 1020 showed good growth at 20^{o}C indicating the application of these inoculants even in the soils with frequent, or sudden temperature fluctuations also. All the selected isolates were tolerant to 7 % (w/v) NaCl, while NII 0943 and NII 1020 tolerated even 10% NaCl again showing an added advantage for the utilization in high – saline soils too. PGPR with inherent genetic ability and physiological adaptation towards the extreme salt concentration, temperatures, drought, soil pH could be employed in the degraded sites for the increased plant production (Maheshwari *et al*., 2012; Yang *et al*., 2009). The acidic pH tolerance of PGPR isolates might have been gained due to the survival on root soils having acidic nature to the CO_2 and organic acid (Curl and Truelove,

1986).Similar physiological characterization of PGPR showing wide range of abiotic stress tolerance have been reported by many researchers (Okanlawon *et al*., 2010; Bhakthavatchalu *et al*., 2013; Anjanadevi *et al*., 2013). The main strategy for stress adaptation of PGPR is a complex multilevel regulatory process in which many genes are involved (Grover *et al*., 2010) and produce heat shock proteins such as GroEL, DnaK, DnaJ, GroES, ClpB, ClpA, ClpX, small heat shock proteins (sHSP), proteases (Munchbach *et al*., 1999), exopolysaccharides (Sandhya *et al*., 2009; Tisdall and Oadea 1982), osmoprotectants such as glutamate, trehalose, proline, glycine beatine, prolinebetaine and ectoine for the modulation of cytoplasmic osmolarity (Talibart *et al*., 1994) and antifreeze proteins, polyols, lipid/fatty acids for cold adaptation (Robinson 2001).

Chapter 6

SUMMARY AND CONCLUSIONS

The current necessity for an alternative to chemical-based fertilizers and pesticides with respect to the environmental and economical point of view, has paved the way to the intensified the research on PGPR technology. The observation and documentation of significant increase and yield on inoculation with these rhizospheric bacteria have been reported for past few decades in the laboratory, greenhouse studies, and to a limited extent in field studies also. Hence, screening strategies for the selection of the best PGPR isolates with high rhizosphere competence plus innate stress tolerance towards the prevailing agro- ecosystems climatic regions are necessary for maximum commercial exploitation of PGPR strains. Forest soils are known for rich microbial diversity, devoid of any industrial and human disturbances.

Over all, in the present study isolation of plant growth promoting rhizobacteria (PGPR) from Western *Ghats* forest soil, characterization for the PGPR attributes under laboratory as well as green net house conditions, talc-based formulation of best isolates with viability evaluation for six months period, characterization of the selected three isolates on the basis of morphological, physiological characteristics and final identification on the basis of 16S rRNA sequence analysis was done. A total of 200 isolates were collected from the rhizospheric region of various plants grown in Western *Ghats* forest soil and 47 isolates were selected on the basis of their phosphate-solubilizing activity on PVK agar. A total of 15 isolates showing more than 300µg/ml of phosphate-solubilisation was then selected for further studies. The key findings from the experimental results are summarised below:

1. Fifteen isolates showed promising phosphate solubilizing activities, which were then further evaluated for IAA, ACC deaminase, phytase, ammonia, HCN, siderophore, chitinase production, N fixation, antifungal activity as well as growth promotion studies on tomato, cowpea, black pepper and cassava crop varieties, which showed as below.

(i) All the 15 strains showed IAA-like auxin production in tryptophan amended growth medium with varied concentrations from low to high production of 16.8-129.1 µg ml^{-1}.

(ii) All the 15 strains showed growth in ACC-supplemented DF medium exhibiting very low to high ACC utilizers from their respective OD_{600} value of 0.08-0.78.

(iii) Chitinase activity was exhibited by one isolate NII 0928, phytase activity was shown by five isolates, *in vitro* antifungal activity by one isolate NII 0906; 11 isolates were positive for siderophore production and nine isolates were positive for HCN production out of the 15 isolates screened. All the 15 isolates were positive for ammonia production with moderate and strong production as shown in the colour of the precipitate settled.

(iv) The growth of seven isolates on N-free agar plate was shown out of the 15 screened. The quantification of fixed nitrogen by the two selected isolates, NII 0928 and NII 1020 were in line with earlier reports on N-fixation.

(v) Fourteen isolates showed significant increase in root length and shoot length of tomato seeds compared to the uninoculated control in tomato-based agar gel-tube under axenic experimental conditions. The root colonization pattern of inoculated isolates was studied qualitatively using SEM analysis and quantitatively using the traditional dilution plate technique. The root colonization pattern from SEM images as well as the good number of colony forming units for the inoculated isolates clearly showed the ability of the isolates to attach strongly over the root surface for their survival. The isolates with increased growth results exhibited a respective increase in the viability represented in colony forming units enumerated using dilution plate method.

(vi) The best nine isolates from the previous tomato-based experiments were then analysed for the growth promotion studies in cowpea under green net house conditions using sterile soil. Among the nine PGPR treatments, highly significant increase in all the biometric

measurements taken was shown for six PGPR treatments. The best PGPR treatments were NII 0943, followed by NII 1020, NII 0928, NII 0906 and NII 0909.The SEM pictures of cowpea root surface clearly depicted the root colonization of isolates in contrast to the uninoculated control root surface as well as the ability of isolates to utilize the root exudates as carbon source for their growth and survival even after 21 days of planting.

(vii) The studies on black pepper stem cuttings with nine isolates showed highly significant growth promotion and total NPK content by four PGPR treatments in terms of all the biometric measurements taken over the uninoculated control. NII 0928 was best in the first set of experiments, followed by NII 0906. In the second batch studies, NII0943 and NII 1020 were statistically on par for all the biometric measurements, except dry weight. NII 0943 showed significant increase in dry weight and total P content over NII 1020.

(viii) The experiments conducted on cassava stem cuttings with three best isolates (from the previous plant based studies) showed highly significant growth promotion in terms of all the biometric measurements taken over the uninoculated control. NII 1020 was highly significant over NII 0943 and NII 0928 for dry weight of stem and plant height. The other biometric measurements taken were all on par for the three PGPR treatments, NII0928, NII 0943 and NII 1020.

2. The maximum increase in the viable cells and high growth rate for all the three best selected isolates was supported by TSB supplemented with 1% glycerol compared to the other two media analysed. Hence, TSB with 1% glycerol was used as growth medium for the bioformulation studies.

3. The storage effect of talc based bioformualtion on the viability of PGPR cells was studied at 4°C and at ambient temperature. The maximum viability was observed at 4°C upto 180 days of storage when compared to ambient temperature storage. The cell viability of NII 0928 and NII 0943 was maintained up to 180 days of ambient storage, while the viability declined

for NII 1020 below the recommended population (10^7 cfu/ g) as per Bureau of Indian Standard (2000).

4. Experiments on the effect of PGPR consortium-based bioformulation of the finally selected three isolates on the growth promotion of black pepper compared to the uninoculated control talc-based formulation and single culture bioformulation conducted in green net house under unsterile soil clearly showed the synergistic, or combinational positive effect of consortium on growth promotion. The consortium showed an increase of root number by 26.9% over NPK control and 13% over the single inoculant NII 0943. The consortium showed an increase of dry root weight of 70.9% over NPK control control, 14.1% over the single inoculant NII 0943 and 15.5% increase over NII 0928. The consortium showed an increase of dry shoot weight of 17.7% over the maximum value attained by the single inoculant NII 0943 and an increase of number of nodes by 43% over the maximum value attained by the single inoculant NII 0928. PGPR consortium showed a significant increase in the root and shoot NP content of black pepper vine over single PGPR as well as uninoculated control treatment, while there was a significant decrease in macro nutrient content over the recommended NPK control treatment.

5. HPLC analysis of culture filtrates revealed the production of multiple organic acids by the efficient phosphate solubilizers. All the three strains varied in the concentrations and types of organic acids secreted into the culture medium. The production of gluconic, citric, formic, succinic and tartaric acids was shown by all the three phosphate solubilizing isolates showing the ability to solubilize tricalcium phosphates through organic acid production.

6. The finally selected three best isolates were then screened for various morphological, physiological and biochemical characteristics. All the three isolates were catalase positive. Two isolates, NII 0943 and NII 1020 were Gram-positive, while NII 0928 was Gram-negative. The carbohydrate utilization profile of the three isolates revealed the high catabolic capability

for different carbohydrate utilization even in the absence of the conventional carbon source. All the three isolates showed growth over a wide range of pH 5.0-10.0, temperature range of 20-40°C and salinity range of 2.5-7.5 % NaCl.NII 0928 and NII 0943 exhibited growth even at 4°C and pH 4.0. Growth of NII 0943 and NII 1020 were observed in 10% NaCl concentrations.

7. The three isolates were genotypically identified on the basis of 16S rRNA gene sequences as *Serratia nematodiphila* NII 0928, *Bacillus tequilensis* NII 0943 and *Kocuria* sp. NII 1020.

The present research work on the isolation and characterization of PGPR isolates from Western *Ghats* forest soil to improve the plant growth revealed the vast functional diversity of PGPRs present in the rhizospheric region of one of the ecological hot spots, which should be further explored and exploited for the benefits of humankind. The growth promoting effect of the selected isolates on tomato, cow pea, black pepper and cassava clearly depicted the broad range of host specificity. The selected three best isolates exhibited multiple PGPR attributes with an added advantage of tolerance towards wide range of pH, temperature and salinity stress accompanied with a strong rhizospheric competency in natural soil serves as a potent tool for further field level studies. Although more research work would be needed to evaluate their efficiency under *in vivo* stressed field experimental conditions, it has been well proved that the relative effectiveness of plant inoculation with single as well as with PGPR consortium showed increased growth promotion and nutrient content of test plants through multiple growth promotive mechanisms harboured by the selected PGPR.

Future research perspectives:

- Quantification of ACC deaminase, chitinase, phytase activity.
- Characterization and quantification of siderophores produced by the isolates.

- Screening for the production of exopolysaccharide (EPS), cytokinin, gibberellins hormone, antioxidant enzymes, amino acids and subsequent further quantification.
- Degradation analysis of organic pollutants present in the rhizospheric region and bioremediation of heavy metals
- Analysis of Induced Systemic Resistance (ISR) to the plants on the treatment with PGPR isolates.
- Mutational analysis of PGPR attributes for the elucidation of the exact mechanism behind growth promotion and rhizosphere colonization.
- Field trials for the commercialization of the PGPR isolates with a point of view on integrated nutrient management of approach involving different combinations of NPK, broad range of host plants, soil types and climatic conditions.

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Appendix I
Culture Media

Pikovskaya agar (g/litre):

Glucose	10.0
$(Ca_3PO_4)_2$	5.0
$(NH_4)_2SO_4$	0.5
KCl	0.2
$MgSO_4$	0.1
$FeSO_4$	0.01
$MnSO_4$	0.01
Yeast extract	0.5
Agar agar	18.0
pH	7.0± 0.2

National Botanical Research Institute's phosphate (NBRIP) liquid growth medium (g/litre):

Glucose	10
$MgCl_2.6H_2O$	5
$MgSO_4.7H_2O$	0.25
KCl	0.2
$(NH_4)_2 SO_4$	0.1
Tricalcium phosphate	5
pH	7.0 ± 0.2

Nutrient broth (g/litre):

Peptic digest of animal tissue	5
NaCl	5
Beef extract	1.5
Yeast extract	1.5
pH	7.0 ± 0.2

Nutrient Agar

Agar of 18g added to 1 L of Nutrient broth

Trypticase soya broth (TSB)(g/litre):

Pancreatic digest of casein	17
Papaic digest of soyabean meal	3
NaCl	5
K_2HPO_4	2.5
Dextrose	2.5
pH	7.3 ± 0.2
TSBG (TSB with 1% glycerol) Glycerol	10ml

Potato Dextrose Broth (g/litre):

Potatoes infusion	200
Dextrose	20
pH	5.6 ± 0.2

DF salts minimal media (per litre):

KH_2PO_4	4.0 g
Na_2HPO_4	6.0 g
$MgSO_4.\ 7H_2O$	0.2 g
Glucose	2.0 g
Gluconic acid	2.0 g
Citric acid	2.0g
$FeSO_4.7H_2O$	1.0 mg
H_3BO_3	10.0 µg
$MnSO_4.H_2O$	11.19 µg
$ZnSO_4.7H_2O$	124.6 µg
$CuSO_4.5H_2O$	78.22 µg
MoO_3	10.0 µg
pH	7.0 ± 0.2

2g of $(NH_4)_2SO_4$ was added to positive control tube.
Filter sterilized ACC with final concentration of 3mM was added to ACC assay tube

Phytase screening medium (g/litre):

Calcium phytate	5
Glucose	10
$(NH_4)_2 SO_4$	0.3
$MgSO_4$	0.5
$CaCl_2$	0.1
$MnSO_4$	0.01
$FeSO_4$	0.01
Agar agar	18
pH	7.0 ± 0.2

Composition of Jensen's medium g/litre

Sucrose	20
Dipotassium hydrogen phosphate	1
Magnesium sulphate	0.5
Sodium chloride	0.5
Ferrous sulphate	0.1
Sodium molybdate	0.005
Calcium carbonate	2
Agar	15

Preparation of Colloidal Chitin (Roberts and Selitrennikoff., 1988)

10g of crab shell chitin was slowly added into 175ml of cold, concentrated HCL and incubated at 4°C for 24hours with vigourous stirring.This suspension was added to 1L cold ethanol for 24 hours incubation at -20°C .The chitin suspension was filtered using Whatman No.1 filter paper and washed with sterile distilled water until the pH becomes neutral. The washed chitin was then dried at 90°C for overnight and crumbed using porcelain mortar.

Preparation of Chitin agar plate (Roberts and Selitrennikoff., 1988)

100mg of colloidal chitin was homogenized after adding to 20ml of distilled water using a glass homogenizer. Nutrient broth of 1.6g and agar of 1.8g were added to 80ml of distilled water. The colloidal chitin suspension was then added to Nutrient broth- agar solution and autoclaved.

CAS agar

CAS agar prepared from three solutions which were sterilized separately before mixing. All the glass-wares were acid washed with 16% HCl.

Fe-CAS indicator solution **(solution 1):**

a) Chrome Azurol S : 60.5mg/50mL distilled Water
b) $FeCl_3.6H_2O$: 10ml(1mM in 10mM HCL)
c) Hexadecyltrimethyl-ammonium bromide : 72.9mg/40mL Distill Water

Basal Agar Medium **(Solution 2):**

Dissolved 30.24 g of PIPES (0.1M) (Piperazine bisdiethene sulphonic acid) in 750 ml of a salt solution containing 0.3 g KH_2PO_4,0.5g of L- asparagine, 0.5 g NaCI, and 1.0 g NH_4C1. The pH was adjusted to 6.8 with 6M NaOH, and water was added to bring the volume to 880 ml. The solution was autoclaved after adding 15 g of agar and then cooled to 50°C.

50% glucose solution (**Solution 3):**

Solution 3 was autoclaved, cooled to 50°C, then 20 ml of the solution was added to the basal agar solution (Solution 2). The indicator solution (Solution 1) was added last, with sufficient stirring to mix the ingredients without forming bubbles.

Appendix II

List of major instruments

Instruments	Model
Autoclave	HVE-50, Hirayama, NewYork
Bioimager	BD Pathway^{TM} Bioimager Attovision 1.5.3-BD Biosciences, USA
Centrifuge	Eppendorf, Germany; Kuboto, Japan, Beckman Coulter, USA
Deep freezer (-86^{0}C)	Operon, Korea
DNA Sequencer	ABI 3100, Applied Biosystems, USA
Electron Microscope	JEOL JSM 5600LV, 115, Japan
Electrophoresis unit	Biorad, India
Gel documentation system	Chemi doc, Biorad, USA
Hot air Oven	Kemi Instruments, India
HPLC	Schimadzu, Japan
Incubator	Infors HT, Ecotron, Switzerland, MCO-2OAIC-SANYO Electric Co Ltd,JAPAN
Laminar air Flow Chamber	Clean air System, India
PCR machine	ep gradient-Eppendorf, India
pH meter	Systronics, India
Scanning electron microscope	Carl Zeiss EVO 18, Germany
UV-VIS Spectrophotometer	UV-160A, Schimadzu, Japan
Weighing balance	Mettler Toledo, Mumbai, India
Kjeldhal Nitrogen Analyser	(Kelplus system classic Dx[VA],Pelican Equipments,India)
Flame Photometer -128	(Systronics, India)

Appendix III

List of Publications in International Journals

1. **Deepa C K,** Syed G Dastagar and Ashok Pandey (2011) Growth enhancement of black pepper (*Piper nigrum*) by a newly isolated *Bacillus tequilensis* NII-0943. *Biologia* 66: 801—806.

2. **Deepa C K**, Syed G Dastager and Ashok pandey (2010). Isolation and characterization of plant growth promoting bacteria from non-rhizospheric soil and their effect on cow pea (*Vigna ungiculata* (L) Walp) seedling growth. *World Journal of Microbiology and Biotechnology* 26: 1233-1240**.**

3. **Deepa C K,** Syed G Dastagar and Ashok Pandey (2010). Plant growth promoting activity in newly isolated *Bacillus thioparus* (NII-0902) from Western ghats forest, India. *World Journal of Microbiology and Biotechnology* 26: 2277- 2283.

4. **Deepa C K,** Syed G Dastagar and Ashok Pandey (2010) Isolation and characterization of novel plant growth promoting *Micrococcus sp* NII-0909 and its interaction with cowpea .*Plant Physiology and Biochemistry* 48 : 987- 992.

5. Syed G Dastager, **Deepa C.K,** Nautiyal C.S, Puneet S.C and Ashok Pandey (2009). Isolation and characterization of Plant growth-promoting strain *Pantoea* NII-186. From Western Ghat Forest soil, India. *Letters in Applied Microbiology* 49: 20-25.

6. Syed G Dastager, **Deepa C.K** and Ashok Pandey (2009) Isolation and characterization of high-strength phenol degrading novel bacterium of *Pantoea* genus. *Bioremediation Journal* 13:171-179.

7. Syed G Dastager, **Deepa C K** and Ashok Pandey (2010). Characterization of plant growth promoting rhizobacterium *Exiguobacterium* NII 0906 for its growth promotion of cow pea (*Vigna ungiculata*). *Biologia* 65:197-203.

8. Syed G Dastagar, Q. S. Raziuddin, **Deepa C K**, Wen-Jun Li and Ashok Pandey(2010). *Pontibacter niistensis* sp. nov., isolated from forest soil *International Journal of Systematic and Evolutionary Microbiology* 60: 2867-2870**.**

9. Syed G Dastagar, **Deepa C K** and Ashok Pandey (2011). Potential plant growth promoting activity of *Serratia nematodiphila* NII-0928 on black pepper (*Piper nigrum*). *World Journal of Microbiology and Biotechnology* 27: 259-265.

10. Syed G.Dastager, **Deepa C.K**, Wen-Jun Li, Shu-Kun Tang and Ashok Pandey (2011) *Paracoccus niistensis* sp nov., isolated from forest soil, India. *Antonie van Leeuwenhoek* 99: 501-506.

Chapter in edited books

1. Syed G Dastager and **Deepa C.K** (2009) Exploiting Bacterial Traits for Plant Growth-Promoting Activity in Agriculture. *Current Topics on Bioprocesses in Food Industry*. Pp 183-192. Asiatech Publishers Inc. New Delhi, India.

Participation in Conferences/Workshops with papers presented as senior author

International

1. **Deepa C.K,** Syed G Dastager, Ashok Pandey (2011). Effect of *Serratia* and *Kocuria* species on biometric characters of Tapioca **(***Manihot esculenta* crantz). International Conference on New Horizons in Biotechnology (NHBT 2011), CSIR NIIST, Trivandrum.India.

2. **Deepa CK,** Himani Jayamurthy, Syed G Dastager, Ashok Pandey (2010).Potential plant growth promoting activity of Serratia nematodiphila NII 0928 on black pepper (Piper nigrum L.)7th BRSI Convention & International Conference on Genomic Sciences & Indo-Italian Workshop on Industrial and Pharmaceutical Biotechnology November 12-14, 2010, Madurai Kamaraj University, Madurai. India.

3. **Deepa C.K,** Syed G Dastager, Ashok Pandey (2009). Isolation and characterization of Plant growth-promoting Rhizobacteria from Western Ghat Forest soil, India. First ASEAN PGPR conference for sustainable agriculture. June 21-24, 2009. Acharya NG Ranga, Agricultural University, Hyderabad. India

4. **Deepa C K,** Syed G Dastager, Suganya S and Ashok Pandey (2008) Screening of plant growth-promoting rhizobacteria from Western Ghat soils. 5th BRSI Convention & International Conference on Bioprocesses in Food Industries. November 6-8, 2008 Osmania University, Hyderabad. India.

Regional

1. **Deepa C.K,** Syed G Dastager, Ashok Pandey (2010). Exploration of plant growth promoting *Rhizobacteria* from Western Ghats soil, India. Proceedings of 22nd Kerala Science Congress, 28-31 January 2010, KFRI, Peechi, pp. 35-36.

Others:

1. Attended the training programme on XXV Winter School on Plant Genetic Engineering held on September 03-17, 2012 at UGC – Networking Resource Centre in Biological Sciences, School of Biological Sciences, Madurai Kamaraj University, Madurai.