

STUDY OF RHIZOBACTERIAL COMMUNITY STRUCTURE AND EVALUATION OF PLANT GROWTH PROMOTING RHIZOBACTERIAL APPLICATION STRATEGIES TO PROMOTE GROWTH AND YIELD IN APPLE

Thesis

by

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(F-2012-23-D)**

Submitted to



**Dr. YASHWANT SINGH PARMAR UNIVERSITY OF
HORTICULTURE & FORESTRY
SOLAN (NAUNI) HP-173 230 INDIA**

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CERTIFICATE-I

This is to certify that the thesis entitled, “**Study of rhizobacterial community structure and evaluation of plant growth promoting rhizobacterial application strategies to promote growth and yield in apple**”, submitted in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY MICROBIOLOGY** to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) is a bonafide record of research work carried out by **Ms Shalini Chandel (F-2012-23-D)** daughter of Sh Diwan Chandel under my guidance and supervision. No part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of investigation has been fully acknowledged.

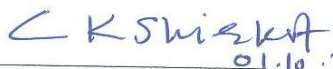
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This is to certify that the thesis entitled, "Study of rhizobacterial community structure and evaluation of plant growth promoting rhizobacterial application strategies to promote growth and yield in apple", submitted by Ms Shalini Chandel (F-2012-23-D) daughter of Sh Diwan Chandel to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.), in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY MICROBIOLOGY** has been approved by the Student's Advisory Committee after an oral examination of the same in collaboration with the external examiner.


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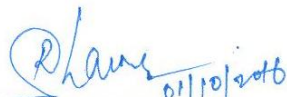
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This is to certify that all the mistakes and errors pointed out by the external examiner have been incorporated in the thesis entitled, **"Study of rhizobacterial community structure and evaluation of plant growth promoting rhizobacterial application strategies to promote growth and yield in apple"** submitted to Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan (H.P.) by **Ms Shalini Chandel (F-2012-23-D)** daughter of Sh Diwan Chandel in partial fulfilment of the requirements for the award of degree of **DOCTOR OF PHILOSOPHY MICROBIOLOGY.**



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ABBREVIATIONS

%	:	Per cent
µg	:	Micro gram
°C	:	Degree centigrade
CAS	:	Chromo azarol-S
cfu	:	Colony forming units
cm	:	Centimeter
CRD	:	Completely randomized design
EDTA	:	Ethylenediaminetetraacetic acid
ER	:	Endorhizosphere
g	:	Gram
<i>gdh</i>	:	Glucose dehydrogenase gene
h	:	Hour
HCl	:	Hydrochloric acid
HCN	:	Hydrogen cyanide
HDTMA	:	Hexadecyltrimethylammoniumbromide
K	:	Potassium
l	:	Litre
M	:	Molar
MEA	:	Malt extract agar
mg	:	Milligram
min	:	Minute
ml	:	Millilitre
mm	:	Millimeter
mM	:	Millimolar
N	:	Nitrogen
NA	:	Nutrient agar
<i>nif</i>	:	Nitrogen fixing gene
nm	:	Nano meter
O.D.	:	Optical density
P	:	Phosphorous
PCR	:	Polymerase chain reaction
PGP	:	Plant growth promoting
PGPR	:	Plant growth promoting rhizobacteria
PGRs	:	Plant growth regulators
ppm	:	parts per million
PSB	:	Phosphate solubilizing bacteria
psi	:	Per square inch
PVK	:	Pikovskaya's medium
rpm	:	Rotation per minute
RS	:	Rhizosphere soil
sp.	:	Species
TCP	:	Tri-calcium phosphate
UV	:	Ultra violet
w/v	:	Weight by volume
v/v	:	volume by volume

Chapter-1

INTRODUCTION

Agriculture contributes to a major share of national income and export earnings in many developing countries, while ensuring food security and employment. Sustainable agriculture is vitally important in today's world because it offers the potential to meet our future agricultural needs, something that conventional agriculture will not be able to do. Recently there has been a great interest in eco-friendly and sustainable agriculture. The microorganisms with the aim of improving nutrients availability for plants are an important practice and necessary for agriculture. Plant growth promoting rhizobacteria are known to improve plant growth in many ways when compared to synthetic fertilizers, insecticides and pesticides. They enhance crop growth and can help in sustainability of safe environment and crop productivity (Glick *et al.* 1999; Podile and Kishore, 2007).

During the past couple of decades, plant growth-promoting rhizobacteria (PGPR) have been used to reduce or replace the use of chemicals in agriculture, horticulture, silviculture, and environmental cleanup strategies. Scientific researches involve multidisciplinary approaches to understand adaptation of PGPR, effects on plant physiology and growth, induced systemic resistance, biocontrol of plant pathogens and biofertilization. This is due to the emerging demand for dependence diminishing of synthetic chemical products, to the growing necessity of sustainable agriculture within a holistic vision of development and to focalize environmental protection (Prathap and Ranjitha, 2015).

Apple (*Malus domestica* Borkh.) is one of the most important fruit crops grown and consumed in the world. Apple culture covers almost the whole region of the temperate climate in the Northern and Southern Hemispheres (Way *et al.* 1990). Among the various temperate fruits grown in hills and mountains of India, apple is considered as an important temperate crop on the basis of its high consumption and nutritional value to consumers. In Himachal Pradesh, apple crop occupies an area of 1,07,700 hectare with a production of 738700 metric tons (NHB, 2014). The apple crop has brought prosperity to state by contributing about 1500 crore in gross domestic production. As per national horticulture database India, average apple production (2013-2014) was 24, 97,000 metric tonnes. In the year 2010, India produced 17, 77, 200 tonnes of apple. Apple cultivation in 2014 occupied an

area of 3, 13,000 hectare of the total 72, 16,000 hectare of land under horticulture indicates the importance of apple industry in Himachal Pradesh (NHB, 2014).

Despite spectacular progress made in area expansion and acceleration of apple production, India's productivity lags behind, when compared to other countries. The prominent reason being the indiscriminate use of chemical fertilizers and emergence of several fungal diseases. Moreover, the use of inorganic fertilizers results in the disturbance of rhizosphere bio-diversity. At present more than 30 per cent of the plantations have completed their productive life. Apple orchard rejuvenation projects are therefore being ambitiously launched by State agencies for restoring six decade old planted apple orchards. The utilization of plant growth promoting rhizobacteria (PGPR) may help to develop an environmentally benign biological approach for managing fungal diseases and enhancing the plant health resulting in increase in yield. The application of plant growth promoting rhizobacteria (PGPR) on horticulture tree species is gaining importance (Aslantas *et al.* 2007; Karlidag *et al.* 2007) as it is a widely accepted fact that PGPR play a key role in plant health and nutrition.

Most microbial diversity of the soil ecosystem is confined to the rhizosphere. The importance of the rhizosphere in soil diversity research is that the root act as “disturbance” (Bazzar, 1996) such that the rhizosphere selects for particular groups or guilds of organism. Rhizobacterial diversity is influenced by both plant and soil type. Although the main diversity of life has been proven to be microbial, the vast majority of soil bacteria still remain unknown due to the fact that only a minor percentage of naturally occurring microorganisms can be cultured (Pace, 1997).

The development and improvement of molecular techniques have provided accurate knowledge of bacteria in their natural environment, refining microbial ecology and generating new questions about the roles and functions of bacteria in the rhizosphere (Lagos *et al.* 2015). Molecular techniques based on DNA analysis provide important tools to evaluate bacterial community composition, identifying the abundance of genes that could be involved in rhizosphere processes. The 16S rRNA gene libraries and profile using amplified 16S rDNA restriction analysis (ARDRA), PCR-RFLP have provided valuable view of microbial diversity that allows comparison of communities in different habitats (Pommier *et al.* 2005, Lawongsa *et al.* 2008; Aranda *et al.* 2011; Passos *et al.* 2014; Franke *et al.* 2015).

In the last ten years, a number of PGPR that have been identified has seen a great boost, mainly because the role of the rhizosphere as an ecological unit has gained importance in the functioning of the biosphere and also because mechanisms of action of PGPR have been deeply studied. A putative PGPR qualifies as PGPR when it is able to produce a positive effect on the plant upon inoculation, hence demonstrating good competitive skills over the existing rhizosphere communities. PGPR influence direct growth promotion of plants by fixing atmospheric nitrogen, solubilizing insoluble phosphates, secreting hormones such as IAA, GAs, and Kinetins besides ACC (1-Aminocyclopropane-1-carboxylic acid) deaminase production, which helps in regulation of ethylene. Induced systemic resistance (ISR), antibiosis, competition for nutrients, parasitism, production of metabolites (hydrogen cyanide, siderophores) suppressive to deleterious rhizobacteria are some of the mechanisms that indirectly benefit plant growth (Jha and Saraf, 2015).

Therefore, keeping this in view, the present study envisages better understanding the dynamics of rhizobacterial community associated with perennial crop such as apple plant and also to prove the hypothesis that the root exudates of apple plant exert some kind of selective pressure on the proliferation of specific kind of rhizobacteria on the surface of the root as well as within the root (endorhizosphere) and PGPR mediated mechanisms of plant growth promotion in order to develop a sustainable PGPR based technology, using single strain formulation with multifunctional PGP traits to address the long-standing desire of farmers/orchardists to ensure that their land remains productive in future. The study aims to determine the structure and functional diversity of PGPR inhabiting the rhizosphere and roots of apple trees and to manage soil health, key issues of apple crop disease management and maximising profitability over the short (minimising the use of chemicals) and long term (increasing the apple yield).

Therefore, the specific objectives of the present study were:

1. Structural and functional rhizobacterial diversity associated with apple replant sites and root rot infected diseased apple orchards.
2. Field evaluation of plant growth promoting rhizobacteria on growth, yield and biocontrol in root rot infected apple orchards.
3. Monitoring the persistence of inoculated PGPR in apple orchards and profiling of PGPR associated with apple rhizosphere.

Chapter-2

REVIEW OF LITERATURE

Plant growth in agricultural soils is influenced by many abiotic and biotic factors (Nelson, 2004). There is a thin layer of soil immediately surrounding plant roots that is an extremely important and active area for root activity and metabolism which is known as rhizosphere. The rhizosphere, is defined as the soil zone under the influence of plant roots, a site of high microbial activity, characterized by a great array of complex and dynamic physical, chemical and biological interactions. In the rhizosphere, microorganisms have an important role in the organic matter transformations and biogeochemical cycles of plant nutrients. In this context, a substantial number of bacterial species interact with their host plants and may exert beneficial effects on plant growth, plant nutrition and disease suppression (Avis *et al.* 2008; Pii *et al.* 2015).

A large number of microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere. Bacteria are the most abundant among them. Plants select those bacteria contributing most to their fitness by releasing organic compounds through exudates (Lynch, 1990) creating a very selective environment where diversity is low (Marilley and Aragno, 1999; Garcia *et al.* 2004; McCully, 2005). Since bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the plants physiology to a greater extent, especially considering their competitiveness in root colonization (Antoun and Kloepper, 2001; Barriuso *et al.* 2008).

Microorganisms that colonize the rhizosphere can be classified according to their effects on plants and the way they interact with roots, some being pathogens whereas others trigger beneficial effects. Rhizobacteria inhabit plant roots and exert a positive effect ranging from direct influence mechanisms to an indirect effect. So, the bacteria inhabiting the rhizosphere and beneficial to plants are termed PGPR (Kloepper, 1980). In the last few years, the number of PGPR that have been identified has seen a great increase, mainly because the role of the rhizosphere as an ecosystem has gained importance in the functioning of the biosphere. Various

species of bacteria like *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been reported to enhance the plant growth (Kloepper *et al.* 1989; Okon and Vanderleyden, 1994).

The use of PGPR offers an attractive way to replace chemical fertilizers, pesticides, and supplements; most of the isolates result in a significant increase in plant height, root length, and dry matter production of shoot and root of plants. PGPR help in the disease control in plants. Some PGPR especially if they are inoculated on the seed before planting, are able to establish themselves on the crop roots. PGPR as a component in integrated management systems in which reduced rates of agrochemicals and cultural control practices are used as biocontrol agents. Such an integrated system could be used for transplanted vegetables to produce more vigorous transplants that would be tolerant to nematodes and other diseases for at least a few weeks after transplanting to the field (Kloepper *et al.* 2004). Selected strains of beneficial PGPR trigger a plant mediated induced systemic resistance (ISR) response that is effective against a broad spectrum of plant pathogens. ISR is a plant-mediated mechanism that resembles classic pathogen-induced resistance, in which non-infected parts of previously pathogen-infected plants become more resistant to further infection (Pieterse *et al.* 2001). In forestry, the potential of inoculating tree roots with PGPR has been recognised. There has been a new focus on investigating the application of PGPR and fungi to commercial forestry operation especially in the areas of enhancing tree growth and survival of tree seedlings through microbially mediated phytohormone production (Tizzard *et al.* 2006).

Nowadays, PGPRs represent an attractive alternative for chemical fertilizers as biofertilizers, phytostimulators, rhizoremediators and phytopathogen biocontrol agents (Lugtenberg and Kamilova, 2009). Despite the perception that PGPR are considered as not dangerous to the environment, their mass application in agriculture have been limited due to their low efficiency in field level applications (Babalola and Oluwamodupe, 2012). There is still a lack of information on PGPR ecology (diversity, competence, distribution, communication, etc.) in the rhizosphere (Compant *et al.* 2005; Lambers *et al.* 2009; Singh *et al.* 2011); however, a considerable interest has been generated in their study and potential use in crop production around the world (Morrissey *et al.* 2004). Currently, it is recognized that the rhizosphere microbiome harbors thousands of different bacterial, archaeal, viruses, fungal and other

eukaryotic taxa. A gram of rhizosphere soil might contain around 10⁹ microbial units and 10⁶ distinct taxa (Torsvik *et al.* 2002; Curtis and Sloan, 2005). The use of molecular techniques have allowed the characterization of unculturable organisms at a level never seen before, since only 1% of soil microorganism have actually been cultured (Ver Berkmoes *et al.* 2009; Van Elsas and Boersma, 2011). Molecular techniques based on DNA analyses provide important tools to evaluate bacterial community composition, identifying the abundance of genes that could be involved in rhizosphere processes.

Ecology of Bacteria in the Rhizosphere; Abundance and Diversity

The rhizosphere is the zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root. This zone is about 1 mm wide, but has no distinct edge. Rather, it is an area of intense biological and chemical activity influenced by compounds exuded by the root, and by microorganisms feeding on the compounds. The rhizosphere harbors a wide variety of bacteria species, and the compositions of bacterial communities differ according to root zone, plant species, plant phenological phase, stress and disease events (Rovira, 1965; Hinsinger *et al.* 2009; Marschner *et al.* 2011). Among them, Proteobacteria are considered to be the most abundant bacterial group in the rhizosphere due to their ability to respond to labile C sources, showing fast growth and adaptation to the diverse plant rhizospheres (Bulgarelli *et al.* 2013; Chaparro *et al.* 2014; Peiffer *et al.* 2013).

Soil microbial communities are often difficult to characterize, mainly because of their immense phenotypic and genotypic diversity. Bacterial populations in upper layers of the soil can contain as many as 10⁹ cells per gram of soil (Torsvik *et al.* 2002). Most of these cells are unculturable. The fraction of the cells making up soil microbial biomass that have been cultured and studied in detail is negligible and often comprises less than 5% of the total population (Borneman *et al.* 1997).

The microbial population in and around the roots includes bacteria, fungi, yeasts and protozoa. Some are free living while others form symbiotic associations with various plants. Rhizosphere microbial populations could be regarded as a stable community around a particular plant species in a specific soil, or alternatively, as a succession of populations. The interaction between these microorganisms and the roots of the plant may be beneficial, harmful or neutral

for the plant, and sometimes the effect of microorganisms may vary as a consequence of soil conditions (Singh and Varaprasad, 2008). There are various approaches to study microbial diversity, which can be broadly divided into (a) cultivation-based methods and (b) cultivation-independent methods (Ahmad *et al.* 2008). Both approaches have their own unique limitations and advantages. Traditional methods to study microbial diversity have been based on cultivation and isolation. For this purpose, a wide variety of culture media have been designed to maximize the variety and populations of microorganisms. A Biolog based method applied for directly analyzing the potential activity of soil microbial communities, called community-level physiological profiling (CLPP), was used to study microbial diversity (Kennedy and Smith, 1995).

Molecular technology has helped to better understand microbial diversity. These molecular techniques include polymerase chain reaction (PCR) or real-time polymerase chain reaction (RT-PCR), which is used to target the specific DNA or RNA in soil. The 16S or 18S ribosomal RNA (rRNA) or their genes (rDNA) represent useful markers for prokaryotes and eukaryotes, respectively. The mixed PCR products can be used for (a) preparing clone libraries and (b) a range of microbial community fingerprinting techniques. Such clone libraries are useful to identify and characterize the dominant bacterial or fungal types in soil and thereby provide a picture of diversity. Moreover, a range of other techniques have been developed to fingerprint soil microbial communities, for example, Density gradient gel electrophoresis or temperature gradient gel electrophoresis amplified rDNA restriction analysis (ARDRA) and ribosomal intergenic spacer length polymorphism (RISA) (Ranjard and Richaume, 2001).

Jha *et al.* (2010) had reported that a good diversity index should encompass both Dominance indices (Example, Simpson index) and Information indices (Example, Shannonwiener index). To comprehend such diversity and to understand their role and distribution in ecology it is advantageous to investigate the combined uses of species richness and diversity .

Passos *et al.* (2014) evaluated the diversity of cultivable plant growth-promoting (PGP) bacteria associated with apple trees cultivated under different crop management systems and their antagonistic ability against *Colletotrichum gloeosporioides*. Samples of roots and

rhizospheric soil from apple trees cultivated in organic and conventional orchards in southern Brazil were collected, together with soil samples from an area never used for agriculture (native field). Bacteria were identified at the genus level by PCR-RFLP and partial sequencing of the 16S rRNA, and were evaluated for some PGP abilities. The most abundant bacterial genera identified were *Enterobacter* (27.7%), *Pseudomonas* (18.7%), *Burkholderia* (13.7%), and *Rahnella* (12.3%).

Tahir *et al.* (2015) reviewed the diversity of rhizosheath-associated bacteria of wheat grown under wheat-cotton and wheat- rice rotation by cultivation, as well as by 454-pyrosequencing of PCR-tagged 16S rRNA gene amplicons. Bacterial isolates predominantly belonging to the genera *Arthrobacter*, *Azospirillum*, *Bacillus*, *Enterobacter* and *Pseudomonas* were obtained. Analysis of pyrosequencing data revealed Proteobacteria, Bacteroidetes and Verrucomicrobiaas the most abundant phyla in wheat-rice rotation, whereas Actinobacteria, Firmicutes, Chloroflexi, Acidobacteria, Planctomycetes and Cyanobacteria were predominant in wheat-cotton rotation. From a total sequences, 10.9% showed 97% similarity with 16S rRNA genes of 32 genera previously shown to include isolates with plant growth promoting activity (nitrogen fixation, phosphate-solubilization, IAA production). Among these, the most predominant genera were *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Bacillus*, *Cyanobacterium*, *Paenibacillus*, *Pseudomonas* and *Rhizobium*.

Jian *et al.* (2015) used a next-generation, Illumina-based sequencing approach to characterize the bacterial community development of apple rhizosphere soil in a replant site (RePlant) and a new planting site (New Plant) in Beijing.. More than 16,000 valid reads were obtained for each replicate, and the community was composed of five dominant groups (Proteobacteria, Acidobacteria, Bacteroidetes, Gemmatimonadetes and Actinobacteria). The bacterial diversity decreased after apple planting.. *Verrucomicrobia* were less abundant in RePlant soil, while *Pseudomonas* and *Lysobacter* were increased in RePlant compared with ReSoil and NewPlant. Their experimental results suggest that planting apple nurseries has a significant impact on soil bacterial community development at both replant and new planting sites, and planting on new site resulted in significantly higher soil urease activity and a different bacterial community composition.

Shanmugam *et al.* (2011) conducted a culture-independent survey of the bacterial diversity in rhizosphere soils of diseased (scab) and disease free apple trees to assess the role of bacteria in disease suppression. Community DNA was extracted from soil samples and amplified by PCR using primers specific for bacterial 16S rRNA gene sequences. Clone libraries were constructed with the PCR products and analysed based on amplified rDNA restriction analysis (ARDRA) patterns. The phylotypes and their frequency distribution in both libraries indicated that the phylotype did not represent a single group. Rarefaction curve, and Shannon and Simpson diversity indices exhibited insignificant diversity differences between the samples in terms of bacterial community composition, whereas more chitinase and β -1,3 glucanase activities were recorded in samples from disease-free trees than from diseased trees. Based on the operational taxonomic units identified in ARDRA, 80 representative clones were selected from the libraries and partially sequenced. Sequence similarity searches with the resulting sequences identified the dominance of uncultured bacteria to the extent of 70% and 72.5% in disease free and diseased rhizospheres, respectively. The foregoing studies conclude a possible role for enhanced microbial activity in terms of enzyme production in tree (apple) health, although no distinct partitioning of composition or significant diversity of bacterial communities inhabiting diseased and disease-free rhizosphere soils was observed at any given time.

Plant Growth Promoting Rhizobacterial Forms

Plant growth promoting rhizobacteria can be classified into extracellular plant growth promoting rhizobacteria (ePGPR) and intracellular plant growth promoting rhizobacteria (iPGPR) (Viveros *et al.* 2010). The ePGPRs exist in the rhizosphere, on the rhizoplane or in the spaces between the cells of root cortex while iPGPRs are located generally inside the specialized nodular structures of root cells. The bacterial genera such as *Agrobacterium*, *Arthrobacter*, *Azotobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Caulobacter*, *Chromobacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Pseudomonas* and *Serratia* belongs to ePGPR (Ahemad and Kibret, 2014). The iPGPR belongs to the family of Rhizobiaceae includes *Allorhizobium*, *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*, endophytes and *Frankia* species both of which can symbiotically fix atmospheric nitrogen with the higher plants (Bhattacharyya and Jha, 2012).

Plant Growth Promotion: Mechanism of Action

Plant growth promotion by plant growth promoting rhizobacteria is a well-known phenomenon and this growth enhancement is due to certain traits of rhizobacteria. There are a number of mechanisms used by PGPR for enhancing plant growth and development in diverse environmental conditions (Fig. 1). According to Kloepper and Schroth (1981) plant growth promoting rhizobacteria mediated plant growth promotion occurs by the alteration of the whole microbial community in rhizosphere niche through the production of various substances. Generally, plant growth promoting rhizobacteria promote plant growth directly (Fig. 1) by either often due to their ability for nutrient supply (nitrogen, phosphorus, potassium and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents, root colonizers, and environmental protectors (Kloepper and Schroth,1981).

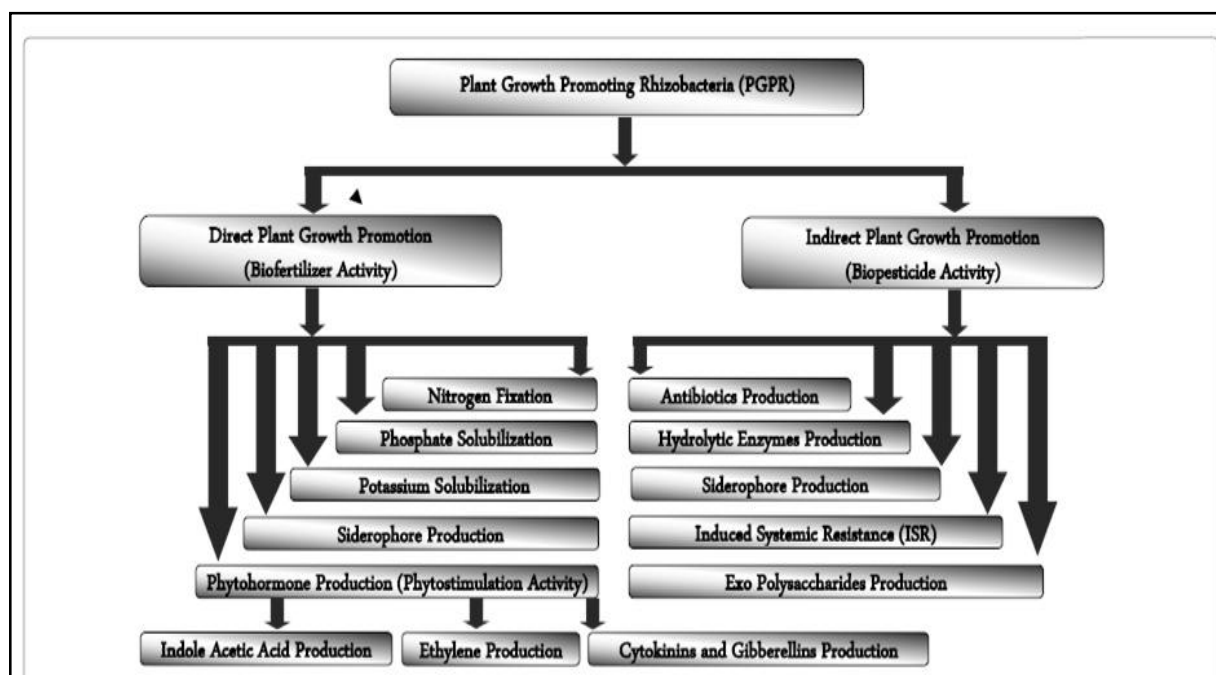


Figure.1. Schematic diagram showing plant growth promoting bacteria affect plant growth directly and indirectly

Based on their mechanism of action, PGPRs can be categorized into three general forms such as biofertilizer, phytostimulator and biopesticide (Fig. 2).

PGPR forms	Definition	Mechanism of action	References
Biofertilizer	A substance that contains live microorganisms which, when applied on the seed, plant surface or soil, colonizes the rhizosphere and promote plant growth through increased supply of primary nutrients for the host plant	Biological nitrogen fixation Utilization of insoluble phosphorus	Vessey (2003) Somers et al. (2004)
Phytostimulator	Microorganism, with the ability to produce phytohormones such as indole acetic acid, gibberellic acid, cytokinins and ethylene	Production of phytohormones	Lugtenberg et al. (2002), Somers et al. (2004)
Biopesticide	Microorganisms that promote plant growth by controlling phytopathogenic agents	Production of antibiotics, siderophores, HCN Production of hydrolytic enzymes Acquired and Induced systemic resistance	Vessey (2003) Somers et al. (2004) Chandler et al. (2008)

Figure. 2. Forms of PGPR and their mechanism of action stimulating plant growth

Microbial Effects on Plant Nutrient Acquisition : Direct Plant Growth Promotion

Rhizobacteria as PGPR can play an important role in promoting nutrient acquisition by plants, favoring factors inducing root biomass accumulation and/or hindering those that could have detrimental effects on root system development. Plant growth promoting rhizobacteria having direct mechanisms that facilitate nutrient uptake or increase nutrient availability by nitrogen fixation, solubilization of mineral nutrients, mineralize organic compounds and production of phytohormones (Arora *et al.* 2012; Bhardwaj *et al.* 2014).

PGPR mediated nitrogen acquisition in host plants

Nitrogen occurs in soil in both organic and inorganic forms and in addition to marked seasonal changes is characterised by a heterogeneous distribution within the soil. Nitrogen inputs through fixation reactions (by either symbiotic microorganisms or potentially through free-living diazotrophs, as discussed in this review) and transformations of N between different pools have important implications for plant growth and for the loss of N from soil systems (Jackson *et al.* 2008). Microbial mediated mineralization of organic forms of N to ammonium (NH_4^+) and its subsequent nitrification to nitrate (NO_3^-) is of major significance to N availability and has influence on root behaviour and rhizosphere dynamics.

Microbial communities are a main component of ecosystems that play critical roles in the biochemical transformations of elements including nitrogen fixation (Madigan *et al.* 2000). Therefore, nitrogen that is available to plants grown for many years without N fertilizers is

considered to be due to biological fixation (James, 2000). Diazotrophs (i.e. N₂-fixing bacteria) are classified as being either symbiotic (*Rhizobia* and *Frankia* species) or as free-living (associative) and/or root endophytic microorganisms (Lethbridge and Davidson 1983; Rao *et al.* 1998; Cocking, 2003). Several groups of symbiotic N₂ fixing bacteria have been identified in soils and flooded systems, such as *Azotobacter*, *Azomonas*, *Beijerinckia*, *Derxia* (aerobic), *Azospirillum*, *Aquaspirillum*, *Thiobacillus*, *Pseudomonas*, *Xanthobacter*, *Rhizobium*, *Methylosinus*, *Mycobacterium* (Microaerobic), *Klebsiella*, *Erwinia*, *Enterobacter*, *Citrobacter*, *Escherichia*, *Bacillus* (facultative anaerobic) and *Desulfovibrio*, *Desulfotomaculum*, *Clostridium* anaerobic, with some being endophytes. Endophytic diazotrophs may have advantage over root-surface associated organisms, as they can colonize the interior of plant roots and establish themselves within niches that are more conducive to effective N₂ fixation and subsequent transfer of the fixed N to host plants (Stewart, 1980; Havelka *et al.* 1982; Zani *et al.* 2000; Vessey, 2003; Zehr *et al.* 2003; Mirza *et al.* 2006; Bashan and de-Bashan, 2010; Reinhold-Hurek and Hurek, 2011)

The process of N₂ fixation, catalyzed by nitrogenase enzymes is essential for maintaining fertility in many ecosystems (Vitousek and Howarth, 1991; Bagwell and Lovell, 2000; Engelhard *et al.* 2000; Steenhoudt and Vanderleyden, 2000). *Rhizobia* develop symbiotic relationships with host legumes and through atmospheric N₂ fixation within nodules can provide up to 90% of the N requirements of the plant (Franché *et al.* 2009; Höflich *et al.* 1994). Free living N₂ fixers also have the potential for providing N to host plants but so far, the direct contribution of N fixation by diazotrophs to the N nutrition of plants and subsequent growth promotion remains in question.

PGPR mediated phosphate acquisition in host plants

Phosphorus (P) is one of the essential macronutrients required for plant growth and development and is intimately involved in a wide range of physiological and biochemical processes. This includes; cellular energy systems, structure and function of nucleic acids, membrane integrity and sugar metabolism (Ehrlich, 1990; Richardson, 2009). However, large areas of soil used for agriculture across the world are deficient in plant-available forms of phosphate which can limit agricultural production (Runge-Metzger, 1995). Cordell *et al.* (2009)

reviewed that of 17.5 million tonnes of P is processed annually from world reserves of rock phosphates 85% is used in the production of fertilizer and suggested that the world may experience a peak in P supply as early as 2033, despite on-going demand for P to support increased food production. For many countries, particularly developing nations where the cost of P-fertilizers is already preclusive, this puts further strain on the long-term sustainability and economic viability of agriculture and highlights the need to consider possible alternatives and future options (Cordell *et al.* 2009b). In addition to the limited global reserves of P and looming supply issues, it has also long been recognized that the efficiency of P-use from fertilizers is low.

Despite most soils containing a large amount of total soil P, only a small proportion is immediately available for plant uptake. The concentration of soluble P in soil is usually very low, normally at levels of 1 ppm or less than 1 ppm due to chemical fixation. This low availability of phosphorous to plants is because of the vast majority of soil P is found in insoluble forms, while the plants can only absorb it in two soluble forms, the monobasic (H_2PO_4^-) and the dibasic (HPO_4^{2-}) ions (Glass, 1989). Phosphate solubilizing microorganisms include different types of microorganisms that convert the insoluble form of phosphorus to soluble form through acidification, secretion of organic acids or protons (Richardson *et al.* 2009), chelation and exchange reactions (Hameeda *et al.* 2008). Bacterial genera like *Azospirillum*, *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas*, *Rhizobium* and *Serratia* are reported as the most significant phosphate solubilizing bacteria (Sturz and Nowak 2000; Sudhakar *et al.* 2000; Alikhani *et al.* 2006).

Vasquez *et al.* (2000) observed that the culture media supplemented with insoluble, tri-basic calcium phosphate and incubated with roots of black and white mangrove became transparent after a few days of incubation. They studied the potential of phosphate solubilizing rhizosphere microbial community both qualitatively and quantitatively in the media containing tri-basic calcium phosphate as a sole phosphorus source and found that the concentration of soluble phosphate in liquid culture increased abruptly with time and the pH in the culture showed a drastic drop.

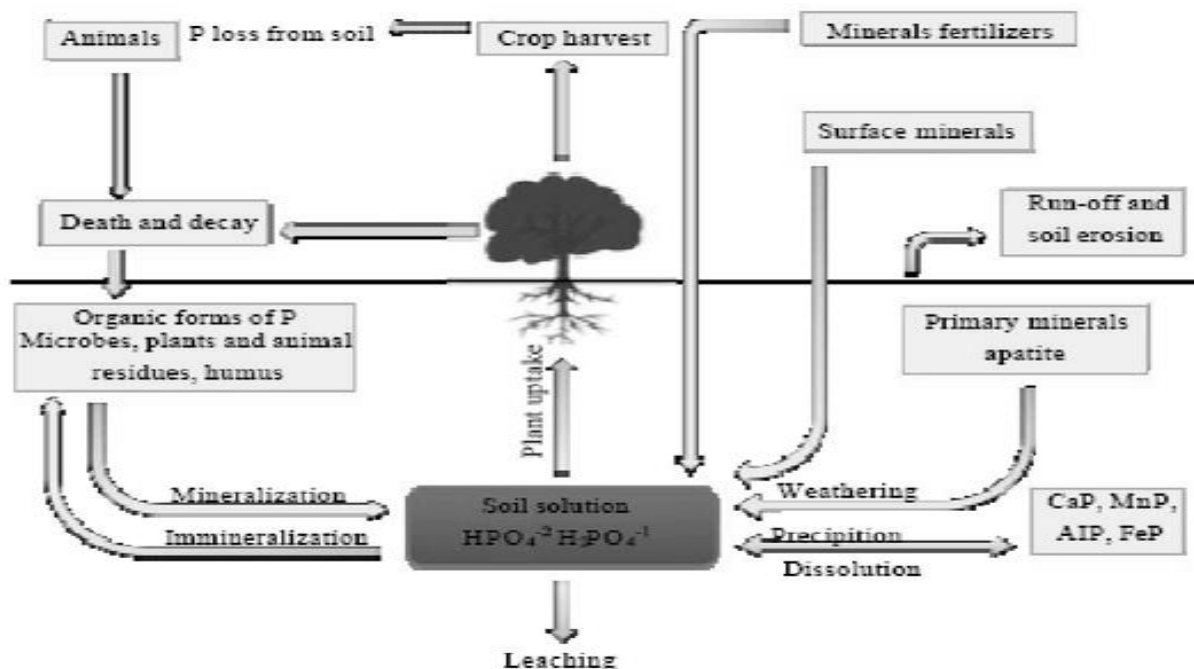


Figure. 3. Solubilization and mineralization of phosphate

Kundu *et al.* (2002) isolated 73 P-solubilizing bacteria from rhizosphere of cowpea, maize, sorghum, cotton and pearl millet and selected these isolates on the basis of their rate of P-solubilization for subsequent studies. They observed that the rate of phosphate solubilization was much faster on tri-calcium phosphate than Mussoorie rock phosphate and there was decrease in pH of medium. They also showed inoculation response on their respective crops in terms of PSB count and plant biomass under pot culture conditions.

Sujatha *et al.* (2004) investigated the solubilization of dicalcium phosphate and tri-calcium phosphate by thermophilic bacteria, actinomycetes and fungi. They observed that dicalcium phosphate was solubilized more readily than tri-calcium phosphate. Their result showed that degree of P-solubilization varied with the incubation period and the organism involved and P-solubilization decreased considerably under alkaline conditions.

Isolates from different cross-inoculation groups of rhizobia obtained from Iranian soils were tested for their ability to dissolve inorganic and organic phosphate. From a total of 446 rhizobial isolates tested for P solubilization by the formation of visible dissolution halos on agar plates, 198 (44%) and 341(76%) of the isolates, solubilized $\text{Ca}_3(\text{PO}_4)_2$ (TCP) and inositol

hexaphosphate (IHP), respectively. The group of *Rhizobium leguminosarum* bv. *viciae* mobilized in liquid TCP Sperber medium significantly ($P < 0.05$) more P (197.1 mg L^{-1}) in 360 h) than other rhizobia tested (Alikhani *et al.* 2006).

Biofertilizers have been used as sources to improve plant nutrients in sustainable agriculture. Han *et al.* (2006) conducted experiments to evaluate the potential of phosphate solubilizing bacteria (PSB) *Bacillus megaterium* var. *phosphaticum* and potassium solubilizing bacteria (KSB) *Bacillus mucilaginosus* inoculated in nutrient limited soil planted with pepper and cucumber. Their results revealed that rock P and K applied either singly or in combination did not significantly enhance soil availability of P and K, indicated their unsuitability for direct application. Phosphate solubilizing bacteria (PSB) was a more potent P-solubilizer than KSB, and co-inoculation of PSB and KSB resulted in consistently higher P and K availability than in the control without bacterial inoculum and without rock material fertilizer.

Khan *et al.* (2006) reviewed that phosphate solubilizing microorganisms (PSM) could play an important role in supplying phosphate to plants in a more environment friendly and sustainable manner. They observed that the performance of PSM *in situ* was contradictory but the solubilization of phosphatic compounds by naturally abundant PSM was very common under *in vitro* conditions.

Rajankar *et al.* (2007) collected 107 samples from saline soil and among these samples, 33 (30.80%) samples showed the ability to solubilize the inorganic insoluble phosphate. From the study, it was observed that the fungi viz; *Aspergillus* spp., *Penicillium* spp. and *Fusarium* spp. had more phosphate solubilizing ability of inorganic insoluble phosphate than bacteria, viz., *B. subtilis* and *B. megaterium*.

Various strains of phosphate solubilizing *Pseudomonas* showed high variability in P-solubilization by utilizing different carbon sources. Naik *et al.* (2008) concluded that these phosphate solubilizing strains play a vital role in plant growth promotion, disease suppression and subsequent enhancement of yield.

Chaiharn and Lumyong (2008) isolated 216 phosphate-solubilizing bacteria from different rice rhizospheric soil in Northern Thailand. These isolates were screened *in vitro* for

their plant growth-promoting activities such as solubilization of inorganic phosphate, ammonia (NH_3), catalase and cell wall-degrading enzyme activity. Their result showed that all the isolates solubilized inorganic phosphate, 77.7 per cent of isolates produced NH_3 and most of the isolates were positive for catalase. They concluded that the isolates exhibited more than two or three plant growth-promoting (PGP) traits.

Singkaravanit and Vichitsoonthonkul (2009) isolated forty-five bacterial and one fungal isolates that showed ability to solubilize inorganic phosphate in Pikovskaya (PVK) medium. They screened these isolates for phosphate solubilizing activity by both plate assay and liquid assay containing rock phosphate as the sole phosphate source. Their results showed that 22 PSB strains have PSI (Phosphate solubilization index) more than 1.5 and also observed a *Bacillus* spp. strain B₂₅ that released and accumulated phosphate at a concentration of 5.44 mg/l in 48 hr of cultivation.

Phosphate solubilizing bacteria (PSB) isolated from rhizosphere soil of 29 crops. Four phosphate solubilizers, were selected for further evaluation, and found that the efficiency of phosphate solubilization was decreased in buffered media compared to non-buffered media. The buffering capacity of the medium reduced the effectiveness of PSBs in releasing P from tri-calcium phosphates (Joseph and Jisha, 2009).

A phosphate-solubilizing bacterial strain NII-0909 isolated from the Western ghat forest soil in India exhibited the plant growth-promoting attributes of phosphate solubilization, auxin production, 1-aminocyclopropane-1- carboxylate deaminase (ACC deaminase) activity and siderophore production. It was able to solubilize ($122.4 \mu\text{g}$ of $\text{Ca}_3\text{PO}_4 \text{ ml}^{-1}$), and produce IAA ($109 \mu\text{g ml}^{-1}$) at 30°C . These results demonstrated that isolates NII-0909 has the promising PGPR attributes to be developed as a biofertilizer to enhance soil fertility and promote the plant growth (Dastager *et al.* 2010).

Mehta *et al.* (2010) isolated *Bacillus circulans* MTCC 8983 from apple rhizosphere. The strain solubilized tri-calcium phosphate and produced substantial amount of soluble phosphorus (957.3 mg/l) in Pikovskaya's (PVK) broth and exhibited the simultaneous production of indoleacetic acid (IAA) ($15.13 \mu\text{g/ml}$), siderophore (57.80%) and growth inhibition against

Dematophora necatrix (46.57%). Regression analysis revealed that phosphate solubilization of *B. circulans* was inversely correlated with pH ($r = -0.98$) and positively correlated with growth ($r = 0.98$), siderophore production ($r = 0.99$), IAA ($r = 0.78$) and antifungal antibiotic activity against *D. necatrix* ($r = 0.87$).

Mehta *et al.* (2014) isolated *Bacillus methylotrophicus* CKAM from root endosphere of healthy apple trees which was selected on the basis of higher P-solubilisation (687 mg/L), nitrogenase activity (237.6 gmole C₂H₄ h⁻¹mg⁻¹ protein), IAA (34 lg/mL), siderophore unit (96.4 %) and antifungal activity against *F. oxysporum* (88.22 %), *Phytophthora sp.* (70.00 %), *D. necatrix* (61.73 %), *S. rolfii* (44.54 %) and *P. aphanidermatum* (62.56 %). We investigated the ability of isolate CKAM to solubilise insoluble P via two possible mechanisms: proton excretion by ammonium assimilation and organic acid production. There were no clear differences in pH and P-solubilisation between glucose–ammonium and glucose–nitrate media. P-solubilisation was significantly promoted with glucose compared with fructose. HPLC study showed that isolate CKAM produced mainly gluconic and oxalic acids with small amounts of 2-ketogluconic, formic acids. During the culture, the pH was reduced with increase in gluconic acid concentration and was inversely correlated with soluble P concentration.

Mechanism of phosphate solubilization

There are two components of P in soil, organic and inorganic phosphates. Inorganic P occurs in soil, mostly in insoluble mineral complexes, some of these appearing after the application of chemical fertilizers. Organic matter, on the other hand, is an important reservoir of immobilized P that accounts for 20-80% of soil P (Richardson, 1994).

Organic Phosphate Solubilization

Organic phosphate solubilization is also called mineralization of organic phosphorus, and it occurs in soil at the expense of plant and animal remains, which contain a large amount of organic phosphorus compounds. The degradability of organic phosphorous compounds depend mainly on the physicochemical and biochemical properties of their molecules, *e.g.* nucleic acids, phospholipids, and sugar phosphates are easily broken down, but phytic acid, polyphosphates,

and phosphonates are decomposed more slowly (McGrath *et al.*1998). Phosphorus can be released from organic compounds in soil by three groups of enzymes:

- Nonspecific phosphatases, which perform dephosphorylation of phosphoester or phosphoanhydride bonds in organic matter.
- Phytases, which specifically cause P release from phytic acid.
- Phosphonatases and carbon-phosphorus (C-P) lyases, enzymes that perform C-P cleavage in organophosphonates.

Inorganic Phosphate Mineralization

Several reports have suggested the ability of different bacterial species to solubilize insoluble inorganic phosphate compounds, such as tri-calcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Goldstein, 1986). In two thirds of all arable soils, the pH is above 7.0, so that most mineral P is in the form of poorly soluble calcium phosphates (CaPs). Microorganisms must assimilate P via membrane transport, so dissolution of CaPs to Pi (H_2PO_4) is considered essential to the global P cycle. Evaluation of samples from soils throughout the world has shown that, in general, the direct oxidation pathway provides the biochemical basis for highly efficacious phosphate solubilization in Gram-negative bacteria via diffusion of the strong organic acids produced in the periplasm into the adjacent environment. Therefore, the glucose dehydrogenase (GDH) may play a key role in the nutritional ecophysiology of soil bacteria. A number of theories have been proposed to explain the mechanism of phosphate solubilization. Important among them are:

- ❖ Acid production theory
- ❖ Proton and enzyme theory

Acid production theory

According to this theory, the process of phosphate solubilization by PSM is due to 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 (Gand 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 (Cunningham and Kuaick, 1992; Illmer and Schinner, 1995; Fasim *et al.* 2002).

The production of citric or gluconic acid and the extrusion of H^+ result from membrane transport mechanisms was described as possible mechanism for dissolving rock-phosphate from hydroxyapatite, iron phosphate, and aluminum phosphate by *Penicillium rugulosum* (Reyes *et al.* 1999). These processes were reported to be influenced by the sources of the nitrogen, phosphate, and carbon.

Goldstein *et al.* (1999) adopted a strategy which could involve specific associations between plant roots and mineral phosphate solubilizing (MPS) bacteria. The most efficient MPS phenotype in Gram negative bacteria resulted from extracellular oxidation of glucose to gluconic acid via the quinoprotein glucose dehydrogenase. They isolated bacterial population that showed no MPS activity and no gluconic acid production and they treated bacterial strains with concentrated solution and glucose dehydrogenase redox cofactor 2,7,9-tricarboxyl-1H-pyrrol[2,3]-quinoline 4,5-dione (PQQ), this resulted in production of high levels of gluconic acid. Their work has led to the conclusion that the resultant acidification of the rhizosphere may play a role in nutrient availability and/or other ecophysiological parameters essential for the survival of plant.

The P-solubilizing capability of gluconic acid was much higher as compared to 2-keto-gluconic acid in the filtrate from strain CC-A174 culture. The process of acidification and chelation by gluconic acid and 2-keto gluconic acid dissolved tricalcium phosphate (TCP) in cultural medium. The chelation property of gluconic acid enables it to form insoluble complex. Insoluble metal forms may be solubilized by protons, with Ca^{++} liberating phosphates (Shekhar *et al.* 2000). Organic acids contribute to the lowering of solution pH as they dissociate in a pH dependent equilibrium, into their respective anion(s) and proton(s) (Welch *et al.* 2002).

Enterobacteria intermedium oxidized glucose to gluconic acid and sequentially to 2-ketogluconic acid (2-KGA). It was observed that with an air supply, the production of 2-KGA markedly increased to about 110 g/l at day 10 in media containing 0.2 M gluconic acid, while it was about 65 g/l without gluconic acid addition and concentration of soluble phosphate

significantly decreased to 200-250 mg/l in media containing 1 per cent CaCO_3 whereas it was about 1000 mg/l without CaCO_3 addition. In the absence of air, the concentration of 2-KGA and phosphate were negligible throughout the culture period (Hwangbo *et al.* 2003).

Glucose is the precursor for synthesis of gluconic acid. It has been suggested that phosphate solubilization in the strains is mediated by glucose or gluconic acid metabolism (Rashid *et al.* 2004). As solubilization of phosphate preceded detection of gluconic acid in the medium, perhaps even low levels of the acid (below the detection level of HPLC) started to dissolve the sparingly soluble phosphate. Alternatively, consumption of gluconic acid by growing cells could also take place. Gluconic acid formation and phosphate solubilization from sparingly soluble phosphorus sources was studied in case of plant growth promoting bacterial strains *A. brasilense* and *A. lipoferum*. It was observed that strains of *A. brasilense* and *A. lipoferum* were capable of producing gluconic acid when grown in sparingly soluble calcium phosphate medium when their usual fructose carbon sources is was amended with glucose (Rodriguez *et al.* 2004).

Ivanova *et al.* (2006) established that lower the concentration of phosphate in the nutritive medium the greater is the conversion percentage of P_2O_5 into soluble forms. Their results showed that *Azotobacter* sp. had maximum extent of phosphate solubilization than *Erwinia* sp. at phosphorite concentration of 0.5 per cent w/v due to production of low molecular mass organic acids which attacked the phosphate structure and transform phosphorus from non-utilizable to the utilizable form for the plants.

Patel *et al.* (2008) demonstrated the role of different organic acids in mineral phosphate solubilization by rhizobacteria depending on the nature of the available carbon source. On sucrose and fructose (non-GDH substrates), *Cirtobacter* sp.DHRSS liberated 170 and 100 μM free phosphate and secreted 2.94 g/L and 2.1g/L acetic acid, respectively. Interestingly, in the presence of GDH-substrates (glucose, xylose and maltose), isolate produced 4.36 g/L gluconic acid and phosphate released was 520 and 570 μM , respectively. Their study revealed that *Citrobacter* sp. DHRSS, isolated from the rhizosphere of sugarcane exhibited mineral phosphate solubilizing (MPS) phenotype on sugar which are non-GDH substrates along with GDH substrates, indicating that it is constitutive and could act on a wide range of aldose sugars.

The mechanism of action of phosphate solubilization in the wild- type strain *Aspergillus tubingensis* and the phenotypic mutants derived from it in the presence of various carbon and nitrogen sources was studied by Relwani *et al.* (2008). They observed that mutants exhibited high P- solubilization as compared to wild type. The P- solubilizing activity was strongly associated with the production of organic acids (*i.e.* succinic and acetic acid) and enzymes such as acid phosphatase and phytase. These results suggested the role of these enzymes in P- solubilization apart from the organic acid exudation and H⁺ pump in *A. tubingensis*.

A mineral phosphate solubilizing bacterium, *Burkholderia cepacia* DA23 has been isolated from cultivated soils. Analysis of the culture medium by high pressure liquid chromatography identified gluconic acid as the main organic acid released by *Burkholderia cepacia* DA23. Gluconic acid production was apparently the result of the glucose dehydrogenase activity and glucose dehydrogenase was affected by phosphate regulation (Song *et al.* 2008).

Trivedi and Sa (2008) screened mutants of *Pseudomonas corrugate* (NRRL B-30409) based on their phosphate solubilization ability, production of organic acids and subsequent effect on plant growth at lower temperatures. Their results showed that two mutants were more efficient than wild type strain for phosphate solubilization activity across a range of temperature from psychotropic (4°C) to mesophilic (28°C) in aerated GPS medium containing insoluble rock phosphate. HPLC chromatography analysis showed that phosphate solubilization potential was mediated by production of organic acid. The study indicated that it is possible to select physiologically efficient strains of *P. corrugate* through mutagenesis and that resultant mutants strains may improve the use of soil phosphate by plants at lower temperatures.

Proton and enzyme theory

Esterase type enzymes are known to be involved in liberating phosphorus from organic phosphatic compounds. PSMs (phosphate solubilizing microorganisms) are also known to produce phosphatase enzyme along with acids which cause the solubilization of P in aquatic environment (Alghazali *et al.* 1986).

Illmer and Schinner, (1995) reported that out of the four efficient phosphates solubilizing microbes, *Penicillium aurathio-griseum*, *Penicillium simplicissimum*, *Aspergillus niger* and *Pseudomonas* sp., only *A. niger* could produce organic acid. Two most probable explanations for this are:

Solubilization without acid production is due to the release of protons accompanying respiration or ammonium assimilation (Dighton and Boddy, 1989; Parks *et al.* 1990). Protons can be pumped into the external medium by various membrane associated pumps which set up ionic gradients for the acquisition of nutrients (Sigler and Hofer, 1991; Gadd, 1993). In addition, protons arise from produced organic acids which also possess an organic acid anion which is usually capable of forming a complex with metal cation (Hughes and Poole, 1991; Burgstaller and Schinner, 1993).

Besides these two mechanisms the production of chelating substances (Luo *et al.* 1993) H_2S , CO_2 (Kapoor *et al.* 1989) mineral acids, siderophores and biologically active substances like indole acetic acids, gibberellins and cytokinins are also correlated with phosphate solubilization. Chelation involves the formation of two or more coordinate bonds between an anionic or polar molecule and a cation, resulting in a ring structure complex (Whitelaw, 2000). Organic acid anions, with oxygen containing hydroxyl and carboxyl groups, have the ability to form stable complexes with cations such as Ca^{2+} , Fe^{2+} , Fe^{3+} , and Al^{3+} , that are often bound with phosphate in poorly forms.

Genetic basis of inorganic phosphate solubilization

The genetic basis of mineral phosphate solubilization *i.e.* the MPS phenotype is not well understood (Goldstein and Liu, 1987). Because the production of organic acids is considered to be the principal mechanism for mineral phosphate solubilization, this assumption has been corroborated by the cloning of gene involved in gluconic acid production *viz.* PQQ (4,5-dihydro-4,5-dioxo-1H-pyrrolo-[2,3-]quinoline-2,7,9 tricarboxylic acid) which is an aromatic, tricyclic ortho-quinone that serves as the redox cofactor for several bacterial dehydrogenases. Among the best known examples are methanol dehydrogenase and glucose dehydrogenase. PQQ belongs to the family of quinone cofactors that has been recognized as the third class of redox cofactors following pyridine nucleotide and flavin-dependent cofactors.

PQQ is a prosthetic group required by several bacterial dehydrogenases, including methanol dehydrogenase (MDH) of Gram negative methylotrophs and some glucose dehydrogenases (GDH). PQQ is derived from two amino acids, tyrosine and glutamic acid (Van Kleef and Duine, 1988; Houck *et al.* 1991). PQQ-dependent glucose dehydrogenase (GDH) can oxidize glucose to gluconate. GDH, is located in the cytoplasmic membrane and require PQQ for the holoenzyme. Gluconate is further oxidized to 2-ketogluconic acid by gluconate dehydrogenase(GADH).

Glucose dehydrogenase (GDH) is a member of quinoproteins, catalyzing the oxidation of glucose to gluconic acid, requiring pyrroloquinoline quinone (PQQ) and also metal ions such as Ca^{2+} (or Mg^{2+} *in vitro*) for its activity.

In order to provide carbon for intracellular metabolism, GDH plays a key regulatory and bioenergetic role in the bacteria. The protons generated in the oxidation contribute directly to the trans-membrane proton motive force (PMF), which results in the uptake of exogenous amino acids and other compounds. Hence, this oxidative glucose pathway might be important for the survival of bacteria in aerobic, low-phosphate and aquatic environment.

Very little is known regarding the genetic regulation governing the mineral phosphate solubilization trait. In fact, the information about the genetic or biochemical mechanisms involved in the synthesis of the GDH-PQQ halo enzyme is scant, and variations between constitutive and inducible phenotypes are observed among several bacterial species (Goldstein, 1994). Glucose, gluconate, manitol, and glycerol are among the possible inducers of the halo enzyme activity (Van Schie *et al.* 1987).

Goldstein and Liu, (1987) cloned a gene from *Erwinia herbicola* that is involved in mineral phosphate solubilization by screening the antibiotic resistant recombinants from a genomic library in a medium containing hydroxyl apatite as the source of P. The expression of this gene allowed production of gluconic acid and mineral phosphate solubilization activity in *E.coli* HB101. Sequence analysis of this gene (Liu *et al.* 1992) suggested its probable involvement in the synthesis of the enzyme pyrroloquinoline quinone (PQQ) synthase, which

directs the synthesis of PQQ, a co-factor necessary for the formation of the holoenzyme glucose dehydrogenase (GDH)-PQQ.

A mineral phosphate solubilizing gene from *Pseudomonas cepacia* has also been cloned and expressed in *E.coli*, and its involvement in gluconic acid production was also demonstrated (Babu-Khan *et al.*1995).

Rodriguez *et al.* (2004) carried out a genetic construction using the broad host range vector pKT230 and plasmid pMCG898, which encodes *Erwinia herbicola* pyrroloquinoline quinone (PQQ) synthase. The final construction was transformed and expressed in *Escherichia coli* MC1061, and the recombinant plasmids were transferred to *Burkholderia cepacia* IS-16 and *Pseudomonas* sp. PSS recipient cells by conjugation. Their results showed that clones containing recombinant plasmids produced higher clearing halos in plates with insoluble phosphate as the unique (P) source, in comparison with those of strains without plasmids.

Tripura *et al.* (2007) cloned a 2.4 kb glucose dehydrogenase gene (*gcd*) of *Enterobacter asburiae* sharing extensive homology to the *gcd* of other enterobacteriaceae members in a PCR-based directional genome walking approach and the expression confirmed in *Escherichia coli* YU423 on both MacConkey glucose agar and hydroxyapatite (HAP) containing media. Mineral phosphate solubilization by the cloned *E. asburiae gcd* was confirmed by the release of significant amount of phosphate in HAP containing liquid medium. Their study concluded that glucose dehydrogenase of a rhizobacterial strain of *Enterobacter asburiae* involved in mineral phosphate solubilization shares properties and sequence homology with other members of enterobacteriaceae.

To determine the factors involved in plant growth promotion by organism, Choi *et al.* (2008) mutagenized wild-type strain B16 using omegon-Km elements (a transposable element designed for *in vitro* insertional mutagenesis and cloning of genes) and isolated one mutant, K818, which was defective in plant growth promotion, in a rockwool culture system. A cosmid clone, pOK40, which complemented the mutant K818, was isolated from a genomic library of the parent strain. Analysis of the DNA sequence of the fragment identified 11 putative open reading frames, consisting of seven known and four previously unidentified pyrroloquinoline

quinone (PQQ) biosynthetic genes. Electro spray ionization-mass spectrometry analysis of culture filtrates confirmed that wild-type B16 produced PQQ, whereas mutants defective in plant growth promotion did not.

PGPR mediated iron acquisition in host plants

Lankford coined the term siderophore in 1973 as a term to describe low molecular weight molecules that bind ferric iron with an extremely high affinity (Lankford, 1973). Term siderophore is derived from a Greek term meaning iron carrier (Ishimaru, 1993). The molecular weight of siderophores range from approximately 600 to 1500 daltons and because passive diffusion does not occur for molecules greater than 600 daltons, siderophores must be actively transported (Ishimaru, 1993). Once actively transported into the periplasm, the iron siderophore complex is bound to a periplasmic binding protein (Braun and Braun, 2002).

Plant growth promoting rhizobacteria promote growth of several annual crops by increased uptake of iron through siderophores. Masalha *et al.* (2000) reported that plants grown in non-sterile soil systems show no iron-deficiency symptoms and have fairly high iron levels in roots, in contrast to plants grown in sterile systems. This supported the possible role of microbial community in iron uptake (Walter *et al.* 1994; Yehuda *et al.* 1996). Crowley *et al.* (1992) and Walter *et al.* (1994) also demonstrated the role of microbial siderophore to combat the chlorosis in host plant system (Sharma and Johri, 2003, Sharma *et al.* 2003).

PGPR may influence the growth of plant roots through the production of siderophores (Richardson *et al.* 2009). Roots of strategy II type plants (e.g. the Graminaeae; Marschner 1995; Robin *et al.* 2008) secreted phytosiderophores (Fe chelators) which bind Fe^{3+} and maintain its concentration in soil solution (Lemanceau *et al.* 2009). At the root surface chelated Fe^{3+} can then be directly taken up as a phytosiderophore-Fe complex (strategy II; Lemanceau *et al.* 2009), whereas in non-graminaceous species (strategy I type plants; Marschner 1995; Robin *et al.* 2008), Fe^{3+} must first be reduced to Fe^{2+} prior to being absorbed by the plant (Lemanceau *et al.* 2009). Rhizobacteria (and fungi) also produce siderophores and it has been shown that plants can absorb bacterial- Fe^{3+} complexes, which includes strategy I species possibly by endocytosis (Bar-Ness *et al.* 1991; Vansuyt *et al.* 2007). The capture of these bacterial complexes by plants

may play a significant role in nutrition and growth, especially in alkaline and calcareous soils where Fe availability is low (Bar-Ness *et al.* 1991; Masalha *et al.* 2000).

PGPR AS BIOSTIMULATOR

The most common phytohormones produced by PGPR are auxins, cytokinins, giberellins and to a lesser extent ethylene, with auxins being the most well characterized (Khalid *et al.* 2004; Patten and Glick, 1996; Arshad and Frankenberger, 1998). Plant growth stimulation by rhizobacteria is attributed to their ability to produce Indole 3-acetic acid (IAA) (Tsavkelova *et al.* 2007) and thus, IAA production is considered as a key trait for the selection of PGPR (Ali *et al.* 2009; Patten and Glick 1996). Members of the genera *Azospirillum*, *Pseudomonas*, *Rhizobium*, *Acetobacter*, *Azotobacter*, and *Bacillus* produce IAA has been effectively used to enhance plant growth and crop productivity (Patten and Glick, 1996; Idris *et al.* 2007).

Idris *et al.* (2007) reported that several routes of tryptophan-dependent IAA biosynthesis in Gram negative are known however, no gene products involved in IAA biosynthesis in Gram-positive bacteria were identified. Therefore they applied a genomic approach and examined the whole-genome sequence of *B.amyloliquefaciens* FZB42 which revealed three candidate genes with apparent homology to genes previously reported to be involved in IAA metabolism: *ysnE*, encoding a protein similar to the IAA acetyltransferase from *Azospirillum brasilense* (Zimmer *et al.* 1991); *dhaS*, similar to indole-3-acetaldehyde dehydrogenase from *Ustilago maydis* (Basse *et al.* 1996); and *yhcX*, encoding a putative nitrilase similar to nitrilase2 from *Arabidopsis thaliana* (Hillebrand *et al.* 1998). All three genes had counterparts in the genome of *B. subtilis* 168 (Kunst *et al.* 1997).

IAA production by PGPR

Leveau and Lindow (2005) isolated several bacteria from the plant surfaces and studied their indole-3-acetic acid (IAA) catabolizing activity. They observed that a strain *Pseudomonas putida* 1290 was able to utilize IAA as sole source of carbon, nitrogen and energy. Their study revealed that the strain 1290 besides being able to destroy IAA, it was also capable of producing IAA in media supplemented with tryptophan.

Arkhipchenko *et al.* (2006) determined the concentration of tryptophan in organic wastes and fertilizers. The detected levels of tryptophan suggested that the application of organic fertilizers can increase the concentration of tryphophan in soil to physiologically relevant values.

Gravel *et al.* (2007) evaluated five bacterial and three fungal isolates for their growth promoting effect on mature healthy tomato plants grown under hydroponic conditions. They observed that *Pseudomonas putida* and *Trichoderma atroviride* showed the activity of production and degradation of IAA. The study revealed that when both the strains were grown in the presence of increasing concentration of Ltryptophan (up to 0.75mM), increase in the fresh weight of roots and shoots of tomato seedlings was observed.

Fatima *et al.* (2009) reported that seven strains isolated from the rhizosphere of wheat, showed significant production of IAA and ranged from 5.5 to 30.6 µg/ml. The study revealed that the strains WPR-51, WPR-42 and WM-3 belonging to *Azotobacter* and *Azospirillum* produced IAA ranging from 19.4 to 30.2 µg/ml and possessed phosphate solubilizing ability. These strains positively affected the germination of wheat as well as increased biomass and root shoot length by inhibiting *Rhizoctonia solani* growth when tested in pot experiments.

Karnwal (2009) tested *Pseudomonas fluorescens* AK1 and *Pseudomonas aeruginosa* AK2 for their ability to produce indole acetic acid in pure culture in the presence and absence of L-tryptophan at 50, 100, 200 and 500 µg / ml concentration. The study revealed that indole production increased with increase in tryptophan concentration (0.5, 1.2, 4.3 and 9.3 µg / ml and 0.2, 0.7, 3.8 and 8.3 µg / ml respectively) for both strains.

Yeon *et al.* (2010) isolated three hundred and seventy four rhizobacteria from soil rhizosphere or rhizoplane contaminated with petroleum and heavy metals. The isolates were screened for plant growth-promoting trait (PGPT), including indole acetic acid (IAA) productivity, 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity and siderophore (s) synthesis ability. They observed that PGPTpossessing rhizobacteria were more abundant in rhizoplane (82%) samples than the rhizosphere soil (75%). Clustering analysis by principle component analysis showed that rhizoplane was the most important factor influencing the ecological distribution and physiological characterization of PGPT-possessing rhizobacteria.

Ashraf *et al.* (2011) isolated twelve bacterial strains from root and rhizosphere samples of different sugarcane growing areas. Ten strains were identified as *Pseudomonas* and two as *Azotobacter*. All isolates showed IAA production in growth medium containing tryptophan as a precursor. Maximum IAA production (4.49mg/L) was detected in isolate A17 whereas, values for IAA production by nitrogen fixing isolates Azoto1 and Azoto2 were comparatively low (0.2 and 0.1mg/l respectively). Beneficial effects of inoculation on sugarcane grown in pots were also observed.

Aziz *et al.* (2012) studied the effect of IAA-producing and non-IAAproducing diazotroph *Bacillus cereus* strains on early growth of shallot (*Allium ascalonicum*) and mustard (*Brassica juncea*) plants. Inoculation with IAA-producing *B. cereus* UPMLH1 significantly increased shallot adventitious roots (root number and length) and shoot growth (19 to 54% increment) whereas inoculation of non IAA-producing *B. cereus* UPMLH24 did not produce significant effect. However, inoculation with IAA-producing and non-IAA-producing strains significantly increased primary roots and shoot growth of mustard plants.

Hassan *et al.* (2015) isolated *Pseudomonas moraviensis* and *Bacillus cereus* from rhizosphere soil of halophytic weed (*Cenchrus ciliaris* L.) of Khewra salt range, and used as bioinoculants. The plant growth promoting rhizobacteria (PGPR) were applied to wheat (*Triticum aestivum*) by seeds soaking, and aqueous solution of tryptophan was added to the rhizosphere soil at 1ug/L, after seed germination. Experiment was conducted both in pots (filled with sterilized soil) under axenic condition and in field under natural condition, for two consecutive years. The inoculation of *Pseudomonas moraviensis* and *Bacillus cereus*, significantly increased the organic matter, P, K, Ca, and NO³-N availability of soil. The inoculation of these PGPR positively enhanced growth and physiology of treated plants, and this affect was further augmented in the presence of tryptophan. Addition of tryptophan with *Pseudomonas moraviensis* and *Bacillus cereus* increased the fresh weight, proline contents and activities of antioxidant enzymes significantly over control. Added tryptophan with both PGPR, improved the number of plants at yield and seeds establishment by improving number of seeds/spike and spike length. Effects of PGPR inoculation alone and with tryptophan were more pronounced in pots grown plants. It is inferred from the results, that tryptophan addition is a

competent source for increasing potential of PGPR, thereby improving wheat growth, and physiology.

Dasgupta *et al.* (2015) screened and identified best three indole-3- acetic acid producing phosphate solubilising rhizobacteria isolated from the rhizosphere soil of Dhaincha (*Sesbania bispinosa*). The isolates showing positive results for phosphate solubilization were further monitored for their Indole-3-acetic acid (IAA) production. Isolate DD4 produced maximum amount of IAA (1260 ppm/Lit on 8th day of incubation). Further investigations for optimization of Indole acetic acid production was carried out at different cultural conditions, such as pH, temperature and concentration of tryptophan. Maximum IAA production was recorded at pH 7, 37° C temperature and 1gm/Lit L-tryptophan concentration. The best 3 IAA producing isolates i.e. DD3, DD4 and DD6 were further identified by 16S rRNA partial gene sequencing as *Escherichia coli* DACG2 (GenBank Accession number JN858966) *Pseudomonas fluorescens* strain DACG3 (GenBank Accession No. KP641168) *Burkholderia sp.* DACG1 (GenBank Accession No. JN639877). These three isolates are needed to be further studied due to their potential to be developed as plant growth promoting rhizobacteria in the field application.

Effect of PGPR inoculation on growth and yield

Plant growth promoting rhizobacteria are beneficial soil bacteria that colonize plant roots and enhance plant growth promotion activity by different mechanisms. In the light of the ability of PGPR to act as biofertilizer and biostimulant, the direct role of PGPR on growth and yield has been reviewed below:

Pandey and Maheshwari (2007) studied the interaction in a plant growth promoting consortium comprising two species *Burkholderia* spp. MSSP and *Sinorhizobium meliloti* PP3 which produced IAA and solubilize inorganic phosphate. The two strains were tested on *Cajanus cajan* in sterile soil and their results showed that inoculation increased the seedling length, yield and weight.

Sharma *et al.* (2007) isolated two phosphate solubilizing strains namely *Pseudomonas fluorescens* and *Bacillus megaterium*. They co-inoculated them into seeds of *Cicer arietinum* and observed enhanced seedling length, radical and plumule length.

Wani *et al.* (2007) isolated 32 bacterial isolates including *Mesorhizobium*, *Azotobacter* and phosphate solubilizing bacteria and tested them for various biochemical activities like IAA production, siderophore, HCN, ammonia and phosphate solubilization. They observed 250 per cent increase in seed yield due to inoculation with *M. ciceri* RC3+A. *chroococcum* A4+Bacillus PSB10 compared to uninoculated control and grain protein content ranged from 180 (*Bacillus* PSB1) to 309 ng g⁻¹ (*M. ciceri* RC3 + *A. chroococcum* A4 +Bacillus PSB10) in inoculated chickpea. The nitrogen content in roots and shoots differed considerably among the treatments.

Karlidag *et al.* (2007) studied plant growth promoting effects of *Bacillus* M3, *Bacillus* OSU-142 and *Microbacterium* FS01 were tested alone or in combination on apple (*Malus domestica* L.) cv. Granny Smith in terms of yield, growth and nutrient composition of leaves in the province of Malatya, Turkey. The presence of M3 and/or OSU-142 and/or FS01 combinations stimulated plant growth and resulted in significant yield increases in Granny Smith. Root inoculation of PGPR strains significantly increased cumulative yield (26.0–88.0%), fruit weight (13.9–25.5%), shoot length (16.4–29.6%) and shoot diameter (15.9–18.4%) compared with the control.

Adesemoye *et al.* (2008) conducted a field study with field corn to test the hypothesis that microbial inoculants (PGPR) that increase plant growth and yield, can enhance nutrient uptake and thereby remove more nutrients (N, P, K) from the field as part of an integrated nutrient management system. Their results showed that inoculants promoted grain yields (kg/ha) 7717 for AMF (Arbuscular Mycorrhiza Fungi), 7260 for PGPR+ AMF, 7313 for PGPR, 5725 for the control group and also enhanced nitrogen content per gram of grain tissues. Significantly higher amounts of N, P and K were removed from the plots with inoculants, based on total nutrient content of grain per plot and hence supported the overall hypothesis and indicated that application of inoculants lead to reduction in buildup of N, P and K in agricultural soils.

Gothwal *et al.* (2008) studied more than thirty rhizobacterial isolates from two ecologically important plant species of Thar desert and evaluated them for their plant growth

promoting effects in terms of seed germination, root, seed length and total biomass production. As a result of this, there was a significant increase in plant growth parameters after inoculation.

Hamdali *et al.* (2008) assessed different plant growth promoting (PGP) abilities of eight rock phosphate (RP)-solubilizing *Actinomycetes* isolates originating from Moroccan phosphate mines. Six of these strains, were able to grow on root exudates of wheat and released phosphate from RP. Growth of potentially phytopathogenic fungi, bacteria and yeasts was significantly inhibited. It was observed that when these strains were grown with wheat plant, in a synthetic minimum medium (SMM) containing insoluble RP as phosphate source, *Streptomyces griseus*-related strain (BH7) was most active RP- solubilizing strain and it stimulated the aerial growth of the plant more than 70 per cent in test tubes and more than 3 per cent in RP soil compared to the uninoculated control treatment.

Ashrafuzzaman *et al.* (2009) isolated and characterized the plant growth promoting rhizobacteria (PGPR) from the rhizosphere soil of rice field for the increased growth of rice. Most of the isolates resulted in a significant increase in plant height, root length and dry matter production of shoot and root of rice seedlings.

Jaleel *et al.* (2009) studied *Pseudomonas fluorescens* as a physiological modulator in the enhancement of medicinally important alkaloids of *C. roseus* and found that *P. fluorescens* has a profound effect on growth and caused a significant enhancement in the production of individual alkaloids like ajmalacine, catharanthine, serpentine and vindoline when compared to untreated control plants.

Karthikeyan *et al.* (2009) studied the individual and combined effects of *Azospirillum brasilense* and *P. fluorescens* on biomass yield and ajmalacine production in *C. roseus*. They found that maximum ajmalacine was recorded in the combined inoculation of *A. brasilense* and *P. fluorescens* in roseal variety of *C. roseus* (0.700mg/g dry wt.) and concluded that the seed priming and seedling treatments of native PGPR's can be used as a good tool in the enhancement of biomass yield and alkaloid contents in medicinal plant cultivation as it provides an ecofriendly approach.

Yazdani *et al.* (2009) reported that use of PSM and PGPR in addition to conventional fertilizer applications (NPK) could improve ear weight, row number and grain number per row and ultimately increased grain yield of *Zea mays* L. They concluded that application of PSM and PGPR together could reduce P application by 50% without any significant reduction of grain yield.

Yanni *et al.* (2010) assessed the ability of biofertilizer inoculants containing *Rhizobium leguminosarum* bv. *trifolii* to enhance production of rice (*Oryza sativa* L.) under actual agricultural conditions in the Nile delta. In the experiment, they observed that by combining superior rhizobial inoculants, grain yield increased up to 47% in farmers' fields, with an average increase of 19.5%. Data on rice straw production, harvest index and the agronomic fertilizer N-use efficiency also indicated positive agronomic benefits of rhizobial inoculation.

Plant growth promoting rhizobacteria (PGPR) can enhance plant growth by alleviating soil stresses. Arzanesh *et al.* (2011) hypothesized that the isolated strains of *Azospirillum* sp. may alleviate the adverse effects of drought stress on wheat (*Triticum aestivum* L.) growth. Three different strains of *Azospirillum lipoferum* (B1, B2 and B3) were used to inoculate wheat seedlings under drought. During the flowering stage, the seedlings were subjected to three drought levels with five different time longevity, including control. Pots were water stressed at 80% (S0), 50% (S1) and 25% (S2) of field capacity moisture in a 25 day-period. They observed that strain B3 increased wheat yield at S1 and S2 by 43 and 109%, respectively as compared to uninoculated treatments.

A potential bacterial strain *Serratia nematodiphila* NII- 0928 isolated from Western ghat forest soil with multiple plant growth promoting attributes was analysed for growth promotion of pepper under green house condition. Seed bacterization with black pepper cuttings in greenhouse trials using Sand: Soil: FYM with three individual experimental sets with their respective control showed clearly the growth promoting activity. The result of this study provided a strong basis for further development of this strain as a bioinoculants to attain the desired plant growth promoting activity in black pepper growing fields (Dastager *et al.* 2011).

Kurek *et al.* (2013) used phosphate solubilizing bacteria isolated from non-rhizosphere as inoculants for plant growth promotion of young apple trees cultivar Ligol (rootstock M26),

which was identified as *Pseudomonas luteola* BN0834 on the basis of biochemical methods and 16S rDNA sequence analysis. *P. luteola* strain BN08-34 was tested for: solubilization of inorganic and organic compounds of phosphorus (calcium phosphate, zinc phosphate, hydroxyapatite and calcium phytate); indole acetic acid (IAA), siderophore production and biosurfactant production; and the ability to grow on a medium without nitrogen added. When the higher number of *P. luteola* BN0834 was introduced into soil without a mineral fertilizer added near the surface of the roots, positive correlations were found between the number of PSM in the apple tree rhizosphere and the content of available P in non-rhizosphere soil and also between the number of PSM in the apple tree rhizosphere and the amount of P, K and Ca in plant leaves. The highest total shoot length was also obtained from *P. luteola* × 10 application.

Ipek *et al.* (2014) studied plant growth promoting effects of *Alcaligenes* 637Ca, *Staphylococcus* MFDCa-1, MFDCa-2, *Agrobacterium* A18, *Pantoea* FF1 and *Bacillus* M3 tested on strawberry cv. 'Aromas' based on yield, number, and weight of fruit, leaf area, vitamin C, total soluble solids (TSS), acidity and ionic composition of leaves under calcareous soil conditions. The results demonstrated that all of bacterial treatments significantly affected all parameters tested. The best result was obtained from 637Ca treatment, which significantly increased fruit yield, number and weight about 47.5, 34.7, and 9.4%, respectively, compared to control. Bacterial treatments including *Alcaligenes* 637Ca, *Staphylococcus* MFDCa-1, MFDCa-2, *Agrobacterium* A18, *Pantoea* FF1, and *Bacillus* M3 to strawberry plants can ameliorate the deleterious effect of high lime on fruit yield, growth and nutrition.

Majeed *et al.* (2015) conducted a study to characterize the native plant growth promoting (PGP) bacteria from wheat rhizosphere and root-endosphere characteristics and evaluated for their beneficial effects on the early growth of wheat (*Triticum aestivum* L.). Among nine bacterial isolates, seven were able to produce indole-3-acetic acid in tryptophan-supplemented medium; seven were nitrogen fixer, and four were able to solubilize inorganic phosphate *in vitro*. Based on 16S rRNA gene sequence analysis, bacterial isolates AJK-3 and AJK-9 showing multiple PGP-traits were identified as *Stenotrophomonas* spp. while AJK-7 showed equal homologies to *Acetobacter pasteurianus* and *Stenotrophomonas* specie. Plant inoculation studies indicated that these Plant growth-promoting rhizobacteria (PGPR) strains provided a significant increase in

shoot and root length, and shoot and root biomass. A significant increase in shoot N contents (up to 76%) and root N contents (up to 32%) was observed over the un-inoculated control.

Indirect plant growth promotion by PGPR

The indirect mechanism of plant growth occurs when PGPR lessen or prevent the deleterious effects of plant pathogens on plants by production of inhibitory substances or by increasing the natural resistance of the host (Nehl *et al.* 1996; Cartieaux *et al.* 2003). PGPR provide different mechanisms for suppressing plant pathogens. These include competition for nutrients and space (Elad and Baker, 1985; Elad and Chet, 1987), antibiosis by producing antibiotics viz., pyrrolnitrin, pyocyanine, 2, 4-diacetyl phloroglucinol (Pierson and Thomashow, 1992) and production of siderophores (fluorescent yellow pigment) viz., pseudobactin which limits availability of iron necessary for growth of pathogens (Lemanceau *et al.* 1992). Other important mechanisms include production of lytic enzymes such as chitinases and β -1, 3-glucanases which degrade chitin and glucan present in the cell wall of fungi (Frindlender *et al.* 1993; Potgieter and Alexander, 1996; Velazhahan *et al.* 1999), HCN production (Defago *et al.* 1990) and degradation of toxin produced by pathogen (Duffy and Defago, 1997). PGPR have attracted much attention in their role in reducing plant diseases. Although the full potential has not been reached yet, the work to date is very promising and may offer organic growers some of their first effective control of serious plant diseases.

PGPR AS BIOCONTROL AGENT

Soils in which a pathogen causes disease symptoms are called conducive soils. Spontaneous control of plant diseases by bacteria in some fields was discovered at several places around the world. Some soils, called suppressive soils contain bacteria that protect plants against fungal diseases despite the presence of disease-causing pathogens in soil (Schroth and Hancock, 1982; Haas and Defago, 2005).

Pathogenic micro-organisms affecting plant health are a major and chronic threat to food production and ecosystem stability worldwide. The term biological control and its abbreviated synonym biocontrol have been used in different fields of biology, but in plant pathology, this term is applied for the use of microbial antagonists (the biological control agent or BCA) to

suppress diseases. Most narrowly, biological control refers to the suppression of a single pathogen (or pest) by a single antagonist in a single cropping system. The use of microbes to control diseases, which is a form of biological control, is an environment-friendly approach (Pal and Gardener, 2006).

Mechanisms of Biocontrol

Microbial control of plant diseases is a complex phenomenon involving not only the biocontrol microbe, the pathogen, and the plant, but also the indigenous microflora, macrobiota such as nematodes and protozoa, and the plant growth substrate such as soil, stonewool, or vermiculite. Many reviews on biocontrol have been written (Thomashow and Weller, 1996; Chin *et al.* 2003; Compant *et al.* 2005; Haas and Defago, 2005).

Competition for nutrients and space, niche exclusion, limiting available Fe supply through production of siderophores, producing lytic enzymes and antibiosis, induced systemic resistance and production of anti-fungal metabolites (AMFs) is the probable means responsible for biocontrol activity of PGPRs (Bloemberg and Lugtenberg, 2001; Jing *et al.* 2007).

Therefore the mechanism of biocontrol can be broadly distinguished into three main categories viz. antibiosis (production of anti-fungal metabolites AMFs), Induced systemic resistance (ISR), competition for nutrients and niches and finally the combinations of all the above modes of action.

Antibiosis: Production of Anti-Fungal Metabolites by PGPR

For a PGPR to be suitable for biocontrol, firstly it must not only synthesize and release the anti-fungal metabolites, but also compete successfully with other organisms for nutrients from the root and for niches on the root to deliver the metabolite along the whole root system. Secondly, the PGPR should produce the anti-fungal metabolite in the right microniche on the root surface. Finally, bacterial mutants (defective in structural genes involved in synthesis of anti-fungal metabolite) should be biocontrol negative (Chin *et al.* 2000; Pliego *et al.* 2008).

Most of the PGPRs are recorded to produce AFMs, of which phenazines, pyrrolnitrin, 2, 4-diacetylphloroglucinol (DAPG), pyoluteorin, viscosinamide and tensin are the frequently

detected classes (Bloembergen and Lugtenberg, 2001; Bhattacharyya and Jha, 2012). Kanosamine and zwittermycin A have also been reported to be produced by *Bacillus cereus* (Milner *et al.* 1996; Emmert *et al.* 2004). Antibiotics more recently discovered in biocontrol strains are d-gluconic acid (Kaur *et al.* 2006) and 2-hexyl-5-propyl resorcinol (Cazorla *et al.* 2006). Volatiles other than hydrogen cyanide, such as 2,3-butanediol, or blends of volatiles are also produced by *Bacillus* spp. (Ryu *et al.* 2003).

P. fluorescens strain, WCS374 has been recorded to suppress *Fusarium* wilt in radish leading to an average increase of 40% in yield (Bakker *et al.* 2007). The individual genes such as *phzO* and *phzH* responsible for the presence of functional group on phenazine compound have been detected (Chin-A-Woeng *et al.* 2001). More recently, informations have been generated on the biosynthesis of pyoluteorin in *Pseudomonas fluorescens* Pf-5 and 2, 4-diacetylphloroglucinol in *P. fluorescens* Q2-87 (Kidarsa *et al.* 2011).

Induced systemic resistance (ISR)

Interaction of some bacteria with the plant roots can result in plants resistant to some pathogenic bacteria, fungi, and viruses. This phenomenon is called induced systemic resistance (ISR) (Van Loon *et al.* 1998; Lugtenberg and Kamilova, 2009). ISR acts through a different signaling pathway to that regulating systemic acquired resistance (SAR). Bacterial determinants that are claimed to produce ISRs include siderophores, the O-antigen of lipopolysaccharide and salicylic acid. Presence of salicylic acid even in nanogram amounts has been indicated to cause an ISR when present (DeMeyer *et al.* 1999; Bloembergen and Lugtenberg 2001; Kloepper *et al.* 2004; Van Loon, 2007; Lugtenberg and Kamilova, 2009).

The mechanism of ISR elicited by PGPR sometimes, overlaps partly with that of pathogen-induced systemic acquired resistance (SAR). Both ISR and SAR represent a state of enhanced basal persistence of the plant that depends on the signaling compounds jasmonic acid and salicylic acid (Van Loon, 2007). Berg (2009) highlighted that pathogens are differently sensitive to the resistance activated by these signaling pathways. These interactions are highly specific on each component: the host plant, the pathogen, as well as the PGPR strain. They recognize each other by chemical signaling: root exudates as well as microbial metabolites can

have this function. The mechanisms of ISR include (1) developmental escape: linked to growth promotion, (2) physiological tolerance: reduced symptom expression, (3) environmental: associated with microbial antagonisms in the rhizosphere, and (4) biochemical resistance: induction of cell wall reinforcement, induction of phytoalexins, induction of pathogenesis-related proteins, and priming of defense responses (resistance).

Recently, interactions between *Bacillus* spp. and plants with special reference to induced systemic disease resistance have been reported by Choudhary and Johri (2009). Several species of *Bacillus* like *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides* and *B. sphaericus* (Ryu *et al.* 2004) are presently recorded to elicit significant reduction in disease incidence on diversity of hosts.

Competition for nutrients and niches

Rhizobacteria can suppress the growth of various phytopathogens in variety of ways like competing for nutrients and space, limiting available Fe supply through producing siderophores, producing lytic enzymes and antibiosis (Jing *et al.* 2007). Chitinases are particularly useful in agriculture as biocontrol agents against fungal phytopathogens because of their ability to hydrolyse the chitinous fungal cell wall (Chaiharn and Lumyong, 2008; Maisuria *et al.* 2008; Suresh *et al.* 2010; Wahyudi *et al.* 2011 and Kumar *et al.* 2012).

Competition of biocontrol bacteria with the pathogen for nutrients and niches in the rhizosphere (CNN) has been suggested for decades as a possible mechanism of biocontrol, but experimental proof was lacking. Kamilova *et al.* (2008) argued that if this mechanism exists such biocontrol strains can be selected. To this end, they applied a mixture of rhizosphere strains onto surface-sterilized seeds, which were subsequently allowed to germinate in a gnotobiotic system (Simons *et al.* 1996). After 1 week, the root tip, which contained the best competitive root colonizers, was removed from the seedling and the bacterial content was briefly allowed to multiply and subsequently applied onto fresh seeds for a new enrichment cycle. After three of such cycles, the isolated bacteria were as good as, or even better biocontrol agents. Mutant studies confirmed the proposed mechanism (Kamilova *et al.* 2005; Validov, 2007).

Enzymes production

Enzymes are the organic catalysts produced generally by micro organisms, differing from other catalysts and constitute the tools which determine the course of the multitude of life process. Various kinds of enzymes are produced by micro organisms. The antagonistic activity against different type of microbes may also be due to the production of lytic enzymes that are produced *in vitro* or *in vivo* by micro organism. An enzyme chitinase and chitobiase produced by some bacteria and fungi like *Mucor*, *Trichoderma* and *Pseudomonas* species possessed lytic effect which was related to antagonistic behavior (Chet, 1984; Gooday, 1986; Uthao and Peberdy, 1991).

Ogbo and Okonkwo (2012) isolated *Enterobacter* strain *Enterobacter asburiae* from roots of maize and possessed multipla plant growth promoting activities viz. Indole-3-acetic acid production, plant polymer hydrolyzing enzymes, pectinase and cellulose as well as ammonia *in vitro*.

Kavroulakis *et al.* (2007) isolated twenty six *Pseudomonas* strains from rhizosphere of apple and pear and screened for the production of various PGPR activities and proteolytic enzyme production viz. protease, chitinase and glucanase. Four out of total isolates were selected on the basis of their higher PGPR attributes and proteolytic activities.

Kumar *et al.* (2012) studied the hydrolytic enzymes viz., chitinase, protease, β -1, 3 glucanase and cellulase from twenty eight *Bacillus* sp. isolated from tomato rhizospheric soil in IIVR farm (DPNSB-1 to 7), IIHR farm (DPNSB-8 to 15), IARI farm (DPNSB-16 to 20) and farm of APHU (DPNSB-21 to 28). Among these strains, IARI isolate of DPNSB-18 exhibited the highest chitinase activity (4.65 IU/ml), IIHR isolate of DPNSB-15 produced highest protease activity (0.79 IU/ml), maximum β -1, 3 glucanase production was noted in *Bacillus* strains viz., DPNSB-14 (IIHR isolate), DPNSB-2 (IIVR isolate) and DPNSB-20 (IARI isolate), ranged from 0.24 IU/ml to 0.39 IU/ml, cellulase production was made by isolates of IIVR, DPNSB-3 (0.75 IU/ml) and DPNSB-1 (0.60 IU.ml) respectively.

Among different lytic enzymes chitinases are particularly useful in agriculture as biocontrol agents against fungal pathogens because of their ability to hydrolyse the chitinous

fungal cell wall (Chaiharn and Lumyong, 2009; Maisuria *et al.* 2008; Suresh *et al.* 2010; Wahyudi *et al.* 2011).

Bhatt and Vyas (2014) isolated and screened bacterial isolates for various PGPR activities viz. P solubilization, production of IAA, HCN, catalase, amylase etc. among these strains 27% produced lipase, 53.0 per cent produced amylase, 50.0 per cent produced chitinase.

Rani *et al.* (2014) isolated 65 bacterial strains, of which 35 were selected for the screening of PGPR traits. Sixteen isolated strains were successfully characterized for the production of enzymes like urease, chitinase, amylase, cellulose, protease and β -1,3 glucanase.

Ramos Solano *et al.* (2010) screened 96 strains of PGPR from the rhizosphere of *N. glauca*. A subset of 442 isolates constituted by the most abundant parataxonomic groups were characterized based on metabolic activities like siderophore and chitinase production regarded as putative PGPR traits.

Lytic enzymes produced by antagonistic bacteria are one of the most potent enzymes for degrading fungal cell walls (Leelasuphakul *et al.* 2006). Oxidative enzymes, peroxidase and polyphenol oxidase, catalyze the formation of lignin and other oxidative phenols, thereby contributing to the formation of defence barriers by reinforcing the cell structure during the defence reaction against pathogens (Thilagavathi *et al.* 2007).

Enzymes such as phenylalanine ammonia-lyase are involved in phytoalexin or phenolic compound biosynthesis. Such enzymes have been correlated with defence against pathogens in several plants (Binutu and Cordell, 2000). Similarly accumulation of phenolic compound in carnation by a *Pseudomonas* sp. thereby decreased *Fusarium* wilt (Van peer *et al.* 1991).

Han and Lee (2005) reported that the simultaneous coinoculation of two or more than two PGPR is more beneficial to the plant as compared to single inoculation. Inoculation with two PGPR strains, *Serratia* sp. and *Rhizobium* sp. into saline soils alleviated the salinity effects on the antioxidant enzymes ascorbate peroxidase and glutathione reductase, along with those on photosynthesis, mineral content and growth.

Biocontrol as a result of PGPR inoculation

Root dip application of *Bacillus subtilis*, *Pseudomonas fluorescens*, *Aspergillus awamori*, *Aspergillus niger* and *Penicillium digitatum* resulted insignificant decline in the rhizosphere population of *Fusarium oxysporum* (Khan and Khan, 2002). The root systems of plants offer different micro-habitats for bacterial growth. Cho *et al.* (2003) studied the endophytic colonization of balloon flower (*Platycodon grandiflorum*) by antifungal strain *Bacillus* sp. C422. The results revealed that *Bacillus* sp. C422 showed antibiotic activities against several phytopathogens by producing the antibiotic iturin A. Sain *et al.* (2005) evaluated ten strains of plant growth promoting rhizobacteria (PGPR), *Pseudomonas fluorescens* and *Bacillus* sp. for biological control of black rot of cauliflower caused by *Xanthomonas campestris* pv. *campestris*.

Han *et al.* (2006) identified an antagonistic strain of *Bacillus* sp. against *Streptomyces scabei* and observed that the culture broth had a suppressive effect on common scab disease in a pot assay, decreasing the infection rate from 75 to 35 per cent. Nikolay *et al.* (2006) reviewed microbially mediated solubilization of insoluble phosphates through the release of organic acids in combination with production of other metabolites such as siderophores, phytohormones and lytic enzymes that take part in biological control against soil borne phytopathogens.

Kavroulakis *et al.* (2007) isolated a strain Fs-K of *Fusarium solani* from endophytic root tissues of tomato plant. Strain Fs-K was able to colonize root tissues and subsequently protect plants against the root pathogen. *F. oxysporum* elicited and induced systemic resistance against the tomato foliar pathogens. Lokesh *et al.* (2007) isolated seven plant growth promoting rhizobacteria which were able to inhibit a wide range of fungal species and were found to be effective against *Fusarium* sp., *Didymella bryoniae* and *Myrothecium* species. The inoculation with phosphate solubilizing bacteria was useful in controlling *Phytophthora* crown blight of pepper under field conditions and increased the yield (Akgul and Mirik, 2008).

Senthilkumar *et al.* (2008) isolated 137 bacterial isolates from surface sterilized root, stem and nodule tissues of soyabean and screened them for antifungal activity. It was observed that *Paenibacillus* sp. (HKA-15) and *Bacillus* sp. (HKA- 121) were potential candidates for biocontrol of charcoal rot fungus as well as for soybean plant growth promotion.

Recep *et al.* (2009) studied the effect of plant growth promoting rhizobacteria as biocontrol agents against *Fusarium* dry rot of potatoes under storage conditions. *B. cepacia* strain OSU-7 resulted in an inhibition zone ranging from 35.33 to 47.37 mm.

Shanmugam *et al.* (2011) developed a talc-based formulations of plant growth promoting rhizobacterial strain S2BC-2 (*Bacillus atrophaeus*) and strain mixture, S2BC-2 + TEPF-Sungal (*Burkholderia cepacia*), inhibitory to the growth of *Fusarium oxysporum* f. sp. *gladioli* (FOG) in gladiolus. The results showed that in comparison to the individual strain, the strain mixture recorded maximum spike and corm production of 100 and 150%, respectively with less vascular wilt and corm rot incidences of 73.6 and 54.8% reduction over the pathogen control in greenhouse when inoculated with FOG. In field experiments, the strain mixture recorded less vascular wilt and corm rot incidences of 48.6 and 46.1% mean reduction over the non-bacterised control, and was almost comparable with that of fungicide (51.5 and 47.1%, respectively).

The capability of a novel bio-organic fertilizer (BIO2) that integrated the biocontrol agent *Bacillus subtilis* N11 and mature composts to control *Fusarium* wilt of banana in pot experiments was investigated. The results showed that the application of the BIO2 significantly decreased the incidence rate of *Fusarium* wilt compared to the control. The colonization of the natural biocontrol agent on banana roots using a GFP marker was also studied (Zhang *et al.* 2011).

Liang *et al.* (2011) isolated and evaluated *Bacillus megaterium* L8, a plant growth-promoting rhizobacterium (PGPR) for its ability to induce resistance in cucumber against seedling damping-off caused by *Pythium aphanidermatum*. *Bacillus megaterium* L8 treatment of cucumber roots effectively suppressed seedling damping-off than did the control as judged by seedling survival rate (83.45 versus 31.68% at 28 days).

Ramyasmruthi *et al.* (2012) isolated 18 bacterial isolates from the rhizosphere of brinjal, capsicum, chilli and screened them for the production of various PGPR activities. These isolates were also found to produce siderophore, IAA, HCN, phosphate solubilisation, NH₃ and catalase. Dual plate assay against *Alternaria alternata* OTA36; *Alternaria brassicola* OCA1; *Alternaria brassicae* OCA3; *Collectotrichum gleosporidose* OGC1 revealed anti-fungal activity by isolate R. The isolate R was identified as *Pseudomonas fluorescens* by biochemical test. Chilli seeds

inoculated with *Pseudomonas* showed 100% germination index and almost 50% reduction in disease incidence by *C. gloeosporioides* OGC1.

Shehata *et al.* (2012) conducted a pot and field experiment to evaluate the effect of *Pseudomonas fluorescens* and *Bacillus subtilis*. The pot experiment evaluated the probable suppressive effect of rhizobacteria as bioagents against *Macrophomina phaseolina*, *Rhizoctonia solani* and *Sclerotium rolfsii* under artificially infested soil. Results showed that co-inoculation of soybean with rhizobacteria led to a significant decrease in pre and post emergence damping-off caused by all pathogens besides enhancing the nodulation. Field experiments carried out in Etay-El Baroud evaluated the promotive and suppressive disease effects of rhizobacteria on nodulation, plant growth and yield of soybean.

Avinash and Rai (2014) studied three standard isolates both *in-vitro* and *in-vivo* against *Fusarium solani*. Maximum inhibition was in case of *Fusarium solani* by *Bacillus cereus* MIC5. *Sarratia* spp. MIC1 antagonized the *F. verticillodes* and *F. solani*. *P. aeruginosa* MIC2 inhibits all tested isolates *F. oxysporum*. *P. aeruginosa* MTCC2581 suppressed the radial growth rate of *F. oxysporum*. The crude extract of *P. aeruginosa* MIC2 developed in chloroform: methanol (9:1) showed a metabolite at R_f - 0.77 which may be 2,4- diacetylphloroglucinol (DAPG), a broad-spectrum antimicrobial agent. Increased cucurbit seeds germination and seedling vigour was observed in *B. amyloliquefaciens* MIC6 (68% & 1576) and *P. aeruginosa* MTCC2581 (70% & 1929) in primed seeds. Further *P. aeruginosa* MTCC2581 can be tested in the field against the *Fusarium* wilt.

Sharma *et al.* (2015) investigated 20 PGP rhizobacterial strains belonging to the genus *Bacillus*, previously isolated from the rhizosphere of several crops, were evaluated for their efficacy against *Phytophthora capsici* causing root and crown rot of tomato. These isolates were characterized for their PGP traits and *in vitro* antibiosis. Liquid based culture of strain S25 was found to be the most promising biocontrol treatment and reduced the disease severity by 83.3%. Conjoint application of four strains also had significant effect in achieving biocontrol efficacy of 66.7%. 16S rDNA and phylogenetic analysis showed that strain S25 exhibits 99% level of similarity with *Bacillus subtilis*. Therefore, it was designated as *B. subtilis* strain CKTR. Hence,

liquid based culture of strain S25 can be used as a promising antagonistic agent for management of *Phytophthora* blight.

Future prospects and challenges

PGPR are an environmentally friendly alternative to chemical fertilizers and pesticides, the use of which is regulated and sometimes forbidden, the market for bioinoculants is therefore expanding. PGPR inoculants can fulfill diverse beneficial interactions in plants leading to promising solutions for sustainable and environment friendly agriculture/horticulture. Application of PGPR in agricultural and horticultural crops has established considerable promises in both the laboratory and greenhouse experiment and to some extent in field as well. However, inconsistent response of PGPR in different environments and on various hosts remains as a significant impediment to their widespread development and application. Paramount to further success of PGPR there is need for better understanding of the ecology of microorganisms, either indigenous or introduced, within the rhizosphere both as individual organisms and through their interaction and directly with host plants.

Recent progress in molecular biology and biotechnology can further help in the understanding of rhizobacterial interactions with crop plants that has encouraged a suitable area of research in PGPR mechanisms relating to rhizosphere colonization. PGPR can be classified as biofertilizers, phytostimulators and biopesticides with certain bacteria having overlapping applications. Therefore, the need of the hour is identify naturally occurring rhizobacterial strains with multifunctional PGP traits, which have a coordinated expression of genes for enhancing the plant growth, yield, nutrient uptake of various crop plants and alleviating the attack of both pests and diseases in field crops in different agro ecosystems. The success and commercialization of PGPR strains is another aspect that needs to be taken into account, which depend on the linkages between the scientific organizations and industries. Commercial success of PGPR strains requires economical and viable market demand, consistent and broad spectrum action, safety and stability, longer shelf life, low capital costs and easy availability of career materials. Quality control in this step is crucial to retain the confidence of farmers on the efficacy of the antagonistic strain. (Richardson, 2001; Nakkeeran *et al.* 2005; Lugtenberg and Kamilova, 2009; Richardson *et al.* 2009; Ali and Hj, 2010; Bhattacharyya and Jha, 2012).

With increasing concern about the natural environment and the understanding that the era of the large scale use of chemicals in the environment needs to come to an end, PGPB offer an attractive alternative that contains the possibility of developing more sustainable approaches to agriculture. Identification of different mechanisms involved in plant-rhizosphere microorganism interaction opened new possibilities to design strategies for improving crop yields. Along this, biotechnology can be applied to further improve strains that have PGPR qualities in order to create transgenic strains that combine multiple mechanisms of action. Our understanding of plant microbe interaction in Rhizosphere must increase before we can presume that utilization of PGPR as biofertilizers will determine a sustainable promotion of host plants growth. 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. PGPRs are excellent model systems which can provide the biotechnologist with novel genetic constituents and bioactive chemicals having diverse uses in agriculture and environmental sustainability.

Chapter-3

MATERIALS AND METHODS

The present investigation entitled “**Study of rhizobacterial community structure and evaluation of plant growth promoting rhizobacterial application strategies to promote growth and yield in apple**” was conducted in the laboratory of Microbiology section of Department of Basic Science at Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh and the studies under field conditions were conducted in the research fields of Department of Fruit Science, UHF, Nauni, RHRS Mashobra and farmers field at Matiana , Thanedhar, Kyari, Kotkhai, Chopal during the year 2013-2015. An account of the material used and methodology adopted is discussed in this chapter.

3.1 MEDIA

Composition of the media used for the study (Atlas, 1995):

3.1.1 Nutrient agar (NA)

Beef extract	:	0.3%
Peptone	:	0.5%
NaCl	:	0.8%
Agar	:	2.0%
pH	:	6.5

3.1.2 Malt extract agar (MEA)

Malt extract	:	2.0%
Agar	:	2.0%
pH	:	5.6

3.1.3 King's medium B

Proteose peptone	:	2.0%
K ₂ HPO ₄ anhydrous	:	0.15%
MgSO ₄ .7H ₂ O	:	0.15%

Glycerol	:	1.5%
Agar	:	2.0%
pH	:	7.2

3.1.4 Nitrogen free medium

K ₂ HPO ₄ anhydrous	:	0.1%
MgSO ₄ .7H ₂ O	:	0.02%
NaCl	:	0.02%
FeSO ₄	:	0.0005%
Soil extract	:	0.5%
Glucose	:	1.0%
Agar	:	2.0%
pH	:	7.6

3.1.5 Pikovskaya's (PVK) broth

Glucose	:	1.0%
Ca ₃ (PO ₄) ₂	:	0.5%
(NH ₄) ₂ SO ₄	:	0.05%
KCl	:	0.02%
MgSO ₄ .7H ₂ O	:	0.01%
MnSO ₄	:	0.0004%
FeSO ₄	:	0.0002%
Yeast extract	:	0.05%
Bromocresol purple	:	0.01%

3.1.6 Pikovskaya's agar

Pikovskaya's broth+ 2.0% agar

3.1.7 Luria Bertani (LB) agar

Tryptophan	:	1.0%
Yeast extract	:	0.5%
NaCl	:	0.5%
Agar	:	2.0%
pH	:	7.5

3.1.8 Chrome- azurol-S- (CAS) agar

CAS	:	0.006%
HDTMA	:	0.007%
HCl	:	0.002%
FeCl ₃	:	0.002%
Agar	:	2.0%

3.1.9 Skim milk agar medium

Pancreatic digest of casein	:	0.50%
Yeast extract	:	0.25%
Glucose	:	0.10%
Skim milk	:	7.00%
Agar	:	2.0%

3.1.10 Starch agar

Starch	:	2%
Peptone	:	5.0%
Beef extract	:	0.3%
Agar	:	2%

3.1.11 Minimal agar medium

Potassium dihydrogen phosphate	:	0.3%
Disodium hydrogen phosphate	:	0.6%
Sodium chloride	:	0.5%
Ammonium chloride	:	0.2%
Magnesium sulphate	:	0.01%
Glucose	:	0.8%
Agar	:	1.5%
pH	:	7.0

3.2 CHEMICALS AND REAGENTS

Analytical grade chemicals and reagents obtained from Hi-Media, BDH or E. Merck were used for most of the investigations.

3.3 MICROBIOLOGICAL METHODS

3.3.1 Microbial strains and their maintenance

Cultures of bacterial strain CKA1, CKAM, G4(3) and RG1(3) (originally isolated from the roots/soil rhizosphere of apple (*Malus domestica* Borkh.) seedlings) and *E.coli* DH5 α were obtained from the culture collection centre of Microbiology section, Department of Basic Science, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh. The bacterial cultures were maintained on solid medium in NA at 4°C and preserved in NB containing 30% (v/v) glycerol at – 20°C. *E.coli* DH5 α was maintained and preserved in LB medium.

3.3.2 Fungal pathogens and their maintenance

Dematophora necatrix, *Pythium aphanidermatum*, *Fusarium oxysporum*, *Sclerotium rolfsii* and *Phytophthora cactorum* and *Corticium salmonicolor* were obtained from the preserved fungal cultures in laboratory of Microbiology, University of Horticulture and Forestry, Nauni, Solan. The culture was maintained on malt extract agar at 4 °C. Sub-culturing was done periodically on same medium at incubation temperature of 24 $\pm$ 1°C.

3.3.3 Incubation

The bacterial culture was grown at 37 $\pm$ 2 °C and fungal culture was maintained at 25 $\pm$ 1 °C except when mentioned otherwise.

3.3.4 Sterilization

Glassware used was thoroughly washed in detergent water, running tap water followed by rinsing in distilled water. The flasks used were tightly plugged with cotton gauge and were covered with aluminum foil. Glassware was then kept in hot air oven at 180°C temperature for one hour. All the media, distilled water solutions etc. were sterilized in autoclave at 15 lbs per square inch pressure of pure steam for 20 minutes, unless mentioned otherwise. Laminar airflow chamber was irradiated by ultra violet (UV) for 15 minutes before use.

3.4 MEASUREMENT OF GROWTH

3.4.1 Preparation of inoculum

A bacterial cell suspension (O.D. 1.0 at 540 nm) of 24h old culture grown on nutrient agar slants at the rate of 10 per cent was used as inoculum in all experiments, unless mentioned otherwise.

3.4.2 Turbidity

Growth was monitored by measuring the change in absorbance of cells in the nutrient broth at 540 nm using un-inoculated broth as blank.

3.4.3 Viable count

Appropriate dilutions of bacterial population were used to seed the medium. The number of viable cells in the initial population was obtained by counting the number of colonies that developed after incubating the plates, and multiplying this figure by dilution factor.

3.5 SAMPLING

Samples (soil/root) were collected from different apple growing locations of Himachal. At each location, different apple orchards were selected based on the following criteria:

- Orchards with new plantation at Matiana (Sablog, Nanni), Kinnaur (Reckong Peo) and Kotkhai (Kufarbagh)
- Orchards with re-plantation at Shimla (RHRS, Mashobra, Dhanta and Jutog), Matiana (Sablog, Nanni, Jhalroo), Kinnaur (Reckong Peo), Kotkhai (Kyari and Kufarbagh), Kotgarh (Shathla and Thanedhar), Chopal and Rohro (Seema and Chajpur)
- Healthy apple orchards at Shimla (RHRS, Mashobra), Matiana (Sablog, Nanni) and Kotkhai (Kyari and Kufarbagh)
- Root rot infected diseased apple orchards at Shimla (RHRS, Mashobra, Dhanta and Jutog), Matiana (Sablog, Nanni, Jhalroo), Kinnaur (Reckong Peo), Kotkhai (Kyari and Kufarbagh) and Kinnaur (Reckong Peo)

Soil/roots samples were taken from four points at 0 to 15cm depth in the area of the basin projection. For maintaining statistical validity, soil sampling was carried out from minimum of ten apple plants/trees/pits within each orchard. The samples from each orchard were pooled to make composite sample. A total of 200 soil and root samples were used for isolation of bacterial population. Sampling was done in the month of February-March of the year 2013. Samples were immediately stored at 4°C in plastic bags loosely tied to ensure sufficient aeration and to prevent moisture loss until assaying of bacterial community structure. The remaining rhizosphere soil was air-dried and sieved (2mm to 5mm mesh size)

prior to soil physicochemical analyses. Available nitrogen, potassium and phosphorus were determined by methods as standardized by Subbiah and Asija (1956); Merwin and Peach, (1951); Olsen *et al.* (1954), respectively. The geographic location and altitude of the sampling sites were determined using global positioning system (GPS) (Table1 and Fig. 4).

3.6 ISOLATION OF RHIZOSPHERIC SOIL AND ROOT ENDOSPHERIC BACTERIAL COMMUNITIES

3.6.1 Isolation of rhizospheric and endophytic rhizobacterial population

Bacterial isolates were isolated from the rhizosphere soil and roots of apple trees during the month of March 2013-2015. Root samples collected from different sites under natural conditions were shaken vigorously to remove the soil tightly adhered to the roots. One gram of this rhizosphere soil was placed in 9 ml of sterilized distilled water under aseptic conditions. To estimate the number of soil bacteria, counts were calculated on the basis of serial 10 fold dilutions in duplicate, using the pour plate method and triplicate samples of 1 gm soil, and an appropriate dilutions (Johnson and Curl, 1972). Colony forming units (cfu) were recorded on nutrient agar plates after 48 hour, the average number per gram oven dry weight of soil was calculated as:

$$\text{cfu (g}^{-1}\text{soil)} = \frac{\text{Bacterial plate count} \times \text{Dilution factor}}{\text{Oven dry wt.of soil}}$$

PSB were enumerated on Pikovskaya's (PVK) medium supplemented with tri-calcium phosphate as insoluble inorganic phosphate source. The population of PSB was determined as given below:

$$\% \text{ P- solubilizers} = \frac{\text{Number of colonies forming halozone on PVK media}}{\text{Total number of colonies on PVK media}} \times 100$$

Siderophore producing bacteria (SPB) were enumerated on Chrome-azurol-S (CAS). The population of SPB was determined as given below:

$$\% \text{ Siderophore producers} = \frac{\text{Number of colonies forming halozone on CAS media}}{\text{Total number of colonies on CAS media}} \times 100$$

Table 1. Site location, geographic and climatic characteristics of apple orchards used for sample collection at Himachal Pradesh

Location	Sampling site	Geographic location	Altitude ^b (m)	Climatic Classification
Kinnaur	Reckong Peo	31.30 ⁰ N 78.10 ⁰ E	1821	Dry Temperate
Shimla	RHRS, Mashobra	31.13 ⁰ N 77.23 ⁰ E	2146	Temperate
	Dhanata	31.1 ⁰ N 77.12 ⁰ E	2290	Temperate
	Jutog	31.1 ⁰ N 77.12 ⁰ E	1729	Temperate
Matiana	Sablog	31.12 ⁰ N 77.24 ⁰ E	2081	Dry Temperate
	Nanni	31.07 ⁰ N 77.21 ⁰ E	2299	Temperate
	Jhalroo	31.11 ⁰ N 77.23 ⁰ E	2100	Temperate
Rohru	Seema	31.12 ⁰ N 77.45 ⁰ E	1583	Temperate
	Chajpur	30.40 ⁰ N 77.20 ⁰ E	2290	Temperate
Kotkhai	Kyari	31.06 ⁰ N 77.32 ⁰ E	1752	Temperate
	Kufarbagh	31.12 ⁰ N 77.28 ⁰ E	2080	Temperate
Chopal	Chopal	30.48 ⁰ N 77.26 ⁰ E	2297	Temperate
Kotgarh	Thanedhar	31.19 ⁰ N 77.29 ⁰ E	2400	Temperate
	Shathla	31.19 ⁰ N 77.29 ⁰ E	1880	Temperate

^b Altitude above sea level

3.6.2 Isolation of endo-rhizobacteria

The root sample was surface sterilized by 0.2 per cent mercuric chloride (HgCl₂) for three minutes followed by repeated washing in sterilized distilled water. The surface sterility of roots was cross checked by incubating the surface sterilized roots in sterilized nutrient broth overnight.

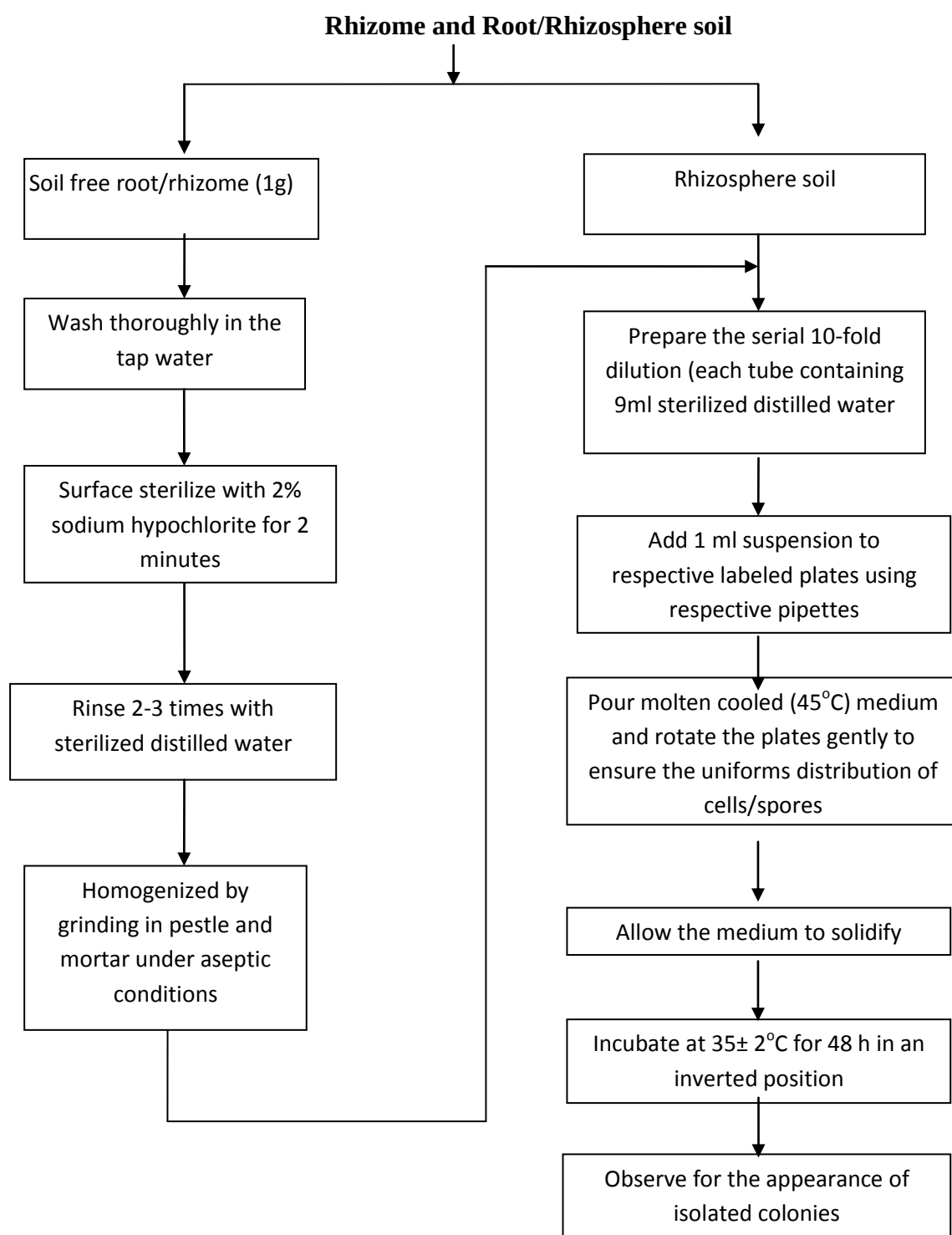


Fig. 4. Generalized flow chart for the isolation of rhizospheric soil and root endophytic bacteria

One gram of surface sterilized root sample was placed in 9 ml of sterilized distilled water and was ground to produce slurry using pestle and mortar under aseptic conditions. The root suspension was diluted in 10 fold series and bacterial count was determined by standard

pour plate technique (Fig. 4). Populations were expressed as colony forming unit (cfu) per gram of the root weight. The isolates were maintained on specific medium for further studies.

3.7 IN VITRO SCREENING FOR MULTIFARIOUS PLANT GROWTH PROMOTING ACTIVITIES

3.7.1 Phosphate Solubilization

3.7.1.1 Qualitative estimation of phosphate solubilization (Pikovskaya, 1948)

The ability of bacteria to solubilize phosphorus was tested by streaking on the PVK agar plates containing known amount of tri-calcium phosphate ($\text{Ca}_3(\text{PO}_4)_2$). The plates were incubated at 37°C for 48h. Each treatment was replicated three times. The bacterial solubilization of phosphorus was exhibited with yellow coloured zones produced around the isolated bacterial colony. The halo diameter around the colony was calculated by subtracting colony size from total size (Fig. 5) Phosphate solubilization index (PSI) was measured using the formula (Edi-Premono *et al.* 1996).

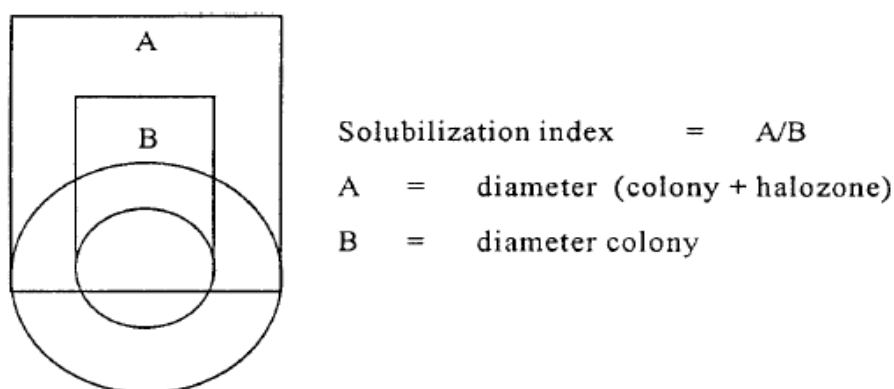


Fig.5. Diagrammatic representation for computation solubilization index

3.7.1.2 Tri-calcium phosphate solubilization in liquid medium

Pikovskaya's medium with composition given in (3.1.5) was used for the solubilization of phosphate. Fifty ml of medium was dispensed in 250 ml of Erlenmeyer flask containing 0.5 per cent tri-calcium phosphate (TCP) and autoclaved at 15 psi for 20 min. The flasks were inoculated with 1 per cent of the 24h old bacterial suspension (OD 1.0 at 540 nm) and incubated at 37°C under shaken conditions for 72h. Simultaneously, two controls of PVK broth were run, one with TCP without inoculum and the other with inoculum, but without TCP. Flasks were withdrawn at 72h and contents were centrifuged at 15000 rpm for 20min at 4°C . Prior to centrifugation, the samples were withdrawn aseptically for determination of

viable number of cells by standard viable plate count technique. The culture supernatant was used for determination of the soluble phosphate.

3.7.1.3 Assay of soluble phosphate estimation (Bray and Kartz, 1945)

The procedure essentially consisted of estimating soluble phosphorus formed by the action of phosphate solubilizing bacteria on tri-calcium phosphate. The soluble phosphorus formed was estimated calorimetrically and the results were extrapolated from standard curve (Appendix I) drawn using potassium di-hydrogen phosphate. An aliquot (0.1-1.0 ml) from the culture supernatant was made to final volume of 25 ml with distilled water and 5 ml ammonium molybdate was added. The mixture was thoroughly shaken. The contents of the flasks were diluted to 20 ml. 1.0 ml of chlorostannous acid was added and its volume was made to 25ml in the volumetric flask. The contents were mixed thoroughly and the blue coloured intensity was measured after 10 minutes at 660 nm. An appropriate blank was kept in which all reagents were added except the culture. P- solubilization was calculated as:

$$\text{P-solubilization} = \frac{T-C}{C}$$

Where,

T = PVK with TCP, inoculated

C = PVK with TCP, un-inoculated

3.7.2 Siderophore Production

3.7.2.1 Qualitative estimation of siderophore by Chrome-azurol-S (CAS) plate assay (Schwyn and Neilands, 1987)

Siderophore production was detected by CAS plate assay method. Sterilized CAS blue agar was prepared by mixing CAS (60.5 mg/50ml distilled water) with 5ml iron solution (1mM $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) and 5ml 10mM HCl. This solution was slowly added to hexadecyltrimethyl ammonium bromide (HDTMA) (72.9 mg/40ml distilled water). Thus, 50 ml CAS dye was prepared and poured into 500 ml nutrient agar and plates were prepared with 25 ml of the medium. Twenty-four hours old culture of the test bacteria was spotted on pre-poured blue coloured CAS agar plates. Plates were incubated for 72h, at 37°C. Formation of a bright zone with a yellowish, pinkish and whitish colour in the dark blue medium indicated the production of siderophore.

3.7.2.2 Quantitative estimation of siderophore using Chrome-azurol-S (CAS) liquid assay method (Schwyn and Neilands, 1987)

0.1 ml of cell free extract of culture supernatant was mixed with 0.5 ml Chrome-azurol-S (CAS) assay solution (Appendix I) along with 10 µl of shuttle solution (0.2M 5-Sulfosalicyclic acid). It was kept at room temperature for ten minutes and absorbance was recorded at 630 nm. The minimal medium was used as a blank and the reference (r) was prepared using exactly the same components except the cell free extract of culture supernatant. The siderophore units were calculated using formula:

$$\text{Per cent siderophore unit} = \frac{A_r - A_s}{A_s} \times 100$$

Where,

A_r is defined as absorbance at 630 nm of reference.

A_s is the absorbance at 630 nm of the test.

3.7.3 ANTIFUNGAL ACTIVITY

3.7.3.1 Agar streak method

In order to test the efficacy of the rhizobacterial antagonists, a loopfull of 48h old culture of each isolate was streaked a little below the centre of the prepoured petriplates of malt extract agar (MEA) and incubated at 37°C. After 24h, a 5mm diameter actively growing test fungal pathogen was placed simultaneously on one side of the streak. A check inoculated with the test pathogen only was kept as control for comparison. Each treatment was replicated 3 times. The plates were incubated at 24±1°C and per cent growth inhibition was calculated according to Vincent, (1947).

$$\text{Per cent growth inhibition } I = \frac{C - T}{C} \times 100$$

Where,

I = Per cent growth inhibition

C = Growth of fungus in control

T = Growth of fungus in treatment

3.7.4 QUANTITATIVE ESTIMATION OF INDOLE-3-ACETIC ACID (IAA) (Gordon and Paleg, 1957)

For the production of auxins, bacterial cultures were grown in Luria Bertani broth (amended with 5 mM L-tryptophan, 0.065 per cent sodium dodecyl sulphate and 1% glycerol) for 72h at 37°C under shake conditions. Supernatant was prepared/collected by centrifugation of cultures at 15,000 rpm for 20 minutes and was stored at 4°C. In measuring the IAA equivalents, 3 ml of supernatant was pipetted into test tube and 2 ml of Salkowski's reagent (2 ml of 0.5 M FeCl₃ + 98 ml 35% HClO₄) was added to it. The tubes containing the mixture were left for 30 minutes (in dark) for the development of pink colour. Intensity of the colour was measured at 535 nm. Concentration of indole-3-acetic acid was estimated by preparing calibration curve using Indole-3-acetic Acid (IAA) (Hi-media) as standard (10-100µg/ml) (Appendix I).

3.7.5 HCN Production (Baker and Schippers, 1987)

The bacterial cultures were streaked on pre-poured plates of King's medium B amended with 4.4g/L glycine. Whatman No.1 filter paper strips were soaked in 0.5% picric acid in 0.2% sodium carbonate and was placed in between the Petri plates. Petri plates were sealed with parafilm and were incubated at 37°C for 1-4 days. Un-inoculated control was kept for comparison. Plates were observed for change of colour of filter paper from yellow to orange brown to dark brown.

3.7.6 Nitrogen Fixing Ability

To test the efficacy of the rhizobacteria as nitrogen fixer, loopful of 24h old culture of each isolate were streaked on nitrogen free glucose agar medium described in (3.1.4), incubated for 72h and the colonies showing growth were selected.

3.7.7 CELL WALL DEGRADING ENZYME PRODUCTION

3.7.7.1 Chitinase assay (Robert and Selitrennikoff, 1988)

3.7.7.1.1 Preparation of colloidal chitin

Colloidal chitin was prepared by the method (Berger and Reynolds, 1958) given below:

1. Powdered chitin digested overnight with concentrated hydrochloric acid at 4°C.
2. After digestion step carefully added distilled water and mix thoroughly.

3. Centrifuge and remove the supernatant carefully (the first two-three wash is highly acidic).
4. Continue washing with distilled until the pH of solution reaches around 4.0
5. Adjust the pH of the colloidal chitin solution by using 2N NaOH (a pH around 6-6.5).
6. Add liquid (10ml of chitin in 100ml media) directly or the chitin suspension in water was centrifuged and the pellet was collected, dried and used at 0.3% in minimal salt media.

The bacterial cultures were spotted on to the prepared minimal agar medium amended with 0.3% colloidal chitin and the plates were incubated at 30°C for 7 days. Development of halo zone around the colony after addition of iodine was considered as positive for chitinase enzyme production.

3.7.7.2 Proteolytic enzymes (Fleming *et al.* 1975)

All bacterial isolates were screened for proteolytic activity by plate assay method on skim milk agar (1% skim milk +100ml of nutrient agar and autoclaved separately). Spot inoculation with 72 h old bacterial culture was done on skim milk agar plate. Plates were incubated at 28°C for 28-48h and observed for proteolysis i.e. clear zone mm (diameter) produced around the spot.

3.8 PHENOTYPIC CHARACTERIZATION OF BACTERIAL ISOLATES

The most effective bacterial isolates exhibiting maximum IAA production and production of siderophore and P-solubilization and also capable of causing maximum inhibition of fungus under *in vitro* conditions were identified on the basis of morphological, cultural and biochemical characteristics by criteria of Bergey's Manual of Systematic Bacteriology (Claus and Berkley, 1986).

3.8.1 Morphological and biochemical characterization

Morphological characteristics of isolates including colony morphology, Gram's reaction and cell shape were investigated for isolates exhibiting multifarious plant growth promoting traits. Biochemical characterization was done using commercial kits (KB009 Hi carbohydrate™ kit and KB013 Hi *Bacillus*™ Identification kit).

3.9 Genetic diversity of rhizobacterial community in apple orchards

Previously sampled soil/root samples shall be used to study genetic diversity of rhizobacterial community in apple orchards.

3.9.1 Genomic DNA extraction by conventional method (Sambrook *et al.* 1989)

Bacterial isolates screened for various PGP trait were grown overnight at 37°C in nutrient broth at 200 rpm. The cells were harvested and processed for DNA isolation.

Requirements:

96-100% Ethanol

Sterile, DNase- free pipette tips and microcentrifuge tubes

RNase A (50 mg/ml)

10 % SDS

Phenol : Chloroform (1:1)

Extraction buffer (20 mg/ml lysozyme, 100mM Tris- HCl, 50mM EDTA, 500 mM NaCl)

TE buffer (10mM Tris HCl, 1mM EDTA)

Procedure

1. 5 ml of overnight grown culture was transferred to a micro-centrifuge tube and centrifuged at 13,000 rpm for 1 minute and supernatant was discarded.
2. Bacterial pellet was suspended in 500µl of extraction buffer and 50 µl of 10 % SDS. Cell pellet was resuspended by vortexing or pipetting.
3. Incubation at 65°C water bath was done for 30 min until the sample lysate becomes clear. During incubation, tube was inverted at every 3 min.
4. After 65°C incubation, 2 µl of RNase A (50mg/ml) was added to sample lysate and mixed by vortexing. Then incubated at room temperature for 5 min.
5. To the lysate equal volume of phenol:chloroform (1:1) was added and mixed well.
6. Centrifuged the above mixture at 10,000 rpm for 5 min at room temperature. Two layers were formed. Upper aqueous layer was collected in new eppendorf tube with the help of pipette.
7. The phenol:chloroform extraction step was repeated.

8. Centrifuged the above mixture at 10,000 rpm for 5 min at room temperature. Two layers were formed. Upper aqueous layer was collected in another new eppendorf tube with the help of pipette.
9. 1/10 volume of 5M NaCl and 2.5 volume of absolute ethanol was added to aqueous phase collected in eppendorf tube.
10. Incubation was done at - 20°C overnight.
11. Centrifuged the above mixture at 12,000 rpm for 20 min at room temperature and supernatant was discarded.
12. The DNA pellet was washed with 1 ml of 70 % ethanol.
13. Centrifuged the above mixture at 12,000 rpm for 5 min at room temperature and supernatant was discarded.
14. DNA pellet was air dried for about 15 min until all the residual ethanol got evaporated.
15. Finally the DNA pellet was suspended in appropriate amount of TE and quantify.

3.9.2 16S rDNA analysis:

The amplification of gene encoding 16S rDNA of rhizobacterial isolates was done using standard PCR reaction employing universal primers (forward 5'GCAAGTCGAGCGGACAGATGGGAGC3' and reverse 5'AACTCTCGTGGTGTGACGGGCGGTG3'). PCR reactions were performed in a 50µL volume containing 5µL 10x PFX buffer, 2µL MgSO₄, 2µL dNTPs (10mM each), 0.7µL PFX polymerase and 1.5µL each of forward and reverse primers. Amplifications were run under the following cycling conditions: 5 min. initial denaturation at 95°C, followed by 30 cycles of denaturing at 95°C for 3 min., annealing at 60°C for 45 s, extension at 72°C for 2 min. and completed with a final extension at 72°C for 10 min.

3.9.3 Primers used in amplified 16S rDNA restriction analysis (ARDRA) fingerprinting

Primers used for the amplification of 16S rDNA mentioned in 3.9.2 were used in the present study for developing ARDRA based fingerprint for identifying the strain using *Hinf*, *HpaII* and *TaqI* restriction enzyme.

3.9.4 Amplified rDNA Restriction Analysis (ARDRA):

Amplified 16S rDNA product of the putative rhizobacteria belonging to different region was distinguished on the bands of restriction digestion by using different restriction enzymes. Restricted digestion was analysed by agarose gel electrophoresis. On the basis of data derived from the restriction profiles, a matrix with binary code was composed in which 1 represented presence value and 0 represented absence value and analyzed by using NTYSIS software for windows. 16S rDNA pattern based on Dice similarity index will be carried out for the grouping of the isolates.

3.9.5 Gel electrophoresis

The amplified PCR product was resolved by electrophoresis using 1.0% agarose gel in 1X Tris-acetate EDTA buffer containing ethidium bromide (0.5µl/ml). DNA ladder of 100 bp was used as marker. The gel was run at 120V for 2h using Bangalore Genei power system. The gels were viewed and images were captured using gel documentation system (Bio-rad). To counter possible stochastic effects of PCR (Wagner *et al.* 1994), five amplifications were carried out on each sample and pooled prior to purification. Amplified PCR products were eluted from the gel using gel extraction kit (Hi Yield Gel/ PCR DNA Extraction Kit from Real Genomics). The PCR product was monitored through gel electrophoresis (1% agarose gel).

3.9.6 Sequence analysis of 16S rRNA Genes

The PCR products of 16S rDNA of dominant isolate from each distinguished group were purified, sequenced and further analyzed for bioinformatics analysis. The BLASTN program (www.ncbi.nlm.nih.gov) was used to find the identity and similarity of each sequences compared to the EMBL database. The Phylogenetic tree were constructed using alignments drawn using ClustalW from the website <http://www2.ebi.ac.uk/clustalw/> (Higgins *et al.* 1994). Phylogenetic analysis was carried out on multiple sequence alignments in order to construct phylogenetic trees by neighbor-joining method using CLC Main Workbench 8.0 (<http://www.clcbio.com>). Bootstrap confidence values were generated using 100 permutations of the data to measure the reliability of the nodes in the phylogenetic tree. The gene sequences were also be submitted to NCBI database.

3.9.7 Gel Extraction (Hi Yield Gel/ PCR DNA Fragment Extraction Kit)

3.9.7.1 Gel dissociation

Agarose gel slice containing relevant DNA fragments was excised and extra agarose was removed to minimize the size of the gel slice. 300 mg of the gel slice was transferred into a microcentrifuge tube. 500 µl of DF buffer was added to the sample and mixed by vortexing. Incubation was done at 55°C for 10-15 min until the gel slice was completely dissolved. During incubation, the tube gets inverted at every 2-3 min and dissolved sample mixture was cooled down to room temperature.

3.9.7.2 DNA binding

A DF column was placed in 2 ml collection tube. 800 µl of sample mixture (from above step) was applied into the DF column and centrifuged at 13,000 rpm for 30 seconds. Flow through was discarded and DF column was placed back in 2 ml collection tube.

3.9.7.3 Wash

Wash buffer 600 µl (ethanol added) was added into DF column and centrifuged at 13,000 rpm for 30 seconds. Flow through was discarded and DF column was placed back in 2 ml collection tube. Centrifuged again for 3 min at 13,000 rpm to dry the column matrix.

3.9.7.4 DNA elution

Dried column was transferred into a new microcentrifuge tube. 15-30 µl of elution buffer or distilled water was added into the center of column matrix. Stand for 2 min until elution buffer or distilled water was absorbed by the matrix. Centrifugation was done for 2 min at 13,000 rpm to elute purified DNA eluted fragment. The amplified products were then sequenced using a commercial sequencing facility (Xcleris lab) to confirm the identity of the bacterial isolate.

3.10 MOLECULAR CLONING AND SEQUENCING OF *gdh* GENE FROM *Bacillus licheniformis* CKA1

3.10.1 Genomic DNA extraction by conventional method (Sambrook *et al.* 1989)

Same as in section 3.9.1.

3.10.2 Cloning of *gdh* gene of *Bacillus licheniformis* CKA1 encoding glucose dehydrogenase enzyme (GDH) using polymerase chain reaction (PCR)

a. Template DNA

The genomic DNA of *Bacillus licheniformis* CKA1 was used as template DNA.

b. Primers for cloning and sequencing of complete *gdh* gene

The complete *gdh* gene of *B. subtilis* T1 was amplified by using gene specific primers for *gdh* gene (Mehta *et al.* 2013). The sequences of the primers are given below:

Forward GDH F: 5'-ATG TAT CCG GAT TTA AAA GGA-3'

Reverse GDH R: 5'-TTA ACC GCG GCC TGC CTG GAA-3'

The above primers were custom synthesized and supplied in lyophilized form by Integrated DNA Technology (IDT), USA. Primers were regenerated by suspending in autoclaved double distilled water to make a stock of 100 μ M, which was further diluted to make working primer solution at a concentration of 20 μ M.

c. PCR reagents

Taq DNA polymerase, dNTP, nuclease free water and PCR buffer etc. were procured from Merck Specialities Private Limited, Mumbai, India.

d. Reaction mixture for PCR

To amplify the *gdh* gene of *B. licheniformis* T1, the PCR was carried out using:

Template DNA (50 ng/ μ l)	1 μ l
Forward primer (20 μ M)	1 μ l
Reverse primer (20 μ M)	1 μ l
dNTP (1.5 mM each)	2 μ l
Taq DNA polymerase (3U/ μ l)	0.3 μ l
PCR buffer (10X)	1X (containing 1.5 mM MgCl ₂)

Final volume was made 25 μ l by adding nuclease free water.

e. Programme of thermocycler (PCR machine)

The PCR was performed using thermocycler (multigene PCR system, Labnet) with following programme:

PCR Conditions (Temperature °C)				
Initial denaturation	Denaturation	Annealing	Extension	Final extension
95 °C	95 °C	55 °C	72 °C	72 °C
2 min	45 sec	45 sec	1 min	10 min
× 1 cycle	× 35 cycles			× 1 cycle

f. Analysis of PCR product

The PCR product was analyzed by 1.0% (w/v) agarose gel electrophoresis (Section 3.6.2). DNA fragments were visualized under UV light after staining with ethidium bromide. Molecular weight of the amplified DNA fragment was determined using 10 µl of Super mix DNA ladder (100 bp size supplied by Thermo Scientific).

g. Purification of PCR product using gel extraction kit (Hi Yield Gel/PCR DNA Extraction Kit)

Gel dissociation

Agarose gel slice containing relevant DNA fragment was excised and extra agarose was removed to minimize the size of the gel slice. 300 ng of the gel slice was transferred into a microcentrifuge tube. 500 µl of DF buffer was added to the sample and mixed by vortexing. Incubation was done at 55 °C for 10-15 min until the gel slice was completely dissolved. During incubation, the tube was inverted at every 2-3 min and dissolved sample mixture was cooled down to room temperature.

A DF column was placed in 2 ml collection tube. 800 µl of sample mixture (from above step) was applied into the DF column and centrifuged at 13,000 rpm for 30 seconds. Flow through was discarded and DF column was placed back in 2 ml collection tube.

Wash

600 µl of wash buffer (ethanol added) was added into DF column and centrifuged at 13,000 rpm for 30 seconds. Flow through was discarded and DF column was placed back in 2 ml collection tube. Centrifuge again for 3 min at 13,000 rpm to dry the column matrix.

DNA elution

Dried column was transferred into a new microcentrifuge tube. 15-30 µl of elution buffer or distilled water was added into the center of column matrix. Stand for 2 min until elution buffer or distilled water was absorbed by the matrix. Centrifugation was done for 2 min at 13,000 rpm to elute purified DNA. Eluted fragment was then sequenced using PCR primers.

h. Cloning of PCR product into pGEM-T vector using cloning kit

Purified PCR product (amplicon) was ligated into pGEM-T easy cloning vector as per manufacturer's instructions (pGEMT-cloning kit, Promega).

Procedure for DNA ligation

The following components were mixed in the order mentioned below in an eppendorf tube:

Sr. No.	Component	Volume
1.	2X ligation buffer	5 µl
2.	pGEMT vector	1 µl
3.	T4 DNA ligase (3U/µl)	1 µl
4.	Insert DNA	3 µl

The above reaction components were incubated overnight at 4 °C in a refrigerator.

Competent cell preparation

- i. A single colony of *E.coli* (DH5α) was inoculated to 2 ml Luria Bertani (LB) broth (without antibiotic) and incubated at 37 °C for overnight (12-14 h) with shaking at 200 rpm.
- ii. 300 µl (1%) of the overnight culture was inoculated to 100 ml of LB medium and incubated at 37 °C for 3 to 4 h till it reached an O.D. of 0.5 to 0.6 measured at 600 nm.
- iii. The culture was transferred to a sterile pre-chilled polypropylene tube and incubated on ice for 30 min.
- iv. Centrifuged at 5000 rpm for 5 min at 4 °C.
- v. The supernatant was discarded and the cell pellet was resuspended in 5 ml of 0.1 M CaCl₂ (ice cold) and incubated on ice for 5 min. Centrifuged at 5000 rpm and 4 °C for 5 min.
- vi. Again the supernatant was discarded and the cell pellet was resuspended in 3 ml of ice cold 0.1 M CaCl₂. The pellet was gently suspended.
- vii. The competent cells were immediately used for the transformation reaction.

Procedure for transformation of competent cells by plasmid DNA and selection of transformants

- i. DH5α competent cells (*E. coli*) were transformed with the ligation products i.e. 200 µl of competent cells was dispensed in 1.5 ml pre chilled eppendorf tube. To it added 10 µl of ligation mixture and incubated in ice for 30 min. Control was also kept where no ligation mixture was added to competent cells.

- ii. Heat shock treatment was given for 90 sec at 42 °C in a water bath, then incubated (chill) in ice for 10 min.
- iii. To it added 0.8 ml of pre warmed LB broth medium and incubated at 37 °C for 1 h with shaking at 200 rpm.
- iv. The transformed cells were spread on Luria Bertani agar plates with ampicillin in (100 µg/ml).
- v. The plates were incubated at 37 °C overnight (12-14 h).
- vi. Colonies grown on the surface of LB agar plates were randomly picked and screened directly for inserts by performing colony PCR with primers for the complete *gdh* gene. Colonies with putative inserts were transferred to numbered patches plates to provide a colony library. Direct colony PCR was then used to recover cloned inserts. A sterilized toothpick was used to smear part of a putative colony from the patch plate/library into a PCR tube. PCR was carried out as described above using primers. All clones containing inserts of the correct size were stored in LB broth containing 30% (v/v) glycerol at -70 °C.

3.12.3 Sequencing and phylogenetic analysis of *gdh* gene of *B. licheniformis* CKA1

Plasmid DNA was isolated with plasmid isolation kit (Axygen PrepTM Plasmid Mini prep Kit) according to manufacturer's instruction from the culture with positive colony PCR results and was further sent for sequencing (Xcleris lab, Ahmedabad, India). The sequence was aligned with corresponding sequences of *gdh* gene of *Bacillus* sp. from the database using BLASTn program (Altschul *et al.* 1997). Multiple alignment with *gdh* gene sequences of genus *Bacillus* was implemented using CLUSTAL W. A neighbour-joining phylogenetic tree was constructed with other *gdh* gene sequences of related taxa retrieved from GenBank using MEGA 6 software. The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein, (1993) with 100 replicates. The sequence was submitted to NCBI GenBank database for registration and assigning of accession number.

3.11 EVALUATION AND ASSESSMENT OF EXTRACTED IAA BY BIOASSAY, THIN LAYER CHROMATOGRAPHY AND HPLC

Bacterial colony of the best IAA producing bacteria was grown in nutrient broth medium at 30°C for 120 h under shake conditions (90rpm). Supernatant was extracted by centrifugation at 10,000 rpm for 30 minutes at 4°C (Chaiarn and Lumyong, 2011).

3.11.1 Extraction of IAA

Mix 100 ml of supernatant with 250 ml of saturated NaHCO₃ solution in separatory funnel

↓
Extract with 300 ml ethyl acetate (2X)

↓
Acidify aqueous layer to pH 2.5 with 5 N HCl

↓
Add equal volume of ethyl acetate and shake vigorously for 5 minutes

↓
Separate the ethyl acetate fraction; and re-extract the aqueous layer (2X) with 300 ml of ethyl acetate solvent

↓
Pool all ethyl acetate fractions and dried over Na₂SO₃

↓
Evaporate the ethyl acetate extract on a rotary evaporator at 40°C

↓
Dissolve residue in 2 ml of methanol for thin layer chromatography

3.11.2 Separation of IAA

IAA extracts thus obtained are then separated by thin layer chromatography (TLC) (Chaiharn and Lumyong, 2011).

Spot 100 µl of the methanol extract on thin layer plate pre spread with silica gel G. Develop in solvent system i.e. isopropanol; water (30:20. v/v) for 12-14 hours. Plates were sprayed with Salkowsky's reagent. Observe development of pink colour spots. Calculate the R_f value as:

$$R_f = \frac{\text{Distance covered by solute}}{\text{Distance run by solvent}}$$

3.11.3 High performance liquid chromatography (HPLC) based analysis of IAA Produced by strain CKA1

IAA produced by most efficient bacterial strain CKA1 was identified by HPLC (Hariharan *et al.* 2014) HPLC analysis of IAA was carried out on a C18 column (5µm; 25 x 0.46cm) by using HPLC grade acetonitrile- water system containing 0.1% trifluoroacetic acid was programmed over 30 min at a flow rate of 0.5mL/min with UV detector at 220 nm at 40 °C. Mobile phase consisted of methanol and water (80: 20 v/v) run at flow rate was analysed by comparison with the elution profiles of those authentic IAA injected separately.

3.12 PREPARATION OF INOCULUM

3.12.1 Preparation of Liquid Formulation

The population density (2.0 O.D at 540 nm) that resulted in formation of 10^8 CFU/ml of strain CKA1 was used for preparation of liquid formulation.

3.13 SEED TREATMENT

Seeds of apple (*Malus domestica* var. Golden Delicious) were collected from District Kinnaur in Himachal Pradesh. Seeds were air dried and kept in cool place.

3.13.1 Stratification

For seed stratification, unsterilized seeds were stored for 50 days at 4°C in moist autoclaved river bed sand in plastic trays.

3.13.2 Seed surface sterilization

Apple seeds were surface sterilized in a 0.2 per cent mercuric chloride solution for five minutes and rinsed several times with sterilized distilled water. Sterilization was checked by placing surface sterilized and unsterilized seeds on nutrient agar plates. The bacterial growth, if any, around the seeds was recorded after 48 h of incubation.

3.13.3 Seed inoculation

Twenty gram of apple seeds (about 300 seeds) were placed in sterilized flask (250 ml) flasks containing 50 ml of the liquid formulation. The flask was gently shaken and seed were kept in the liquid based formulation of bacterial culture. The seeds remained immersed in the formulation for twelve hours before sowing. The seeds were immediately emptied into the sterilized petriplate, and allowed to air dry. The number of bacterial cells per seed as determined by standard spread plate technique.

3.14 SEEDLING TREATMENT

Root system of apple seedlings was immersed in liquid formulation of different bacterial strain for two hours before plantation. Once planted the seedlings were drenched with the liquid formulation. Twenty five and fifty ml of liquid formulation was used for drenching one apple seedling in plant growth chamber, net house conditions and nursery conditions, respectively.

3.15 SOIL DRENCHING

Apple trees in orchard were treated with the liquid culture by drenching the soil in basin of apple trees. One litre of liquid culture diluted to 5 litres was used to drench basin of one apple tree and 250ml of liquid culture was used to drench apple seedlings.

3.16 NET HOUSE AND POLYHOUSE STUDIES

3.16.1 Type and preparation of potting mixture

Soil obtained from a depth of 0-20 cm from a fallow area in forest block of the Department of Silviculture and Agroforestry, UHF, Solan was sieved through 2 mm sieve and used for pot culture experiment. The sand, soil and farm yard manure (FYM) was mixed in a ratio of 1:1:1 in order to make the potting mixture. The mixture was then filled in the pots and moistened to one third saturation capacity.

3.16.2 Sowing of seeds

Seeds were sown in pots containing potting mixture. Thirty stratified seeds treated or untreated with liquid based inoculant were sown at equidistance and covered with moss grass till emergence of plumule. After 4 days of seedlings emergence thinning was done and 4 plants per pot were maintained. Five replicate pots per treatment with 4 plants in each pot were placed in a randomized block design in net house and poly house.

3.17 FIELD TRIALS

3.17.1 Apple seedlings and trees in orchards

Apple seedlings and trees of different cultivars of the age group of 15-20 years were selected randomly at different location mentioned having apple orchard. Trial lay out was done in a randomized block design with three replications per treatment with three trees in each replication.

3.18 Chemical analysis of soil

Freshly prepared potting mixture for experimentation in net house, soil from apple nursery, and soil from apple orchards at different location mentioned above were analyzed for some chemical characteristics.

3.18.1 Available NPK

Nitrogen, phosphorus, and potassium were determined by the procedures outlined by Alkaline potassium permanganate method of Subbiah and Asija, (1956); extraction with 0.05 M NaHCO₃ (pH 8.5) and development of colour by phosphomolybdate (Olsen *et al.* 1954), and ammonium acetate method (Merwin and Peach, 1951), respectively.

3.19 SEED GERMINATION STUDIES

Germination studies were conducted in plant growth chamber, in net house and in nursery under conditions.

3.19.1 Germination per cent

Germination per cent was calculated as the number of seeds sown and the number of seeds germinated expressed in percentage.

3.20 ANALYSIS OF APPLE SEEDLING

Apple seedlings obtained from plant growth chamber, net house and in nurseries under field conditions were analyzed for different traits such as: shoot characteristics, root characteristics, and per cent nutrient (NPK).

3.20.1 Shoot characteristics

3.20.1.1 Shoot length

Shoot length was recorded in centimeters from the ground level to the apical bud of stem.

3.20.1.2 Shoot diameter

Shoot diameter of seedlings was measured in millimeters using electronic vernier caliper at three equidistant positions along the length of the seedling from collar to apical bud.

3.20.2 Leaf surface area

Leaf surface area was measured with the help of surface area meter.

3.20.3 NPK content in seedling

Oven dried plant samples were ground and sieved for estimation of total nitrogen, phosphorus and potassium content by following standard procedure (Piper, 1966; Jackson, 1973)

3.20.3.1 Digestion of samples

The digestion of 0.20 g samples for estimating nitrogen was carried out in concentrated H₂SO₄ by adding digestion mixture of following chemicals:

Potassium sulphate = 400 parts

Mercuric oxide = 3 parts

Copper sulphate = 20 parts

Selenium powder = 1 part

For the estimation of other elements i.e. P and K, the digestion was carried out in diacid mixture prepared by mixing nitric acid and perchloric acid in the ratio of 4:1, taking all relevant precautions as suggested by Piper, (1966).

3.20.3.2 Estimation of nutrient elements

The nitrogen was estimated in Kjeltac Auto 1030 Analyzer (Tecator AB, Sweden). Phosphorus was determined by Vanado molybdo-phosphoric yellow colour method (Jackson, 1973) by using spectrophotometer and potassium was determined by flame-photometer (Jackson, 1973).

3.21 EFFECT OF PGPR INOCULATION ON FRUIT SET, FRUIT QUALITY, CHEMICAL CHARACTERISTICS AND YIELD OF APPLE IN THE FIELDS

The experiment was carried out during 2013 to 2015 in orchards at experimental field of Regional Horticulture Research Station Mashobra, Shimla, farmer field at Matiana (Nanni, Sablog), Kotgarh (Shathla) and (Kyari) Kotkhair in order to evaluate the yield and growth of young apple trees (*Malus domestica* Borkh, cv. Starking delicious). In the first year of field experiment (2013), 36 apple trees were selected and divided in 4 application groups including 3 trees with three replication in completely randomized design. Application strategies of *Bacillus licheniformis* CKA1 liquid formulation include foliar spray ($\times 10^{10}$ cfu g⁻¹ of CKA1 formulation) at full bloom, root inoculation alone ($\times 10^{10}$ cfu g⁻¹ of CKA1 formulation) in month of February and July (1 litre mixed with 4 litre of water) and foliar application in combination with root inoculation. The field experiment was conducted in the same orchard using the same apple trees in 2014 and 2015. Growth promoting effects of bacterial treatments were evaluated by determining cumulative yield (kg/tree), average fruit weight (g) and total soluble solids (TSS, %) and shoot length (cm) and diameter (mm) on 100

annual shoots in 2012–2013. In addition, the effect of the bacterial treatments on the plant nutrient element (PNE) contents of leaves was evaluated in 2013–2015.

3.22 STUDY BACTERIAL ANTAGONISM OF *Dematophora necatrix* AT ROOT LEVEL

3.22.1 Apple Seedlings

The experiment was conducted to evaluate at root level, the role of strain CKA1, CKAM, G4(3), RG1(3) and conjoint application of all four strains in direct antagonism (prevention) of white root rot apple seedlings. The potting mixture was inoculated with wheat grain based culture of *Dematophora necatrix*.

3.22.1.1 Preparation of *Dematophora necatrix* inoculum

The mass culture of *Dematophora necatrix* for the inoculation of seedlings was prepared on wheat grains. Wheat grains were first soaked in water for 12 h and thereafter boiled for 30 minutes. Boiled wheat grains were supplemented with sucrose (50g) and sand (200g) per 2 kg of grains after excess water had been drained off and mixed thoroughly. The mixture was autoclaved at 15 lbs per square inch pressure for 30 minutes in heat resistant polypropylene bags plugged with non-absorbent cotton regularly for three consecutive days. Sterilized medium in polypropylene bags was inoculated with 3-4 fungal bits of week old *Dematophora necatrix* culture under aseptic conditions and incubated at 25±1°C.

3.22.2 Preparation of *Phytophthora cactorum* inoculum

For mass culture of *P.cactorum* corn meal sand culture (135 g of soil, 15 g of corn meal and 20 ml of potato juice) was used. The corn meal sand culture was sterilized in an autoclave at 121°C on three separate occasions lasting 60 min each (103 kPa). Mycelium containing discs of fungal pathogen grown on PDA for 7 days were mixed with wheat sucrose sand culture and corn meal sand culture in 500 ml sterile Erlenmeyer flasks and allowed to develop for 4 weeks in an incubator at 25°C. This culture was used for pathogen challenge of one year old apple seedlings

3.22.3. Pathogenicity test

Pathogenicity of fungus was tested on apple seedlings grown in pots. The soil around the plant trunk was removed gently to expose the roots and the inoculum of *Dematophora necatrix* and *P.cactorum* were mixed separately in the soil surrounding the roots and the roots

were again covered with the soil. Separate controls were maintained, which involved all similar operations except the addition of fungal culture. Treated pots were regularly watered and observed for symptom appearance.

3.22.3.1 Computation of disease parameters

3.22.3.1.1 Disease incidence

Disease incidence was computed using formula (Ramamoorthy *et al.* 2002):

$$\text{Per cent disease incidence} = \frac{\text{Number of diseased plants}}{\text{Total number of plants observed}} \times 100$$

3.22.3.1.2 Disease severity

Disease severity was calculated in terms of foliar disease severity scale and root disease severity scale (Bora *et al.* 2004):

$$\text{Percent disease severity} = \frac{\Sigma(\text{Rating} \times \text{Number of plants rated})}{\text{Total number of plants} \times \text{highest rating}} \times 100$$

3.22.3.1.3 Foliar disease severity scale:

0	:	no symptoms;
1	:	≤ 25% of leaves with symptoms;
2	:	26-50% of leaves with symptoms;
3	:	51-75% of leaves with symptoms;
4	:	76-100% of leaves with symptoms

3.22.3.1.4 Root disease severity scale:

0	:	no symptoms;
1	:	≤ 25% root system showing symptoms;
2	:	26-50% root system showing symptoms;
3	:	51-75% root system showing symptoms;
4	:	76-100% root system showing symptoms

Efficacy of treatments was calculated using Abbott's formula, and statistical analyses were done using Duncan's multiple range test at 5% significance level.

3.23 REISOLATION AND ENUMERATION OF ENDORHIZOBACTERIA AND RHIZOSPHERE SOIL BACTERIA

The bacterial population was determined in apple seedlings grown in plant growth chamber, net house and nurseries under field conditions as per protocol developed. (Fig .4)

3.23.1 Reisolation of endorhizobacteria

The endophytic bacterial count was determined by taking whole root system. The roots were washed with tap water to free the rhizosphere soil. Washed roots were surface sterilized by 0.2 per cent mercuric chloride (HgCl_2) solution for 3 minutes and rinsed several times with sterilized distilled water. Surface sterilization of roots was cross checked by incubating the surface sterilized roots in sterilized nutrient broth. The bacterial growth, if any, around the roots were recorded after 24 h of incubation. One gram of root sample was placed in 9 ml of sterilized distilled water and was ground to produce slurry using mortar and pestle under aseptic conditions. The root suspension was diluted in 10 fold series and bacterial count was determined by standard spread plate technique. Populations were expressed as colony forming units (cfu) per gram of wet root weight. Isolates were maintained on specific medium for further studies.

3.23.2 Reisolation of rhizosphere soil bacteria

Rhizosphere bacterial population was determined in 1.0 g of rhizosphere soil. Apple seedlings collected from net house and field were shaken vigorously to remove the soil tightly adhered to the roots. One gram of soil was placed in 9 ml sterilized distilled water under aseptic conditions. The soil suspension was diluted in 10 fold series and the bacterial count was determined by using standard spread plate technique. Populations were expressed as colony forming units (cfu) per gram of dried soil.

3.24 COMPARATIVE STUDIES OF REISOLATED BACTERIAL STRAINS WITH REFERENCE ISOLATE (STRAIN CKA1)

A comparative study of reisolated bacterial strains along with reference strain strain CKA1 was carried out based on morphological similarity; ARDRA based fingerprinting using standard methods as described previously.

3.25 STATISTICAL ANALYSIS

All the experiments were conducted under statistical frame work in triplicates along with equal number of appropriate controls. Appropriate statistical/mathematical tools were

utilized as per the objectives and cross- section variation of the data. Data were analyzed using SPSS 16.0 (Analytical Software).

3.25.1 Variability analysis

In order to compare the consistency, the coefficient of variation has been worked out for each of the observed characteristics under each site as given below:

$$cv = \frac{\sigma}{\bar{X}} \times 100$$

Where,

$\bar{X}$ = Mean value

σ = Standard deviation

3.25.2 Analysis of variance technique (ANOVA)

Completely Randomized Block Design (CRD) and CRD factorial were applied to know the average performance of various characters on P- solubilization, antifungal activity, per cent siderophore unit and IAA production using SPSS16.0. To have pair wise comparison of treatment means, critical difference at 5 per cent level of significance (LSD) has been used.

3.25.3 Principal component analysis

To know the influence of rhizosphere soil on distribution of PGPR at different sites (i.e. healthy and root rot infected diseased apple orchard; replant and new plantation sites), principal component analysis (PCA) was carried out. The variables were normalized by division of their standard deviation and the Eigen values and Eigen vectors of the correlation matrix and the percentages of variance for each component were calculated.

3.26 Diversity indices

The establishment of inoculated strain CKA1 in soil at different locations were evaluated using diversity indices. Simpson's index of Dominance (D) is in general the measures of diversity that accounts for both richness and proportion of individual (Atlas and Bartha, 1998).

The dominance indexes was calculated by using the following formulas (Ventorino *et al.* 2007).

- (i) Simpson's index of Dominance (D) $D = \sum P_i^2$

Chapter-4

RESULTS AND DISCUSSION

This chapter deals with the experimental results and discussion and most likely causes and effects, underlying patterns, resulting in the predictions, evidence or even a line of reasoning, which support interpretation, agree or perhaps contradict previous work, in the light of the available literature. The salient findings obtained during the course of investigation are presented under following main headings:

- 4.1 Culturable rhizobacterial population associated with healthy and root rot infected diseased apple orchards of Himachal Pradesh**
- 4.2 Selection and screening of representative rhizobacteria from healthy and root rot infected diseased apple orchards for multifarious plant growth promoting traits**
- 4.3 Principal component analysis of rhizobacteria from healthy and root rot infected diseased apple orchards**
- 4.4 Genetic diversity of representative plant growth promoting rhizobacteria from healthy and root rot infected diseased apple orchards using amplified ribosomal DNA restriction analysis**
- 4.5 Culturable rhizobacterial population associated with apple trees grown at replant and new plantation sites of Himachal Pradesh**
- 4.6 Selection and screening of representative rhizobacteria from replant and new plantation sites of apple orchards for multifarious plant growth promoting traits**
- 4.7 Principal component analysis of rhizobacteria from replant and new plantation sites of apple orchards**
- 4.8 Genetic diversity of representative plant growth promoting rhizobacteria from replant and new plantation sites of apple orchards using amplified ribosomal DNA restriction analysis**

- 4.9 Biochemical and 16S rDNA based phylogenetic characterization of plant growth promoting rhizobacteria isolated from apple
- 4.10 Effect of *Bacillus licheniformis* CKA1, *B. methylotropicus* CKAM, *Serratia plymuthica* G4 (3) and *B. methylotropicus* RG1 (3) inoculation on growth of apple seedlings/plants under net house and nursery field conditions
- 4.11 Effect of *Bacillus licheniformis* CKA1, *B. methylotropicus* CKAM, *Serratia plymuthica* G4 (3) and *B. methylotropicus* RG1 (3) inoculation on health of apple seedlings (white root rot and collar rot disease management in apple)
- 4.12 Optimization of cultural conditions for IAA production by *Bacillus licheniformis* CKA1
- 4.13 Cloning and sequencing of glucose dehydrogenase (GDH)- encoding gene (*gdh*) in *Bacillus licheniformis* CKA1
- 4.14 Effect of *Bacillus licheniformis* CKA1 inoculation on vegetative growth of apple cultivars in orchards at different location
- 4.15 Plant growth response (vegetative and nutrient parameters) of apple plants to *Bacillus licheniformis* CKA1 inoculation in orchards at different location after three year of plantation
- 4.16 Effect of *Bacillus licheniformis* strain CKA1 inoculation on soil nutrient content in apple orchards at different location
- 4.17 Plant growth response (yield, pomological traits, fruit content and plant nutrient content) of apple trees to *Bacillus licheniformis* CKA1 inoculation in apple orchards
- 4.18 Population dynamics of inoculated *Bacillus licheniformis* CKA1 at different apple orchards

4.1 CULTURABLE RHIZOBACTERIAL POPULATION ASSOCIATED WITH HEALTHY AND ROOT ROT INFECTED DISEASED APPLE ORCHARDS OF HIMACHAL PRADESH

Plant growth promoting rhizobacteria are the soil bacteria that colonize plant roots and facilitate the growth and development of plants directly by either assisting in resource acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels, or indirectly by decreasing the inhibitory effects of various pathogens on plant growth and development in the forms of biocontrol agents. They are able to multiply and occupy all the ecological niches found on the roots at all stages of plant growth and shares positives relationship with the plants (Aranda *et al.* 2011). The bacteria lodging around/in the plant roots (rhizobacteria) are more versatile in transforming and mobilizing the nutrients compared to those from bulk soils (Hayat *et al.* 2010). Therefore, the rhizobacteria are the dominant deriving forces in recycling the soil nutrients and consequently, are crucial for soil fertility (Glick, 2012). PGPR, therefore, have potential to reduce the application of agro-chemicals and maintain biotic diversity in plant associated bio-community.

PGPR ecology/diversity seeks to know which bacteria exist in the rhizosphere and to understand how they interact with each other and with the plant. Such studies are limited by the fact that only 1-10 per cent of rhizosphere bacteria have been cultured (Hugenholtz and Pace, 1996). Investigating the diversity of rhizosphere microorganisms is important for understanding the impacts of new agricultural technologies upon soil ecology, nutrient transformations and plant succession and to manipulate these associations to increase crop production (Germida *et al.* 1998). Numerous studies have compared microbial communities among soils from different ecosystems (Axelrood *et al.* 2002), different land use histories (Benizri and Amiaud, 2005) and different long-term cropping regimes (Larkin, 2003). Few studies (Rumberger *et al.* 2007; Hernesmaa *et al.* 2005) have focused on structural and functional diversity of microbial populations associated with woody plants, while simultaneously addressing the influence of physical geographical parameters.

Among the various temperate fruits grown in hills and mountains of India, apple (*Malus domestica* Borkh.) is considered as an important temperate crop on the basis of its high consumption and nutritional value. Attempts to enhance apple productivity, in the Trans-Himalayan region has resulted in rapid expansion in area under apple cultivation. However, the strict physio-geographic conditions pose several hurdles. One such hindrance

is the variation in plant health, nutritional status and yield of apple in healthy and diseased apple trees. The present investigation focuses on evaluating culturable rhizospheric bacterial populations associated with apple trees grown at healthy and root rot infected diseased apple orchards of Himachal Pradesh. The findings revealed a significant difference in rhizobacterial diversity among healthy and diseased apple. The results have been presented and discussed in details in the following sections.

4.1.1 Isolation and enumeration of culturable rhizobacterial population associated with apple trees in healthy and root rot infected diseased apple orchards

Rhizospheric soil sample were collected in the month of February–March from different sampling sites of apple at different locations. Samples were drawn from each tree's basin from four different points and pooled to create on composite sample for isolation purposes. The population of culturable bacteria capable of growth on nutrient medium were counted and reported as colony forming units (cfu) per gram of whole root system or cfu per gram of rhizosphere soil on dry weight basis. The number of soil bacteria was calculated on the basis of serial tenfold dilution in triplicate (Johnson and Curl, 1972).

4.1.1.1 Culturable rhizospheric population associated with healthy apple orchards at different locations

The graphical presentation of total culturable bacteria (cfu/g), total P-solubilizers (%), total siderophore producers (%) and total rhizobacterial population growing on N-free medium (%) obtained from rhizospheric soil of healthy apple orchards at different sampling sites is presented in Fig. 6(A), 7(A), 8(A) and 9(A), respectively. The results revealed that culturable rhizobacterial population (colony forming unit) was observed at all the sampling sites which ranged from 47.20×10^5 cfu g⁻¹ to 127.50×10^5 cfu g⁻¹ soil on nutrient agar medium. The results are in concomitance with study carried out by Mehta *et al.* (2013), where they reported the presence of diverse population of phosphate solubilizing plant growth promoting rhizobacteria associated with apple trees in the Trans-Himalayan region of Himachal Pradesh. Guo *et al.* (2014) also carried out community analysis of plant growth promoting rhizobacteria for apple trees and reported nitrogen-fixing bacteria, phosphate-solubilizing bacteria, silicate bacteria and antagonistic bacteria.

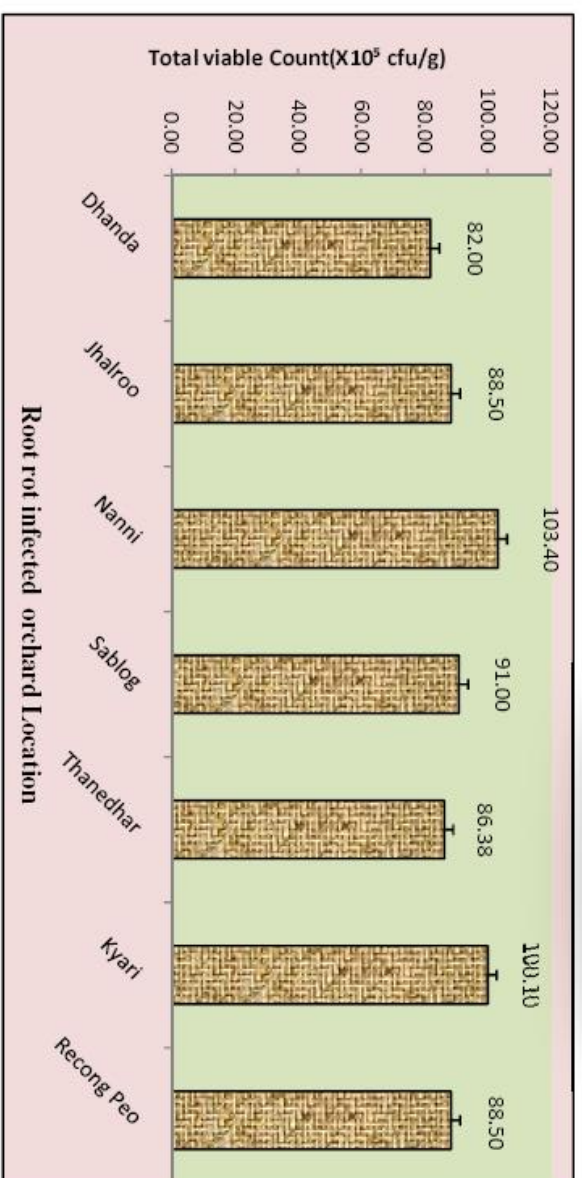
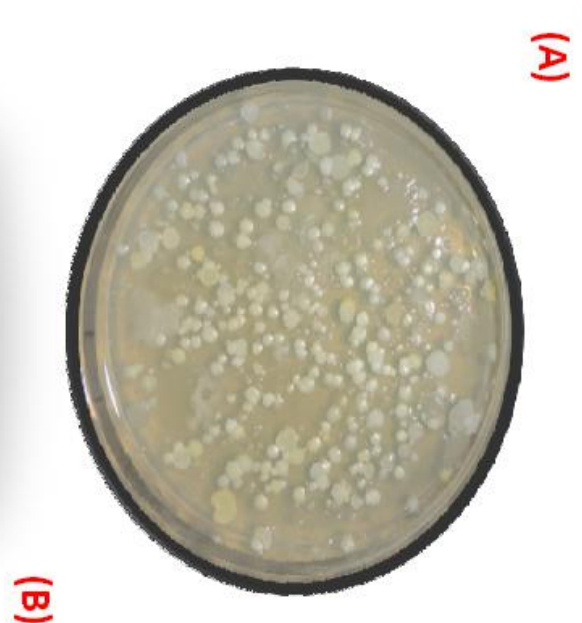
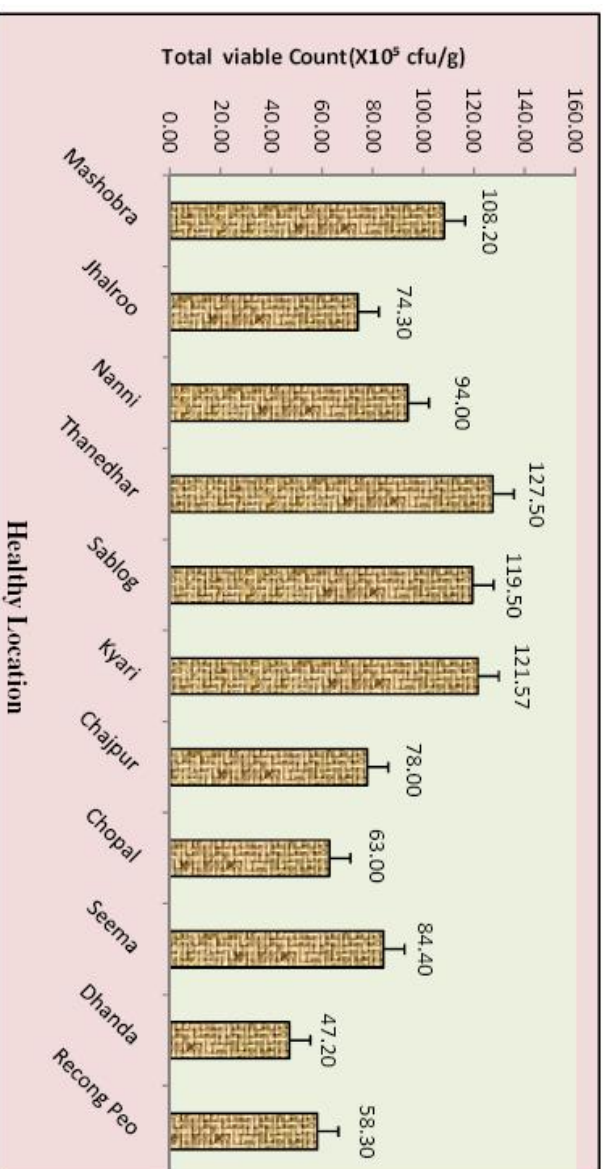
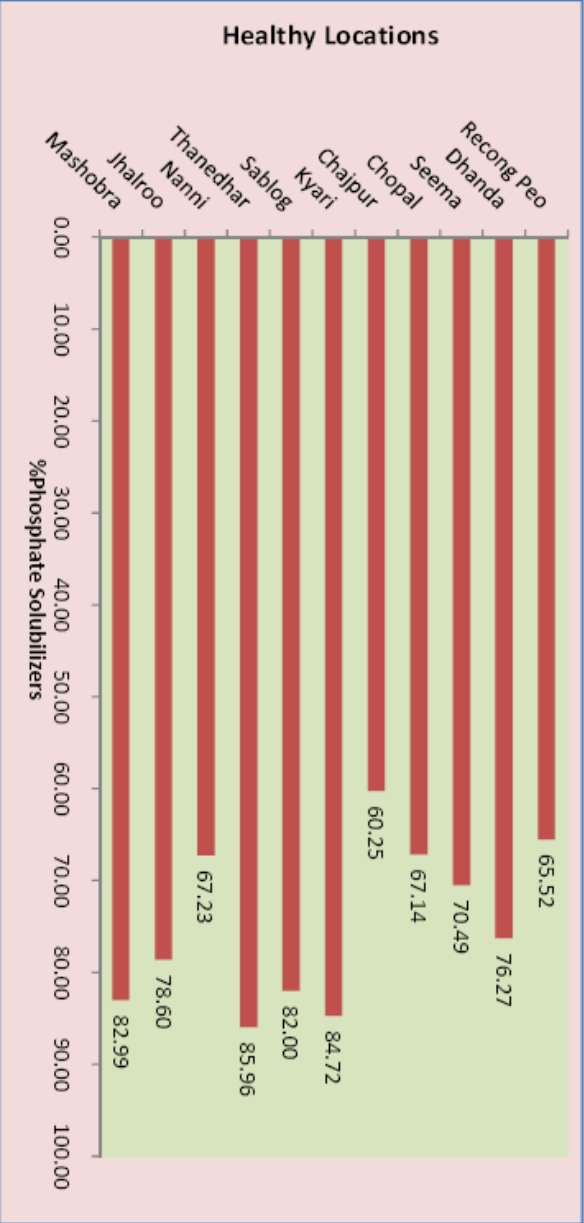
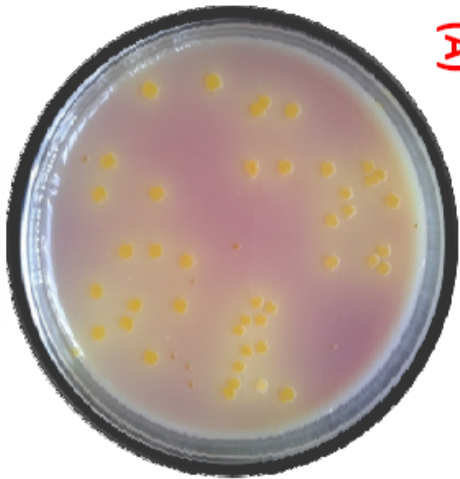


Figure 6. A comparison of total viable count of culturable bacterial population isolated from (A) healthy orchard (B) root rot infected diseased apple orchard at different locations

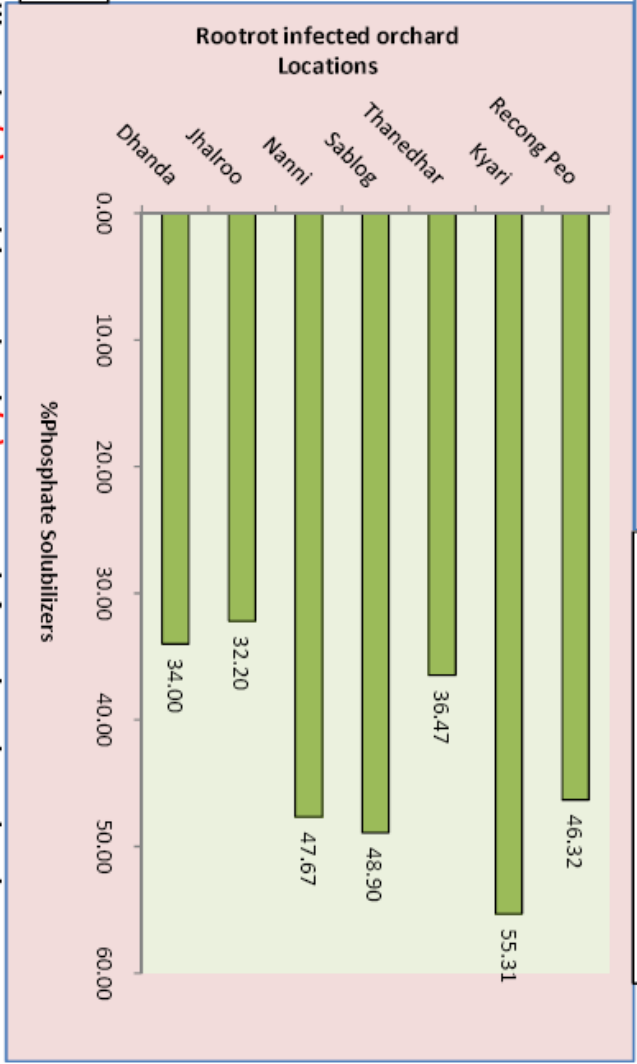


Average P- solubilizers at Healthy location =76.41 per cent

(A)

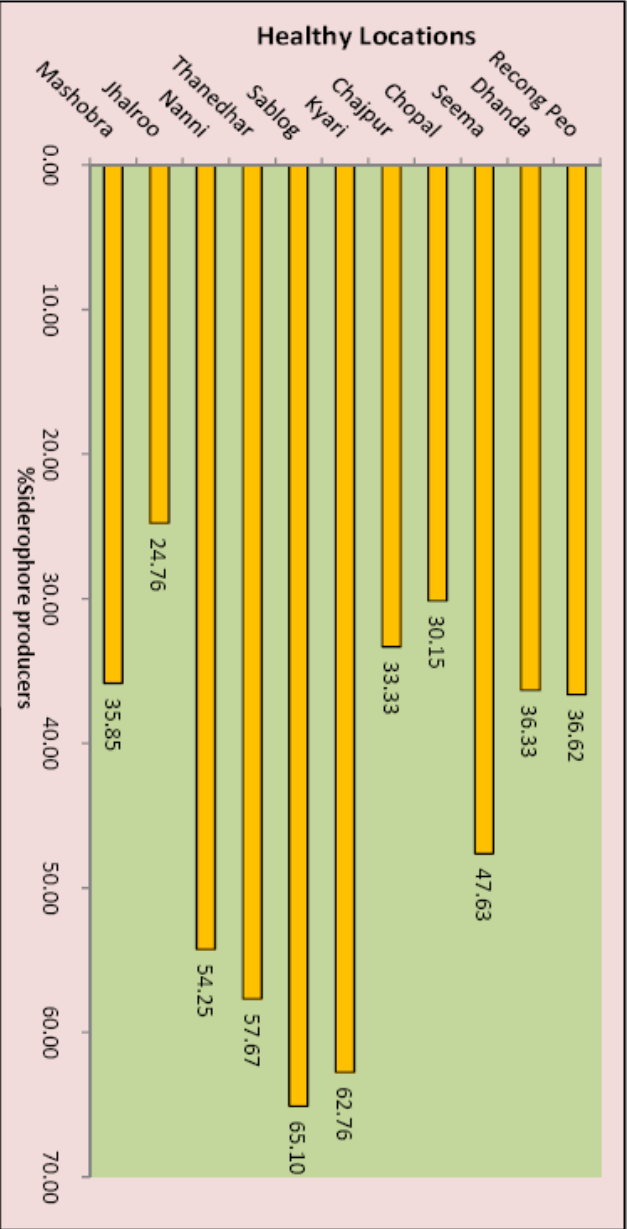


(B)



Average P- solubilizers at root rot infected location =43.73 per cent

Figure 7. A comparison of diversity among phosphate solubilizers in (A) healthy orchard (B) root rot infected diseased apple orchard at different locations



Average Siderophore producers at Healthy location =47.08 per cent

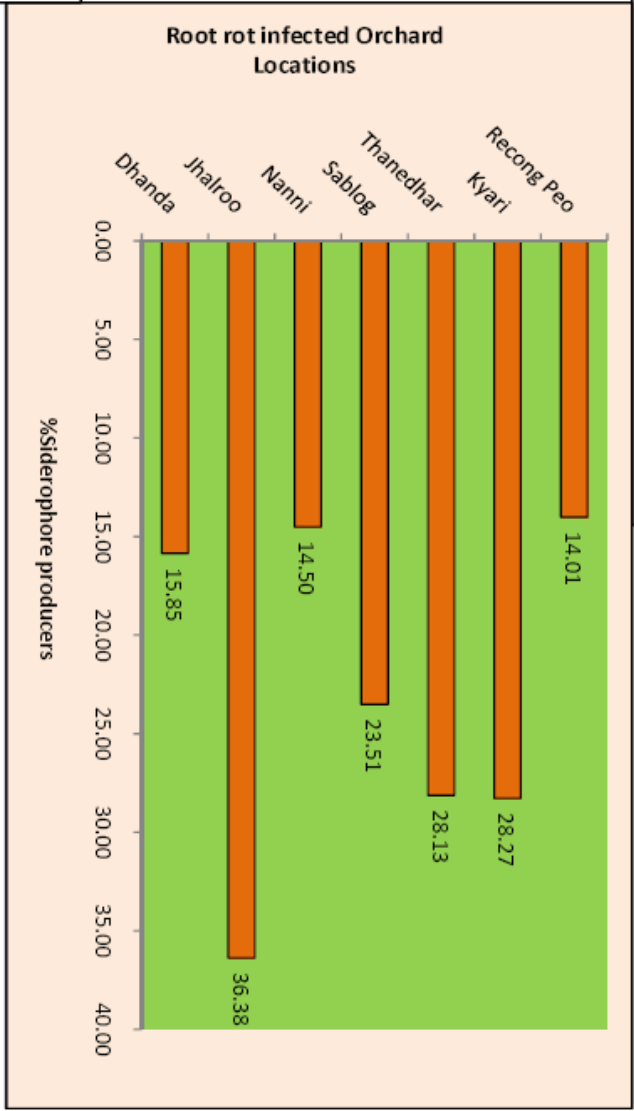
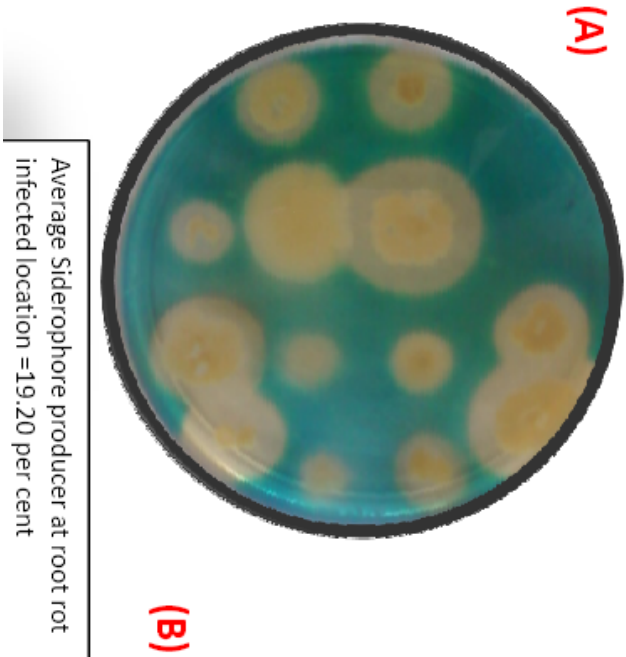


Figure 8. A comparison of diversity among siderophore producers in (A) healthy orchard (B) root rot infected diseased apple orchard at different locations

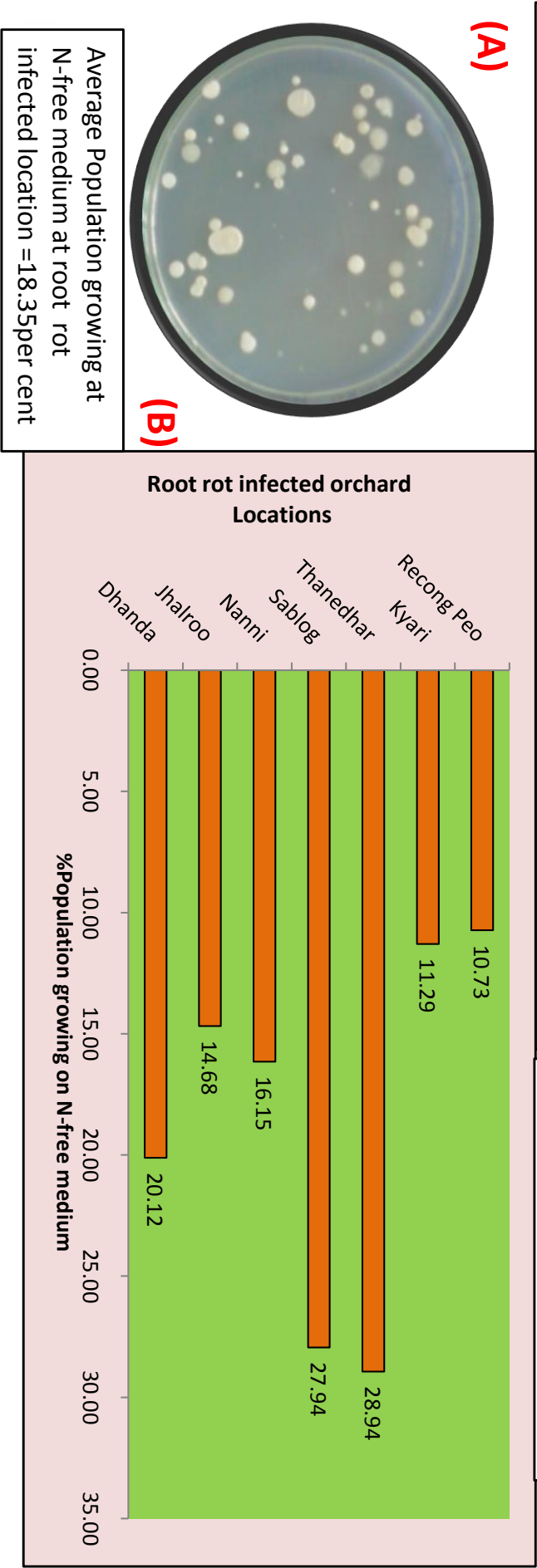
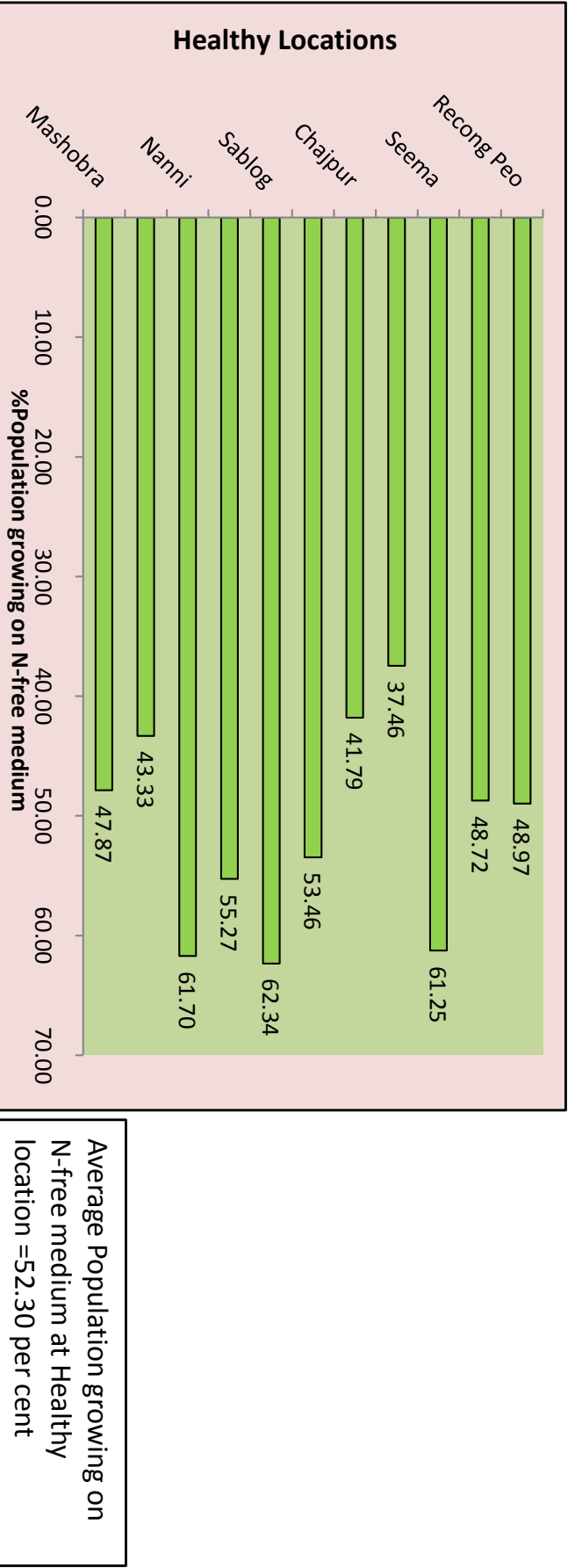


Figure 9. A comparison of diversity among per cent population growing on N-free medium in (A) healthy orchard (B) root rot infected diseased apple orchard at different locations

The results in the present investigation revealed that the total culturable rhizobacterial population, on the nutrient agar medium at RHRS, Mashobra was 108.20×10^5 cfu g⁻¹. Of the total population obtained, 89.90 per cent were P-solubilizing, 35.85 per cent were siderophore producers and 47.87 per cent were able to grow on nitrogen free medium.

Total number of culturable bacteria isolated from apple rhizospheric soil of Jhalroo resulted in a population of 74.30×10^5 cfu g⁻¹, out of which 78.60 per cent were P-solubilizers, 24.76 per cent were siderophore producers and 43.33 per cent of the population was able to grow on nitrogen free medium.

The total viable bacterial population recovered from rhizosphere soil of Nanni resulted in 94.00×10^5 cfu g⁻¹ of culturable bacteria. The soil samples showed the presence of 67.23 per cent P-solubilizers, 54.25 per cent siderophore producers and 61.70 per cent culturable rhizobacterial communities able to grow on N free medium.

Location Thanedhar accounted for the total of 127.50×10^5 cfu g⁻¹ of culturable bacteria, out of which 85.96 per cent were P-solubilizers, 57.67 per cent were siderophore producers and 55.27 per cent of the population was able to grow on nitrogen free medium.

The total number of culturable bacteria inhabiting the rhizospheric soil of apple trees at location Sablog was 119.50×10^5 cfu g⁻¹. The presence of 82.00 per cent P-solubilizers, 65.10 per cent siderophore producers and 62.34 percent bacteria able to grow on medium deficient with nitrogen was observed.

Total culturable bacterial population present in the apple rhizosphere soil sample of Kotkhai contain 121.57×10^5 cfu g⁻¹ on the nutrient agar medium. Of the total population obtained, 84.72 per cent were P-solubilizing, 62.76 per cent were siderophore producers and 54.46 per cent were able to grow on nitrogen free medium.

Total number of culturable bacteria recovered from rhizospheric soil of Chajpur resulted in 78.00×10^5 cfu g⁻¹, out of which 60.25 per cent were P-solubilizers, 33.33 per cent was siderophore producers and 41.79 per cent of the populations were able to grow on nitrogen free medium.

The total viable bacterial population recovered from rhizosphere soil of Chopal resulted in 63.00×10^5 cfu g⁻¹. The soil samples showed the presence of 67.14 per cent P-solubilizers, 30.15 per cent siderophore producers and 37.46 per cent bacteria were able to grow on N free medium.

Total culturable bacterial population present in the apple rhizosphere soil sample of Seema contain 84.40×10^5 cfu g⁻¹ on the nutrient agar medium. Of the total population obtained, 70.49 per cent were P-solubilizing, 47.63 per cent were siderophore producers and 61.25 per cent were able to grow on nitrogen free medium.

Total number of culturable bacteria recovered from rhizospheric soil of Dhanda resulted in 47.20×10^5 cfu g⁻¹, out of which 76.27 per cent were P-solubilizers, 36.33 per cent was siderophore producers and 48.72 per cent of the population was able to grow on nitrogen free medium.

Rhizosphere soil sample of location Reckong Peo contained 58.30×10^5 cfu g⁻¹ of culturable bacteria on the nutrient agar medium. Of the total population comprised of 65.52 per cent P-solubilizing, 36.62 per cent siderophore producers and 48.97 per cent able to grow on nitrogen free medium.

The extreme habitats harbor microbial diversity, which may be fundamental for the maintenance and conservation of global genetic resources. It has been previously well documented that one of the most important indexes of soil quality is considered to be the diversity of microbial community (Singh *et al.* 2013). Standard culture technique to characterize microbial ecology involves isolation and characterization of microorganisms using commercial growth medium such as LB medium, nutrient agar and TSA (Krey *et al.* 2013). Plate counts provide quantitative yields and specific information and diversity (Torsvik *et al.* 1990; Ruiz *et al.* 2005). The finding in the present study, indicate higher percentage of P-solubilizers followed by siderophore producers and putative N- fixers in the rhizosphere region, might be due to the root exudates that exert a selective pressure on the proliferation of specific microbes. It has been reported that the soil attached to root system is a hotspot for microbial abundance and their intense activity is due to the presence of root exudates and rhizodeposits around the plants (Smalla *et al.* 2006).

4.1.1.2 Culturable rhizospheric population associated with root rot infected diseased apple at different locations

For the determination of total viable bacterial population present in rhizospheric soil samples of root rot infected diseased apple orchards, isolations were carried out on nutrient agar plate and the viable count ranged from 82 to 103×10^5 cfu g⁻¹ at different sampling location. A detailed account of comparison of total rhizobacterial population at different locations is presented in Fig. 6(B)).

The total viable bacterial population from soil samples of location Dhanta was detected using serial dilution method on nutrient agar medium that accounted for 82×10^5 cfu g⁻¹ of viable count. Culturable bacterial population harboring P-solubilizing bacteria resulted in 34 per cent on Pikovskaya's medium whereas siderophore producers on CAS medium resulted in 15.85 per cent of the total rhizospheric population. Growth in nitrogen free medium accounted for 20.12 per cent of the total population.

The total viable bacterial population present in rhizospheric soil samples of location Jhalroo obtained by isolation method on nutrient agar plate resulted in 88.50×10^5 cfu/g. The culturable bacterial population harbouring P-solubilizing bacteria resulted in 36.38 per cent on Pikovskaya's medium and 24.75 per cent siderophore producers on nutrient agar amended with CAS medium. Growth in nitrogen free medium resulted in 14.68 per cent of the total population.

Total number of culturable bacteria recovered from rhizospheric soil of Nanni, resulted in 103.40 cfu g⁻¹ $\times 10^5$ of culturable bacteria, out of which 78.60 per cent were P-solubilizers, 14.50 per cent were siderophore producers and 16.15 per cent of the population was able to grow on nitrogen free medium.

The total viable bacterial population recovered from rhizosphere soil of Sablog resulted in 91.00×10^5 cfu g⁻¹ of culturable bacteria. The soil samples showed the presence of 48.90 per cent P-solubilizers, 23.51 per cent siderophore producers and 27.94 per cent bacteria able to grow on N free medium.

Total culturable bacterial population present in the apple rhizosphere soil sample of Thanedhar contained 86.38×10^5 cfu g⁻¹ of soil on the nutrient agar medium. Of the total population obtained, 70.49 per cent were P-solubilizing, 47.63 per cent were siderophore producers and 61.25 per cent were able to grow on nitrogen free medium.

Total number of culturable bacteria recovered from rhizospheric soil of Kyari, resulted in 100.10×10^5 cfu g⁻¹, out of which 55.31 per cent were P-solubilizers, 28.27 per cent were siderophore producers and 11.29 per cent of the population was able to grow on nitrogen free medium.

Total culturable bacterial population present in the apple rhizosphere soil sample of Recong Peo contain 88.50×10^5 cfu g⁻¹ of soil on the nutrient agar medium. Of the total population obtained, 46.32 per cent were P-solubilizing, 14.01 per cent were siderophore producers and 10.73 per cent were able to grow on nitrogen free medium.

The results in Fig. 6, 7, 8 and 9 revealed that the culturable rhizobacterial population of phosphate solubilizers, siderophore producers and nitrogen fixing bacteria varied at all the locations and relatively their quantity (per cent of total viable count) was higher in rhizospheric soil of healthy trees when compared to the diseased rhizosphere at the same location. Apple rhizosphere soil bacterial population has been reported to vary between 9.8×10^5 cfu/g to 1.7×10^6 cfu/g soil (Rumberger *et al.* 2007). In another study, the viable rhizosphere soil bacterial count ranges between 49.4 to 96.8×10^6 cfu/g soil of apple trees (Mehta *et al.* 2013). Such variation in the population of PGPRs between the locations with higher number in healthy apple trees/ soil samples, might also be accounted by the root exudates from healthy and diseased trees and environmental conditions. As the rhizosphere is a site of intense microbe-plant interaction in which the effect of plants on PGPR in rhizosphere and vice versa are interdependent (Mukergi *et al.* 2006), it seems possible that more number of phosphate solubilizers, siderophore producers and nitrogen fixing PGPRs might contribute for the healthiness of apple trees. However further detailed studies in the role of enhanced population of PGPRs on survival and prevalence of pathogens are required, to further confirm the causes of the healthiness of apple trees.

The presence of lesser population of P- solubilizers, siderophore producers and putative nitrogen fixers among all the root rot infected diseased locations investigated in the present study might be due to the interactions of pathogenic microbes with host, especially in

the root ecosphere. In root rot infected diseased apple orchards the root system is small with discolored feeder roots and few functional root hairs (Wilson *et al.* 2004). As a result of which the concentration of root exudates in the rhizosphere of root rot infected diseased trees is in much less concentration which possibly results in lesser population of nutrient mobilizing rhizobacteria as has also been observed in the present findings. Root exudates contain many kinds of compounds including sugars, organic acids, amino acids, phenolic compounds, and some secondary metabolites (Bais *et al.* 2006), which are used as substrates for bacteria (Campbell and Greaves, 1990).

4.1.2 A comparative overview of culturable rhizobacterial population associated with apple trees in healthy and root rot infected diseased apple orchards

A comparison of functional traits among rhizobacterial populations from healthy and root rot infected diseased apple orchards revealed significant variation, as is evident from the data appended in figures 7, 8 and 9. Phosphate solublizers at both healthy and root rot infected diseased apple orchards accounted for maximum contribution towards the total rhizobacterial population, which was closely followed by siderophore producers and putative N-fixers. However, detailed comparison revealed that among the P-solublizing rhizobacteria, in healthy apple orchards an overall average of 76.41 per cent were P-solubilizers, whereas only 43.73 per cent were observed at root rot infected diseased apple orchards. A similar trend was observed in case of siderophore producers and putative N-fixers. Siderophore producers in healthy apple orchards accounted for average of 47.08 per cent, whereas in root rot infected diseased apple orchards their contribution was 19.20 per cent. An overall average contribution of rhizobacterial population growing on nitrogen free medium in healthy orchards was 52.37 per cent, whereas 18.35 per cent were observed in root rot infected diseased apple orchards. Microorganisms can contribute to the nutrition of plants through a number of mechanisms, including direct effects on the availability of nutrients or plant growth promoting substances, which are synthesized by the bacterium, or by facilitating the absorption of certain nutrients from the environment. Soil microorganisms may affect the growth of plants using any one or more of these mechanisms (Ahmad *et al.* 2006; Farina *et al.* 2012). Results in the present study are in concomitance with research carried out by several researchers who reported siderophore-producing bacteria in rhizosphere of apple (Passos *et al.* 2014); tobacco rhizosphere (Tian *et al.* 2009); sunflower (*Helianthus annus* L.) rhizosphere (Ambrosini *et al.* 2012).

The findings in the present study highlight the fact that the presence of higher population of nutrient mobilizers can be positively correlated towards the overall health of an apple orchard. The plausible reason, for the lower population densities of functional rhizobacteria in root rot infected diseased apple orchards can be because of fact that there is major interaction occurring between plant pathogens and host in the rhizosphere niche. It is well known that pathogens are studied most effectively within an ecological context of interactions among microbes, their hosts, and the environmental conditions in which they live (Harvell *et al.* 1999). A basic knowledge of the composition and distribution of the microbial communities associated with healthy as well as diseased organisms is essential for the diagnosis and eventual treatment and prevention of plant diseases by using disease suppressive microbial communities. Regulation and exploitation of microbial diversity is widely recognized as a component of sustainable and integrated pest management approaches for plant disease control (Shanmugam *et al.* 2011).

4.2 SELECTION AND SCREENING OF REPRESENTATIVE RHIZOBACTERIA FROM HEALTHY AND ROOT ROT INFECTED DISEASED APPLE ORCHARDS FOR MULTIFARIOUS PLANT GROWTH PROMOTING TRAITS

A total of sixty six and thirty one bacterial isolates obtained from healthy and root rot infected diseased apple orchards respectively, with atleast one of the plant growth promoting traits i.e. phosphate solubilization, siderophore production and growth on nitrogen free medium were selected and screened for other multifarious plant growth promoting activities (Table 2 ; Plate1). All the selected bacterial isolates exhibited variation in production of different plant growth promoting traits viz. IAA production, HCN production, P-solubilization, and antifungal activity against *Phytophthora sp.*, *Pythium aphanidermatum*, *Dematophora necatrix*, *Fusarium oxysporum* and *Sclerotium rolfsii*. The methodology used for screening of PGPR in the present investigation is in concomitance to work carried out by earlier researchers (Mehta *et al.* 2010; Ramos- Solano *et al.* 2010; Sofi, 2011; Mehta *et al.* 2013; Chauhan *et al.* 2014; Sharma, 2015; Minakshi, 2015) who also reported multifarious plant growth promoting activities from different PGPR associated with varied crops and trees.

4.2.1 Plant growth promoting traits of culturable bacterial isolate from healthy and root rot infected diseased apple orchards

A total of sixty six and thirty one rhizospheric isolates obtained from the healthy and root rot infected diseased apple orchards respectively at different locations of Himachal

Pradesh were screened for multifarious plant growth promoting traits. All the culturable bacterial isolates showed variation in their ability to produce IAA, HCN, siderophore, solubilize phosphorus and antifungal activity against different fungal pathogens (Table2; Plate 1).

4.2.1.1 Tricalcium phosphate solubilization by representative bacterial isolates from healthy apple and root rot infected diseased apple orchards in Pikovskaya's broth medium

The phosphate solubilizing activity of the selected bacterial isolates was compared on the basis of their P-solubilization ($\mu\text{g/ml}$) in PVK broth medium. The results of P-solubilization for isolates obtained from healthy and root rot infected diseased apple orchards showed significant variation in quantitative P-estimation (Table 2 and 4; Plate 1).

4.2.1.1.1 Tricalcium phosphate solubilization by representative bacterial isolates from healthy apple orchards

Among the nine isolates of location RHRS, Mashobra, maximum P- solubilization was observed for isolate M(7) ($325.00\mu\text{g/ml}$) followed by M(8) ($85.00\mu\text{g/ml}$). However, no P-solubilization was observed for isolate M(5).

Of the four isolates obtained from location Dhanta, maximum P- solubilization was observed for isolate D(1) ($59.00\mu\text{g/ml}$). The isolate D(3) solubilised minimum TCP with release of $29.20\mu\text{g/ml}$ phosphorous.

Location Jhalroo resulted in selection of six isolates and maximum P-solubilization ($67.00\mu\text{g/ml}$) was observed for isolate J(1) followed by J(3) ($35.00\mu\text{g/ml}$) and minimum TCP solubilization ($8.40\mu\text{g/ml}$) was observed for isolate J(5).

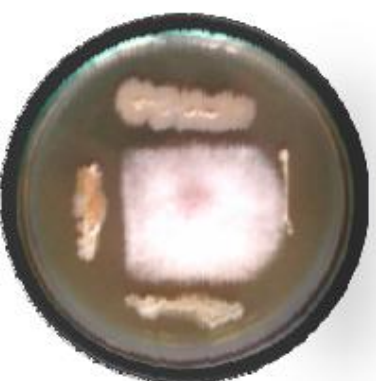
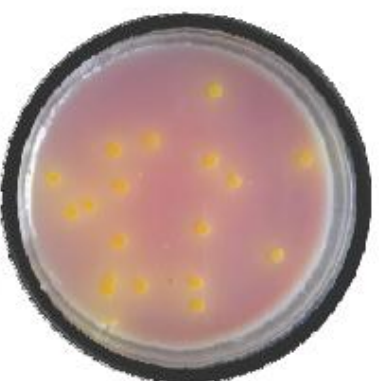
Of the seven isolates obtained from location Nanni, maximum P- solubilization was observed for isolate N(1) ($293.00\mu\text{g/ml}$) followed by N(5) ($77.70\mu\text{g/ml}$). However, for one isolate N(3) P-solubilization was not detected.

Among seven isolates of the location Sablog, maximum P- solubilization ($81.30\mu\text{g/ml}$) was observed for isolate S(6) followed by S(4) ($48.00\mu\text{g/ml}$). The isolate S(5) solubilised minimum TCP with release of $13.07\mu\text{g/ml}$ phosphorous.

Table 2: Screening of culturable bacterial isolates from healthy apple orchards for multifarious plant growth promoting activities

Location	Isolates	Quantitative P-estimation (µg/ml)****	Quantitative estimation (Per cent siderophore unit)**	Growth on Nitrogen free medium	HCN Production ^y	IAA Production (µg/ml)
RHRS, Mashobra	M(1)	11.55	66.69	+	+++	60.00
	M(2)	12.5	34.21	-	+	17.00
	M(3)	19.20	0.00	-	-	19.00
	M(4)	41.60	75.12	+	++	20.00
	M(5)	0.00	0.00	+	+	38.00
	M(6)	36.00	29.28	+	-	34.00
	M(7)	325.00	58.57	-	+	23.00
	M(8)	85.00	131.00	+	+++	28.00
Dhanta	D(1)	59.00	0.00	+	-	73.00
	D(2)	0.00	18.89	-	-	0.00
	D(3)	29.20	20.92	+	+	13.00
	D(4)	33.00	12.08	+	-	26.00
Jhalroo	J(1)	67.00	84.25	-	-	59.00
	J(2)	28.00	38.00	-	+	7.00
	J(3)	35.00	20.90	+	++	30.00
	J(4)	0.00	0.00	+	-	58.00
	J(5)	8.40	150.00	+	-	54.00
	J(6)	10.00	0.00	-	+	52.00
Nanni,	N(1)	293.00	60.21	-	+	25.00
	N(2)	80.73	60.23	+	+++	0.00
	N(3)	0.00	0.00	+	-	18.00
	N(4)	14.43	134.00	+	+	49.75
	N(5)	77.70	66.11	+	+	16.00
	N(6)	50.00	56.41	++	+	35.00
	N(7)	40.00	53.00	-	-	13.00
Sablog	S(1)	16.00	26.10	-	+++	96.00
	S(2)	43.00	69.10	-	+	34.00
	S(3)	25.30	75.31	-	+	19.00
	S(4)	48.00	10.46	+	+	52.00
	S(5)	13.07	42.20	+	-	26.00
	S(6)	81.30	131.00	+	-	29.00
	S(7)	36.00	0.00	+	-	34.00
Thanedhar	T(1)	46.00	75.31	+	++	19.00
	T(2)	15.00	16.73	-	+	13.00
	T(3)	50.00	25.10	+	-	8.00
	T(4)	28.00	0.00	-	+	7.00
	T(5)	70.00	87.86	-	++	21.00
	T(6)	45.00	72.12	-	+	34.00
	T(7)	16.00	0.00	-	+	66.00
Reckong Peo	K(1)	43.00	18.50	+	+	9.00
	K(2)	46.00	54.00	-	+	17.00
	K(3)	48.00	80.00	-	+	23.00
Kyari	Kot(1)	47.60	33.43	+	+	8.00
	Kot(2)	27.00	0.00	-	+	33.00
	Kot(3)	57.00	56.00	+	-	70.00
	Kot(4)	55.00	0.00	+	+++	63.00
	Kot(5)	34.00	15.33	+	+	8.00
	Kot(6)	48.00	19.00	+	-	26.00
	Kot(7)	320.60	0.00	+	+	21.00
	Kot(8)	43.80	19.49	-	+	24.00
Chajpur	Cj(1)	27.44	34.51	-	+	0.00
	Cj(2)	31.43	100.40	-	-	13.00
	Cj(3)	46.00	25.10	-	+	66.00
	Cj(4)	71.00	56.70	+	+	21.00
	Cj(5)	27.00	0.00	+	+	33.00
Seema	R(1)	38.00	0.00	+	+	21.00
	R(2)	24.00	43.41	+	++	18.00
	R(3)	44.00	28.00	-	-	9.00
	R(4)	56.00	0.00	+	++	0.00
	R(5)	76.00	46.00	-	+	13.00
Chopal	C(1)	38.00	54.39	+	+	149.00
	C(2)	81.60	101.40	-	+++	24.00
	C(3)	47.00	0.00	+	+	83.00
	C(4)	38.00	16.73	+	-	17.00
	C(5)	412.00	33.4	+	+	12.00
	C(6)	39.20	0.00	+	+	14.00

%Siderophore Unit = Where, Ar=Absorbance of Reference at 630 nm As=Absorbance of test at 630 nm * P- solubilized = T-C; Where, T: P-solubilized in test, C: P-solubilized in control ; ^yHCN : - No colour change of the filter paper from yellow to brown; + change in colour of filter paper from yellow to orange brown;



Protease activity

Siderophore Production

P-Solubilization

HCN Production

Chitinase activity

(2)

(b)

(c)

(d)

(c)

(f)

Antifungal activity

(a) *F. oxysporum* (b) *P. aphanidermatum* (c) *Phytophthora* sp. (d) *S. rolfii* (e) *C. salmonicolum* (f) *D. necatrix*

Plate1. Screening of culturable bacterial isolates for multifarious plant growth promoting activities

Among the six isolates of the location Sablog, maximum P- solubilization (19.00 µg/ml) was observed for isolate SD(2) followed by SD(6) (14.50 µg/ml). The isolate SD(5) solubilized minimum TCP with release of 5.00 µg/ml phosphorous.

In liquid PVK medium, out of four isolates of location Thanedhar, maximum P- solubilization was observed for bacterial isolate TD(3) (58.40 µg/ml) followed by isolate TD(4) (46.00 µg/ml) and minimum TCP solubilization (5.30 µg/ml) was observed for isolate TD(2).

Among the three isolates of the location Reckong Peo, maximum P- solubilization (50.50µg/ml) was observed for isolate KD(2). The isolate KD(1) solubilised minimum TCP with release of 14.50 µg/ml phosphorus.

Total four isolates obtained from location Kyari, with maximum P-solubilization observed for isolate KyD(1) (32.00 µg/ml) followed by KyD(2) (23.50 µg/ml). However, minimum P-solubilization was observed for isolate KyD(4) (8.50 µg/ml).

The results of the present study are in concomitance with observation of P- solubilization by rhizobacteria which were reported to mobilizing P from tri calcium phosphate (Alikhani *et al.* 2006; Vazquez *et al.* 2000; George *et al.* 2013; Mehta *et al.* 2014). Similar range of phosphate solubilization was reported by Pandey *et al.* (2006); Chaung *et al.* (2007); Banerjee *et al.* (2010); Duangpaeng *et al.* (2013). The finding in the present study are in concomitance with the findings of Passos *et al.* (2014) who reported cultivable plant growth-promoting (PGP) bacteria with phosphate solubilization ability associated with apple trees cultivated under different crop management systems.

4.2.1.2 Quantitative estimation of siderophore production by representative bacterial isolates from healthy and root rot infected diseased apple orchards

Another important trait of PGPR is the production of siderophore. PGPR produces a range of siderophore which have a very high affinity for iron. Therefore, the low availability of iron in the environment would suppress the growth of pathogenic organisms including plant pathogenic fungi (Whipps, 2000). The siderophore production of bacterial isolates were compared on the basis of percent siderophore unit (%SU) on nutrient broth amended with CAS dye (Table 2 and 3; Plate1). Variation in quantitative estimation methods was observed for isolates obtained from healthy and root rot infected diseased apple orchards.

4.2.1.2.1 Quantitative estimation of siderophore production by representative bacterial isolates from healthy apple orchards

Among the eight isolates of healthy apple at location RHRS, Mashobra, maximum per cent SU (131.00%) was obtained for isolate M(8) followed by M(4) (75.12) and minimum (29.28%) for isolate M(6). However, no siderophore production was observed for isolate M(3) and M(5).

Location Dhanta resulted in selection of four isolates and maximum per cent SU (20.92%) was observed for isolate D(3) minimum % SU(12.08%) for isolate D(4). Isolate D(1) was not able to produce siderophore.

Location Jhalroo resulted in selection of total six isolates, out of which isolate J(5) resulted in maximum %SU (150.00%) followed by J(1) and minimum %SU (20.90%) was observed for isolate J(3). However two isolates J(4) and J(6) were not able to produce siderophore.

Total seven isolates were obtained from location Nanni, maximum %SU was observed for isolate N(4) (134.00 %) followed by N(5) (66.11%). However, no siderophore production was observed for isolate N(3).

Among the seven isolates of the location Sablog, maximum %SU (131.00%) was observed for isolate S(6) followed by S(3) (75.31%). The isolate S(4) produces minimum siderophore with %SU of 10.46%. Isolate S(7) could not siderophore.

In total only seven isolates were obtained from location Thanedhar. Maximum % SU (87.86%) was observed for T(5) and minimum % SU(16.73%) for isolate T(2). Isolate T(4) and T(7) were not able to produce siderophore.

Location Recong Peo resulted in selection of three isolates and maximum % SU (80.00%) was observed for isolate K(3) and isolate K(1) produces minimum siderophore with % SU of 18.50%.

Eight isolates were recovered from location Kyari, maximum % SU was observed for isolate Kot(3) (56.00%) followed by Kot(1) (33.43%). However, three isolate Kot(2), Kot(4) and Kot(7) were not able to produce siderophore.

Table 3: Screening of culturable bacterial isolates from root rot infected diseased apple orchards for multifarious plant growth promoting activities

Location	Isolates	Quantitative P-estimation (µg/ml)****	Quantitative estimation (Per cent siderophore unit)**	Growth on Nitrogen free medium	HCN Production †	IAA Production (µg/ml)
Dhanta	DD(1)	34.00	14.50	+	-	5.40
	DD(2)	27.30	0.00	-	-	17.00
	DD(3)	38.43	0.00	-	-	11.00
	DD(4)	13.97	21.50	-	-	9.40
	DD(5)	60.30	11.30	+	+	7.00
	DD(6)	11.00	0.00	-	-	3.00
	DD(7)	23.37	13.00	-	-	5.00
Jhalroo	JD(1)	5.30	0.00	-	-	3.00
	JD(2)	16.00	25.00	-	+	26.00
	JD(3)	39.20	12.06	-	-	41.00
Nanni	ND(1)	6.00	0.00	+	+	0.00
	ND(2)	33.50	21.50	-	-	14.00
	ND(3)	19.00	56.30	-	-	21.00
	ND(4)	18.50	11.50	-	-	0.00
Sablog	SD(1)	11.00	27.00	+	-	4.80
	SD(2)	19.00	12.00	+	-	7.00
	SD(3)	7.50	9.00	-	-	11.00
	SD(4)	8.50	18.80	-	+	13.00
	SD(5)	5.000	29.20	-	-	16.00
	SD(6)	14.50	18.50	-	+	6.00
Thanedhar	TD(1)	31.50	0.00	-	-	14.00
	TD(2)	5.30	4.20	-	+	13.00
	TD(3)	58.40	62.70	-	-	31.00
	TD(4)	46.00	27.10	+	+	0.00
Reckong Peo	KD(1)	14.50	0.00	-	+	16.00
	KD(2)	50.50	42.10	+	-	21.00
	KD(3)	36.30	0.00	-	-	0.00
Kyari	KyD(1)	32.00	15.30	+	+	24.00
	KyD(2)	23.50	26.60	+	-	12.00
	KyD(3)	11.00	29.70	-	-	6.50
	KyD(4)	8.50	31.50	+	-	4.00

%Siderophore Unit = $\frac{Ar}{As} \times 100$ Where, Ar=Absorbance of Reference at 630 nm As=Absorbance of test at 630 nm * P- solubilized = T-C; Where, T: P- solubilized in test, C: P-solubilized in control ; †HCN : - No colour change of the filter paper from yellow to brown; + change in colour of filter paper from yellow to orange brown

Among the five isolates of the location Chajpur, maximum % SU (100.40%) was observed for isolate Cj(2) followed by Cj(4) (56.70%). The isolate Cj(3) produced minimum siderophore with %SU of 25.10 % and no siderophore production was observed in isolate Cj(5).

In liquid nutrient broth medium, maximum siderophore production was observed for bacterial isolate R(5) (46.00%) followed by isolate R(2) (43.41%) of location Seema. Two isolates R(1) and R(4) were not able to produce siderophore.

Location Chopal resulted in selection of six isolates producing maximum %SU (101.40%) for isolate C(2) and minimum % SU (16.73%) was observed for isolate C(4). However, isolate C(3) and C(6) were not able to produce siderophore.

4.2.1.2.2 Quantitative estimation of siderophore production by representative bacterial isolates from root rot infected diseased apple orchards

Seven isolates in total were obtained from location Dhanta, where maximum %SU was observed for isolate DD(4) (21.50%). However, siderophore production was not observed for isolates DD(2), DD(3) and DD(6).

Among the three isolates of the location Jhalroo, maximum % SU (25.00%) was observed for isolate JD(2). The isolate JD(1) was not able to produce siderophore.

Of the four isolates obtained from location Nanni, maximum % SU (56.30%) was observed for ND(3). Isolate ND(1) was not able to produce siderophore.

Location Sablog resulted in selection of six isolates and maximum % SU (29.20%) was observed for isolate SD(5) and isolate SD(3) produces minimum siderophore of 9.00 % SU.

Location Thanedhar resulted in the isolation of four isolates with maximum % SU for isolate TD(3) (62.70%) while isolate TD(1) did not show siderophore production.

Among three isolates of the location Recong Peo, only one isolate KD(2) was able to produce siderophore with 42.10 %SU.

In liquid nutrient broth medium, maximum siderophore production was observed for bacterial isolate KyD(3) (29.70%) where as isolate KyD(1) produces minimum siderophore with 15.30% SU.

Siderophores provides a competitive advantage to producer organism over fungal pathogens for the absorption of available iron. The role of siderophore in the control of diseases had been reported by Baker *et al.* (1986). In a study reported by Srivastava *et al.*

(2014), a total of 45.45 per cent isolates displayed positive results of siderophore production which supports our findings that only 47.08 per cent and 19.20 per cent selected bacterial isolates from healthy and root rot infected diseased apple, respectively showed siderophore production.

Similarly siderophore-producing bacteria have been documented by other studies (Adesina *et al.* 2007; Tian *et al.* 2009). Ambrosini *et al.* (2012) evaluated the diversity of cultivable siderophore-producing bacteria in association with the sunflower (*Helianthus annuus* L.) rhizosphere.

4.2.1.3 IAA production by representative bacterial isolates from healthy and root rot infected diseased apple orchards

Biosynthesis of IAA is not limited to higher plants even organisms such as bacteria are able to make physiologically active IAA that might have pronounced effects on plant growth and development. The representative bacterial isolates showed variation in production of IAA (Table 2 and 3).

L-tryptophan is considered as the IAA precursor in bacteria, because its addition to IAA producing bacterial cultures promotes and increases IAA synthesis (Tsavkelova *et al.* 2007). Root exudates are natural source of L-tryptophan for rhizosphere microflora, which may enhance auxin biosynthesis in the rhizosphere. Production of IAA in the presence of a suitable precursor such as tryptophan has been reported for several PGPR belonging to the genera *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Pantoea*, *Pseudomonas*, and *Serratia*. 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 (Bhatt and Vyas, 2014).

IAA production in culture supernatants of representative bacterial isolates grown in Luria bertani broth showed 93.93 per cent isolates from healthy apple orchards while 6.06 per cent lack the ability of IAA production from tryptophan. The maximum IAA production (149.00 µg/ml) was observed in case of isolate C(1) of healthy apple orchard of Chopal and minimum IAA production was observed in case of isolate J(2) of healthy apple orchard of location Jhalroo. No IAA production was observed in four isolates viz D(2), N(2), Cj(1) and R(4) of healthy apple at location Dhanta, Nanni, Chajpur and Seema, respectively.

In the present study, results of root rot infected diseased apple orchards showed a large variation in IAA producing bacterial population with respect to all locations. Salkowski reaction of culture supernatants of selected bacterial isolates grown in Luria bertani broth showed that 87.09 per cent isolates from root rot infected diseased apple were observed as their ability to produce IAA while 12.09 per cent lack the ability. The highest IAA production by the isolates was observed in rhizosphere soil of location Jhalroo JD(1) (41.00 µg/ml) followed by isolate of Thanrdhar TD(3) (31.00 µg/ml). But the amount of IAA production in root rot infected diseased was significantly low (41.00 µg/ml). as compared to bacteria screened from healthy apple (149.00 µg/ml by C(1) of Chopal). 4 isolates (ND(1), ND(4), TD(4) and KD(3)) from of root rot infected diseased apple at three location i.e. Nanni, Thanedhar and Recong Peo respectively were not able to produce IAA. This might be due to the reason that rhizobacterial population in root rot infected diseased apple orchard is low as compared to population of pathogens. This indicates that healthy orchards have high population of IAA producing rhizobacteria which might have a direct correlation with the enhanced growth in apple plants.

The negative IAA producing ability of the most of the isolates from root rot infected diseased apple trees and few isolates from healthy trees might be due to loss of genetic and physiological apparatus of IAA biosynthesis of those isolates (Wahyudi *et al.* 2011). Furthermore, the activity of the indole pyruvic decarboxylase, an enzyme which determines IAA biosynthesis in *Bacillus* sp., is variously connected to protein structure or different level of ipde gene expression (Patten and Glick, 2002).

Many evidences, based on research carried out by several investigators suggested that IAA may be the first PGP traits compared to ACC deaminase activity, siderophore production and phosphate solubilization traits used for screening rhizosphere and endophytic bacteria for various crops. Plant PGP agents such as bacterial IAA contributes to circumvent the host defence by depressing the IAA signalling in the plant; IAA can also have a direct effect on bacterial survival and its resistance to plant defence. It can be speculated that IAA production trait is part of the strategy used by IAA producing bacteria to circumvent the plant defence system. IAA is a plant hormone with no apparent function in bacterial cells however it might improve the fitness of the plant–bacterium interaction (Etisami *et al.* 2015).

4.2.1.4 Antifungal activity of representative culturable bacterial isolates against different fungal pathogens from healthy and root rot infected diseased apple orchards

Table 4 and 5 revealed that antifungal activity of selected culturable bacterial isolates from healthy and root rot infected diseased apple orchards against fungal pathogen viz. *Phytophthora* sp., *Fusarium oxysporum*, *Dematophora necatrix*, *Sclerotium rolfsii*, *Pythium aphanidermatum*.

Representative bacterial isolates exhibited variation in antifungal activity against all the fungal pathogens (Plate 1).

Among the sixty six isolates of healthy apple orchards, 77.27% of them were able to inhibit the growth of *D. necatrix*, 30.00% were able to inhibit *Phytophthora* sp., 86.36% isolates showed inhibition against *F.oxysporum*, 63.63% of them were able to inhibit the growth of *S. rolfsii* and 77.27% showed antagonistic activity against *P.aphanidermatum*. Maximum percent growth inhibition was exhibited by isolate N(2) (68.33%) of Nanni, Cj(3) (47.10%) of Chajpur, D(1) (66.60%) of Dhanta, R(1) and R(5) (52.00%) of Seema, against *D. necatrix*, *Phytophthora* sp., *F. oxysporum*, *S. rolfsii* and *P. aphanidermatum* respectively.

Of the thirty one isolates obtained from diseased apple orchards, 35.48% showed inhibition against *D.necatrix*, 16.45% were able to inhibit the growth of *Phytophthora* sp., 45% isolates against *F.oxysporum*, 35.25% against *S.rolfsii*, 41.93% against *P.aphanidermatum*. Maximum percent growth inhibition was exhibited by isolate JD(1) (46.50%) of Jhalroo, JD(1) (46.20%) of Jhalroo, TD(4) (51.10%) of Thanedhar, TD(4) (68.50%) of Thanedhar, KyD(4) (46.00%) against *D. necatrix*, *Phytophthora* sp., *F. oxysporum*, *S. rolfsii* and *P. aphanidermatum*, respectively.

Rhizobacteria are soil bacteria that colonize plant roots; they are able to multiply and occupy all the ecological niches found on the roots at all stages of plant growth (Antoun and Prévost, 2006). Such bacteria may negatively interact with plants, directly by competing for nutrients. Alternatively, the relationship between rhizobacteria and the host plant can be positive. For example, the bacteria may compete with pathogens for survival in the rhizosphere or they may promote mutualistic relationships with plants they were associated, allowing nutrient exchange and stimulating antibiotic production against phytopathogenic agents (Siddiqui, 2006; Conrath *et al.* 2002).

Table 4: Screening of culturable bacterial isolates from healthy orchards for antifungal activity against different fungal pathogens

Location	Isolates	Antagonistic activity against fungal pathogens(% Growth inhibition)**				
		<i>Dematophora necatrix</i>	<i>Phytophthora sp.</i>	<i>Fusarium oxysporum</i>	<i>Sclerotium rolfsii</i>	<i>Pythium aphanidermatum</i>
RHRS, Mashobra	M(1)	46.50	-	-	-	CI
	M(2)	57.14	-	54.00	43.50	45.70
	M(3)	-	-	CI	-	-
	M(4)	CI	-	CI	-	CI
	M(5)	47.00	-	CI	51.00	49.20
	M(6)	50.00	-	47.50	48.70	48.00
	M(7)	CI	-	CI	-	CI
	M(8)	67.50	CI	CI	47.30	47.00
Dhanta	D(1)	-	-	66.60	-	62.80
	D(2)	CI	-	CI	-	-
	D(3)	CI	-	CI	CI	-
	D(4)	-	CI	53.30	CI	CI
Jhalroo	J(1)	CI	CI	47.32	CI	-
	J(2)	50.00	CI	-	48.43	48.50
	J(3)	CI	-	CI	-	CI
	J(4)	CI	-	-	-	CI
	J(5)	CI	-	47.50	CI	CI
	J(6)	67.70	CI	CI	47.30	50.00
Nanni	N(1)	CI	-	CI	-	CI
	N(2)	68.33	CI	47.32	48.88	50.00
	N(3)	CI	-	-	CI	CI
	N(4)	62.30	CI	CI	46.60	51.30
	N(5)	66.11	CI	CI	CI	CI
	N(6)	52.50	-	-	49.43	-
	N(7)	CI	-	CI	-	CI
Sablog	S(1)	CI	-	-	-	CI
	S(2)	CI	-	CI	45.00	-
	S(3)	66.11	-	CI	CI	CI
	S(4)	67.71	-	CI	CI	CI
	S(5)	CI	-	53.30	CI	CI
	S(6)	66.45	CI	CI	CI	48.50
	S(7)	46.00	-	47.50	48.70	48.50
Thanedhar	T(1)	-	-	CI	-	CI
	T(2)	CI	-	CI	-	CI
	T(3)	CI	-	CI	47.60	48.00
	T(4)	50.00	-	-	48.43	48.50
	T(5)	-	-	CI	-	-
	T(6)	CI	-	CI	47.00	-
	T(7)	CI	46.20	CI	46.00	-
Reckong Peo	K(1)	-	-	CI	-	-
	K(2)	CI	-	45.00	47.50	CI
	K(3)	-	-	CI	-	CI
Kyari	Kot(1)	CI	-	CI	CI	CI
	Kot(2)	CI	-	45.80	CI	CI
	Kot(3)	-	-	CI	-	62.80
	Kot(4)	CI	CI	CI	-	CI
	Kot(5)	45.00	-	CI	47.50	46.00
	Kot(6)	CI	-	53.30	CI	CI
	Kot(7)	-	-	CI	-	-
	Kot(8)	46.50	-	CI	CI	-
Chajpur	Cj(1)	CI	-	CI	-	CI
	Cj(2)	CI	46.20	CI	48.32	46.20
	Cj(3)	CI	47.10	CI	-	CI
	Cj(4)	60.50	CI	51.75	47.60	48.80
	Cj(5)	CI	CI	48.80	CI	-
Seema	R(1)	CI	-	CI	52.00	-
	R(2)	48.00	-	46.70	-	47.50
	R(3)	-	-	CI	51.00	CI
	R(4)	-	-	-	CI	CI
	R(5)	CI	CI	48.70	52.00	48.00
Chopal	C(1)	-	-	46.50	CI	CI
	C(2)	CI	-	CI	CI	46.00
	C(3)	-	-	-	-	47.50
	C(4)	-	-	-	-	-
	C(5)	-	CI	CI	46.00	CI
	C(6)	46.50	CI	49.00	47.50	CI

**% Growth inhibition = $\frac{C-T}{C} \times 100$ Where, C: growth of fungus in control; T: Growth of fungus in test , CT=Contact Inhibition

Table 5: Screening of culturable bacterial isolates from root rot infected diseased apple orchards for antagonistic activity against fungal pathogens

Location	Isolates	Antagonistic activity against fungal pathogens (% Growth inhibition)**				
		<i>Dematophora necatrix</i>	<i>Phytophthora sp.</i>	<i>Fusarium oxysporum</i>	<i>Sclerotium rolfsii</i>	<i>Pythium aphanidermatum</i>
Dhanta	DD(1)	-	-	CI	-	CI
	DD(2)	-	-	-	-	-
	DD(3)	-	-	-	CI	CI
	DD(4)	CI	-	47.00	-	-
	DD(5)	-	-	-	-	-
	DD(6)	CI	-	-	-	-
	DD(7)	-	-	-	CI	CI
Jhalroo	JD(1)	46.50	46.20	CI	CI	-
	JD(2)	-	-	CI	-	CI
	JD(3)	CI	-	46.00	57.25	CI
Nanni	ND(1)	-	-	CI	CI	-
	ND(2)	-	-	-	-	CI
	ND(3)	CI	-	46.00	50.00	-
	ND(4)	CI	-	-	-	CI
Sablog	SD(1)	-	-	CI	-	CI
	SD(2)	-	-	-	-	-
	SD(3)	CI	-	46.00	-	CI
	SD(4)	-	CI	-	-	-
	SD(5)	-	-	-	47.50	-
	SD(6)	-	-	CI	-	-
Thanedhar	TD(1)	CI	-	-	-	CI
	TD(2)	CI	-	-	-	-
	TD(3)	-	-	-	-	-
	TD(4)	-	-	51.10	68.50	-
Kinnaur	KD(1)	CI	-	46.00	-	CI
	KD(2)	-	-	-	CI	CI
	KD(3)	-	-	-	-	-
Kyari	KyD(1)	-	-	-	CI	-
	KyD(2)	-	-	-	-	-
	KyD(3)	-	-	CI	-	-
	KyD(4)	CI	-	48.50	-	46.00

**% Growth inhibition = $\frac{C-T}{C} \times 100$ Where, C: growth of fungus in control; T: Growth of fungus in test

The findings in the present study are in concomitance with reports documented by several researchers who showed that the interactions exist not only between host and the soil borne fungal pathogen, but also with rhizobacteria having antagonistic phytopathogenic activity (Van Loon, 1997; Van Loon *et al.* 1998; Pieterse *et al.* 1996, 1998, 2001; Gozzo, 2003; Kurkcuoglu *et al.* 2007; Beneduzi *et al.* 2008; Ambrosini *et al.* 2012; Souza *et al.* 2013).

4.3 PRINCIPAL COMPONENT ANALYSIS OF RHIZOBACTERIA FROM HEALTHY AND ROOT ROT INFECTED DISEASED APPLE ORCHARDS

Principal Component Analysis (PCA) was carried to evaluate correlations between apple associated rhizobacterial populations and different plant growth promoting traits in

healthy and root rot infected diseased apple orchards. The variables were normalized by division of their standard deviation, the Eigen values and Eigenvectors of the correlation matrix and percentages of variance for each component were calculated.

PCA plots showing relationship between apple rhizobacterial isolates (isolated from (a) healthy and (b) root rot infected diseased apple orchards) and plant growth promoting traits viz. phosphate solubilization, siderophore production, IAA production are presented in Fig. 10 Results revealed that principal component 1 (phosphate solubilization) and principal component 2 (siderophore production) at both the locations accounted for more than 70% of the total variance.

A comparative analysis of per cent variance as a result of component 1 (phosphate solubilization) revealed that, a significantly higher variance (54.18%) in root rot infected diseased apple orchards was observed. While only 38.20% variance was observed in healthy apple orchards. The results therefore, suggest that higher variation in population of phosphate solublizers is present at the location with root rot infected diseased apple trees, thus indicating the rhizobacterial mediated P-mobilization phenomenon is not a limiting factor in root rot infected diseased apple orchards. However, comparative analysis of the variance as a result of component 2 (siderophore production) revealed an opposite trend. Higher variance (33.74%) was observed in healthy apple orchards while only 25.45% variance was observed in root rot infected diseased apple orchards, therefore suggesting lower variance of siderophore producers in root rot infected diseased apple orchards. Higher population variance of siderophore producers at healthy locations, suggest that the iron chelating properties of siderophore producers did not allow the pathogens to survive in healthy tree rhizosphere. Thus the findings indicate population of siderophore producers is critical when comparing relationship between apple rhizobacterial isolates (isolated from healthy apple and root rot infected diseased apple orchards) and plant growth promoting traits. Majority of PGPR organisms are known to produce phytohormones, exhibit antifungal activity and phosphate solubilizing properties (Khamna *et al.* 2010; Dasgupta *et al.* 2015; Krey *et al.* 2013; Etisami *et al.* 2015). Siderophore production by PGPR has been proposed to explain the suppressive and antagonistic effect against different fungal pathogens (Buysens *et al.* 1996; Kirner *et al.* 1998; Shirivastav *et al.* 2004).

PCA plots showing relationship between apple rhizobacterial isolates (isolated from (a) healthy and (b) root rot infected diseased apple orchards) and antagonistic abilities against

different fungal pathogens are presented in Fig.11. Results revealed that principal component 1 (rhizobacterial populations exhibiting antagonism to *Dematophora necatrix*) and principal component 2 (rhizobacterial populations exhibiting antagonism to *Phytophthora* sp.) at both the locations accounted for more than 50% of the total variance. Thus, highlighting the fact that among the different fungal pathogens (*Dematophora necatrix*, *Phytophthora* sp., *Fusarium oxysporum*, *Sclerotium rolfsii* and *Pythium aphanidermatum*) evaluated in the present study, the antagonism of rhizobacterial populations against *Dematophora necatrix* and *Phytophthora* sp. is of critical importance.

A comparative analysis of per cent variance as a result of component 1 (rhizobacterial populations exhibiting antagonism to *Dematophora necatrix*) revealed that, a significantly higher variance (35.45%) in healthy apple orchards was observed. While only 28.43% variance was observed in root rot infected diseased apple orchards. The results therefore, suggest that lower population variance of rhizobacterial antagonists to *Dematophora necatrix* are present at the location with root rot infected diseased apple trees, thus indicating the fact that, the fungal pathogen *Dematophora necatrix* significantly contributes to the disease in root rot infected diseased apple orchards. Further analysis of the data appended in Fig.11 reveals that per cent variance as a result of component 2 (rhizobacterial populations exhibiting antagonism to *Phytophthora* sp.) followed a trend opposite to that as observed with component 1. A variance of 20.05% was observed in healthy apple orchards as compared to 24.42% variance in rhizobacterial populations exhibiting antagonism to *Phytophthora* sp. in root rot infected diseased apple orchards.

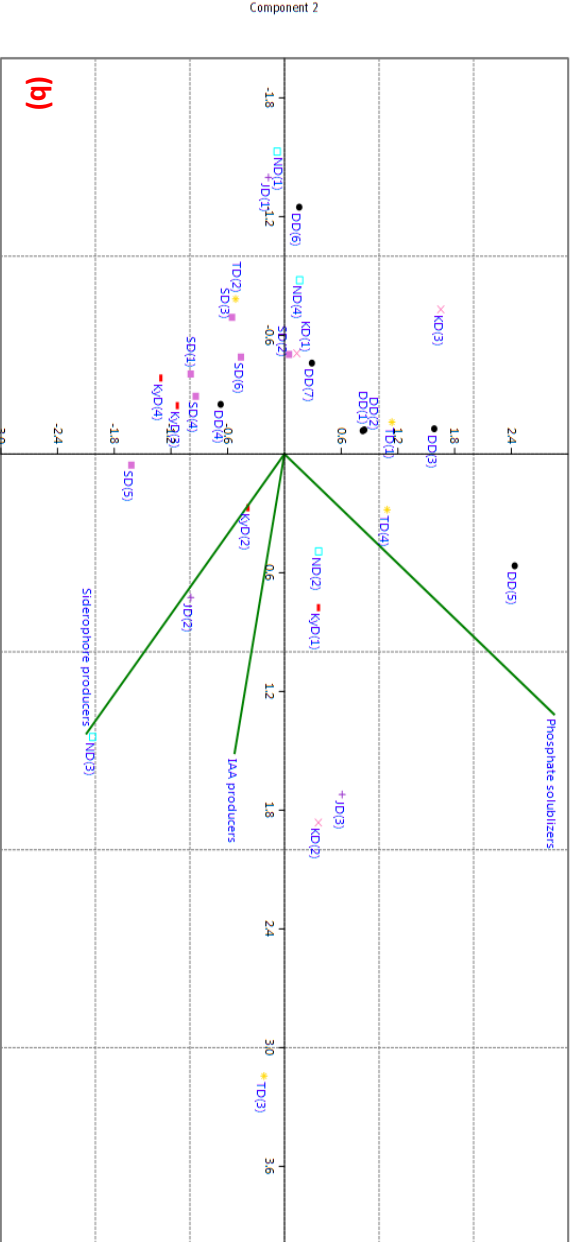
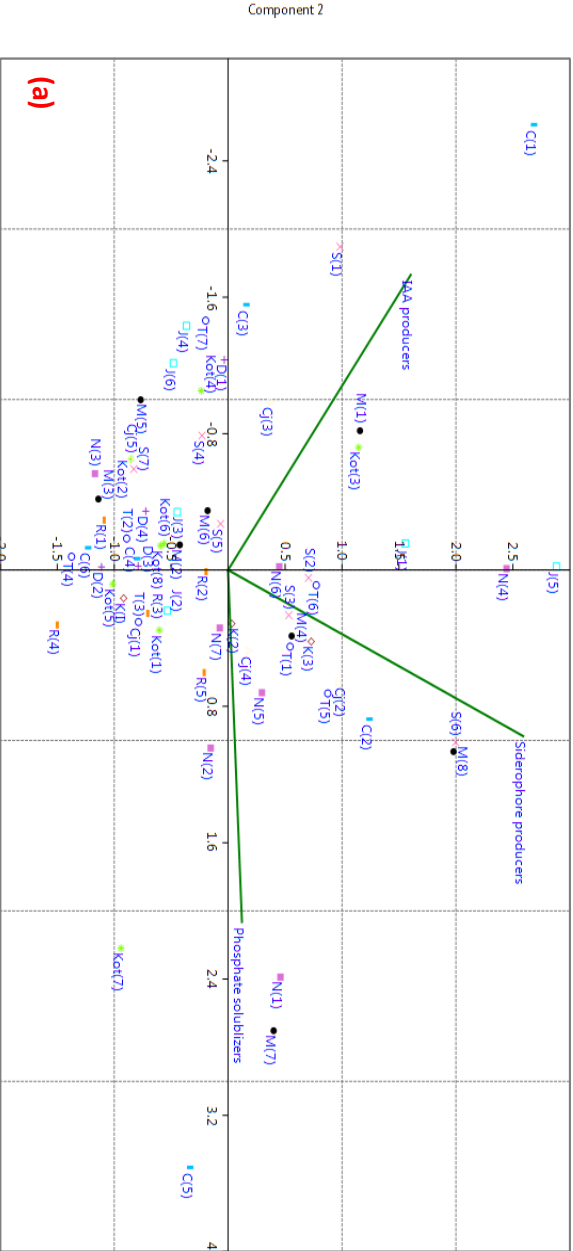
Because little is known about the interactions between beneficial microorganisms and their host plants which may induce systemic resistance against phytopathogenic fungi (Kurkcuoglu *et al.* 2007). Therefore, based on the PCA analysis, the finding in the present study clearly help in establishing a correlation between antagonist rhizobacterial population and phtopathogenic fungi. It is well known that pathogens are studied most effectively within an ecological context of interactions among microbes, their hosts, and the environmental conditions in which they live (Harvell *et al.* 1999). Therefore, a basic knowledge of the composition and distribution of the microbial communities associated with healthy as well as diseased apple orchards, as evaluated in the present study, is essential for the diagnosis and eventual treatment and prevention of plant diseases by using disease suppressive microbial communities.

4.4 GENETIC DIVERSITY OF REPRESENTATIVE PLANT GROWTH PROMOTING RHIZOBACTERIA FROM HEALTHY AND ROOT ROT INFECTED DISEASED APPLE ORCHARDS USING AMPLIFIED RIBOSOMAL DNA RESTRICTION ANALYSIS

To assess and compare the genetic diversity of culturable PGPR isolates from healthy and root rot infected diseased apple orchards at different locations, amplified ribosomal DNA restriction analysis (ARDRA) and DNA sequence analysis of 16S rRNA gene was conducted. Vaneechoutte *et al.* (1995) concluded that the restriction profile produced by ARDRA can be used as a robust library for particular species. All the 66 and 31 isolates obtained from healthy and root rot infected diseased apple tree rhizosphere respectively were further analysed using PCR amplification and digested with the restriction endonucleases in order to look for species variation among the morphotype selected. The aim of the present study was to decipher the diversity of rhizobacterial isolates within the diverse zone of healthy and root rot infected diseased apple orchards and their PGP attributes. Research on genetic diversity of microbes from rhizosphere of various crops such as wheat, apple, rice and maize have been documented by many researchers (Lawongsa *et al.* 2008; Guleria *et al.* 2014; Upadhyay *et al.* 2009).

In the present study, three tetra cutter restriction endonucleases, *HpaII*, *Hin fI* and *Taq I* were employed for the restriction analysis of PCR products. PCR amplification of the 16S rDNA of representative isolates from healthy and root rot infected diseased apple orchards resulted in a single fragment of 1400 bp, corresponding to the predicted size of the amplification product of 16S rDNA. The 16S rDNA amplicons were digested with restriction enzymes (Plate 2 and 3). Using these restriction enzymes three to nine fragments were observed for almost all of the isolates in the size ranging from 100-1000 bp.

Analysis of *Taq I*, *Hpa II* and *Hin fI* generated finger print patterns resulted in the grouping of isolates belonging to the same species together. In accordance with the dendrogram of genetic similarity using Dice similarity coefficient index, all the 66 isolates from healthy apple were grouped into 15 different phylotype at 75 % similarity level (Fig.12). Majority of the isolates were grouped into phylotype II, III, VII and VIII; phylotype X and XII comprised of four isolates each whereas phylotype IV and V comprised of two isolates each. Phylotypes VI, IX, XIII, XIV and XV were represented by single isolate each and thus formed out groups in the dendrogram constructed.



Components 1 and 2 account for 71.94 % variance

Components 1 and 2 accounts for 79.63 % variance

Principal Components	Healthy apple orchards	Root rot infected apple orchards
	% variance	
Component 1	38.20 (1.15)	54.18 (1.63)
Component 2	33.74 (1.01)	25.45 (0.76)
Component 3	28.06 (0.84)	20.38 (0.61)

Figure in parenthesis indicate Eigen values

Component 1: Phosphate solubilizers

Component 2: Siderophore producers

Component 3: IAA producers

Figure. 10. PCA plots showing relationship between apple rhizobacterial isolates (isolated from (a) healthy apple orchards and (b) root rot infected diseased apple orchards) and plant growth promoting traits viz. phosphate solubilization, siderophore production, IAA production

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All 31 isolates from root rot infected diseased apple were grouped into 8 different phylotype at 75% similarity level (Fig.14). Majority of the isolates were grouped into phylotype II, III and IV; phylotype VII comprised of three isolates and phylotype I and VI comprised of two isolates each, whereas phylotype V and VIII were represented by single isolate each and thus formed otgroups in the dendrogram constructed.

4.4.1 Phylogenetic analysis of reprasantative isolates from healthy and root rot infected diseased apple orchards

Representative isolates belonging to different ARDRA phlotypes, showing high growth promoting potential, were selected for the identification purposes. Isolates were identified by sequencing and phylogenetic analysis of 16S rRNA genes which shows a diverse group of bacterial isolates from helthy and disease apple orchards. 16S rRNA sequence analysis indicate that all isolates were diverse in terms of species texas.

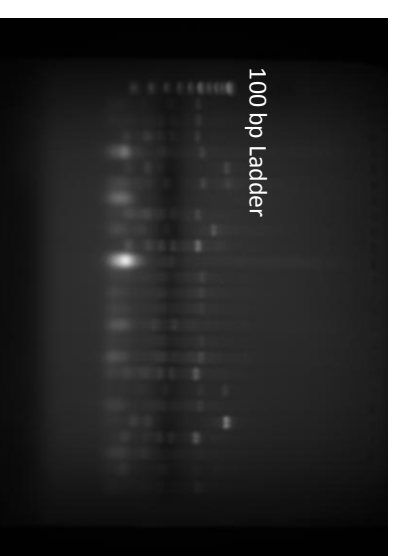
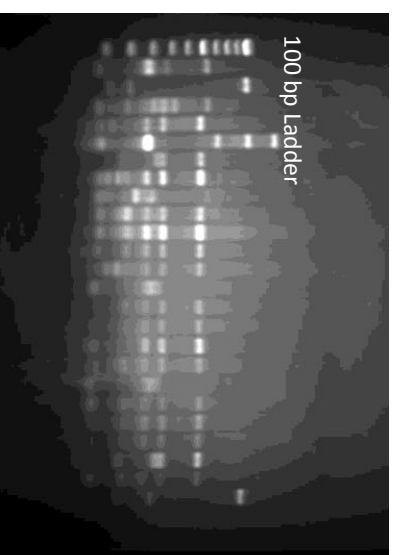
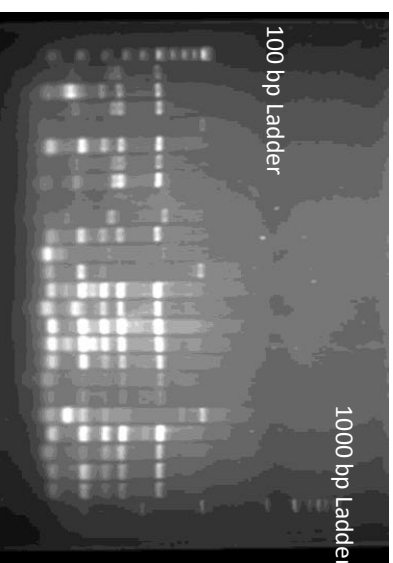
The BLASTn search revealed the presence of diverse type of bacteria belonging to genus *Bacillus*, *Cosenzaea*, *Plesiomonas*, *Providencia* and *Tatumella*, in the rhizosphere of healthy apple orchards at different locations. The isolates exhibited nucleotide similarity with the nearest relatives in the NCBI GenBank database which ranged from 85-99%. A detailed account of the identities of the isolates has been presented in Table 6. Similarly, sequence analysis of isolates from root rot infected diseased apple orchards, based on BLASTn search revealed the presence of bacteria belonging to genus *Bacillus*, *Pantoea*, *Leminorella* and *Morganella* at different locations. The isolates exhibited nucleotide similarity with the nearest relatives in the NCBI GenBank database which ranged from 93-99% and has been presented in Table 7.

To best of our knowledge, there are only a handful reports in literature that report these novel genera, isolated from rhizosphere of healthy apple and root rot infected diseased apple trees (Mehta *et al.* 2010; Shanmugam *et al.* 2011; Passos *et al.* 2014; Jian *et al.* 2015,). Figure 13 and 15 represents the circular phylogram of apple rhizospheric bacterial isolates from healthy and root rot infected diseased apple orchards, constructed using neighbor joining method with 100 bootstrap samplings. Phylogenetic analysis confirmed the affiliation of the selected PGPR from healthy apple into *Bacillus*, *Cosenzaea*, *Plesiomonas*, *Providencia* and *Tatumella* revealed by clustering of each genus to its corresponding group. Phylogenetic tree revealed that out of total fifteen strains, ten strains were clustered closely with different species of genus *Bacillus* with high bootstrap confidence values. The genus *Bacillus* was reprinted by species *Bacillus subtilis*, *Bacillus aerius*, *Bacillus soli*, *Bacillus anthracis* and

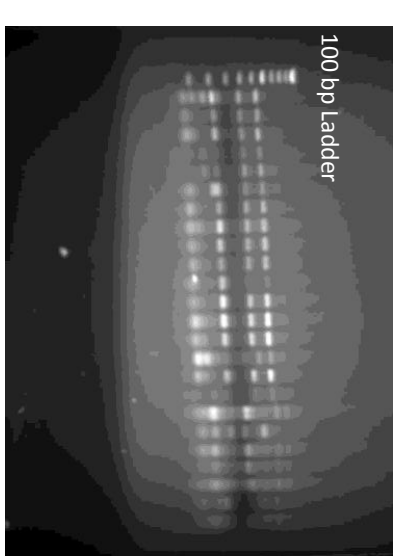
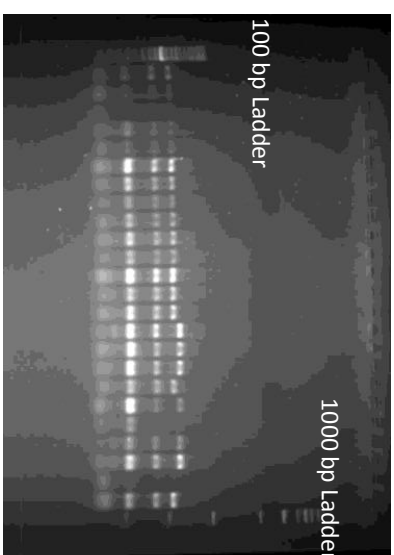
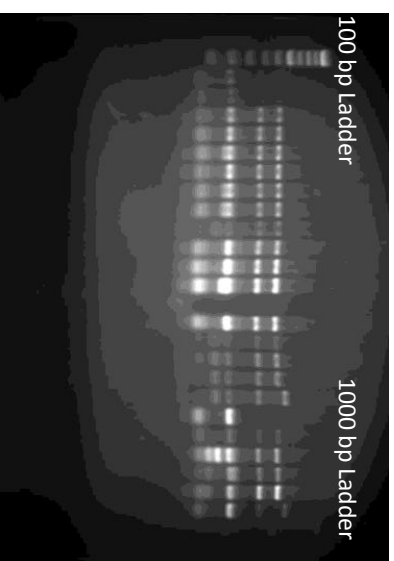
i)	A1 Ladder; A2 M(1); A3 M(2); A4 M(3); A5 M(4); A6 M(5); A7 M(6); A8 M(7); A9 M(8); A10 D(1); A11 D(2); A12 D(3); A13 D(4); A14 J(1); A15 J(2); A16 J(3); A17 J(4); A18 J(5); A19 J(6); A20 N(1); A21 N(2); A22 N(3); A23 N(4); A24 N(5); A25 N(6); A26 Ladder	B1 Ladder; B2 N(7); B3 S(1); B4 S(2); B5 S(3); B6 S(4); B7 S(5); B8 S(6); B9 S(7); B10 T(1); B11 T(2); B12 T(3); B13 T(4); B14 T(5); B15 T(6); B16 T(7); B17 K(1); B18 K(2); B19 K(3); B20 Kot(1); B21 Kot(2); B22 Kot(3); B23 Kot(4); B24 Kot(5); B25 Kot(6); B26 Ladder	C1 Ladder; C2 Kot(7); C3 Kot(8); C4 Cj(1); C5 Cj(2) C6 Cj(3); C7 Cj(4); C8 Cj(5); C9 R(1); C10 R(2); C11 R(3); C12 R(4); C13 R(5); C14 C(1); C15 C(2); C16 C(3); C17 C(4); C18 C(5); C19 C(6)
ii)	A1 Ladder; A2 M(1); A3 M(2); A4 M(3); A5 M(4); A6 M(5); A7 M(6); A8 M(7); A9 M(8); A10 D(1); A11 D(2); A12 D(3); A13 D(4); A14 J(1); A15 J(2); A16 J(3); A17 J(4); A18 J(5); A19 J(6); A20 N(1); A21 N(2); A22 N(3); A23 N(4); A24 N(5); A25 N(6); A26 Ladder	B1 Ladder; B2 N(7); B3 S(1); B4 S(2); B5 S(3); B6 S(4); B7 S(5); B8 S(6); B9 S(7); B10 T(1); B11 T(2); B12 T(3); B13 T(4); B14 T(5); B15 T(6); B16 T(7); B17 K(1); B18 K(2); B19 K(3); B20 Kot(1); B21 Kot(2); B22 Kot(3); B23 Kot(4); B24 Kot(5); B25 Kot(6); B26 Ladder	C1 Ladder; C2 Kot(7); C3 Kot(8); C4 Cj(1); C5 Cj(2) C6 Cj(3); C7 Cj(4); C8 Cj(5); C9 R(1); C10 R(2); C11 R(3); C12 R(4); C13 R(5); C14 C(1); C15 C(2); C16 C(3); C17 C(4); C18 C(5); C19 C(6)
iii)	A1 Ladder; A2 M(1); A3 M(2); A4 M(3); A5 M(4); A6 M(5); A7 M(6); A8 M(7); A9 M(8); A10 D(1); A11 D(2); A12 D(3); A13 D(4); A14 J(1); A15 J(2); A16 J(3); A17 J(4); A18 J(5); A19 J(6); A20 N(1); A21 N(2); A22 N(3); A23 N(4); A24 N(5); A25 N(6); A26 Ladder	B1 Ladder; B2 N(7); B3 S(1); B4 S(2); B5 S(3); B6 S(4); B7 S(5); B8 S(6); B9 S(7); B10 T(1); B11 T(2); B12 T(3); B13 T(4); B14 T(5); B15 T(6); B16 T(7); B17 K(1); B18 K(2); B19 K(3); B20 Kot(1); B21 Kot(2); B22 Kot(3); B23 Kot(4); B24 Kot(5); B25 Kot(6); B26 Ladder	C1 Ladder; C2 Kot(7); C3 Kot(8); C4 Cj(1); C5 Cj(2) C6 Cj(3); C7 Cj(4); C8 Cj(5); C9 R(1); C10 R(2); C11 R(3); C12 R(4); C13 R(5); C14 C(1); C15 C(2); C16 C(3); C17 C(4); C18 C(5); C19 C(6)

Plate 2 ARDRA fingerprints of culturable bacterial communities in rhizosphere soil of apple trees of healthy apple orchards, with (i) *HinfI* (ii) *HpaII* (iii) *Taq I* restriction enzymes

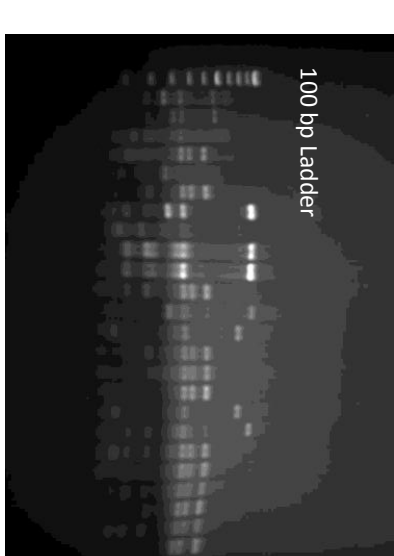
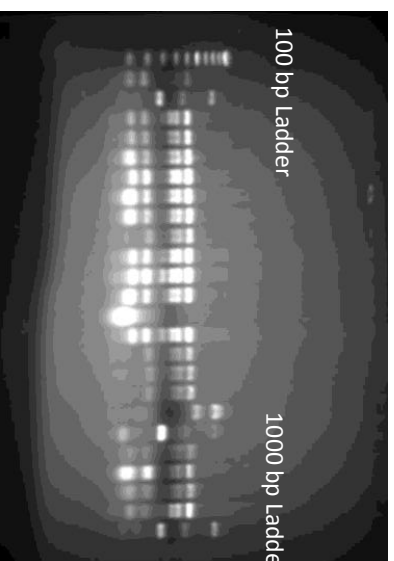
(i)



(ii)



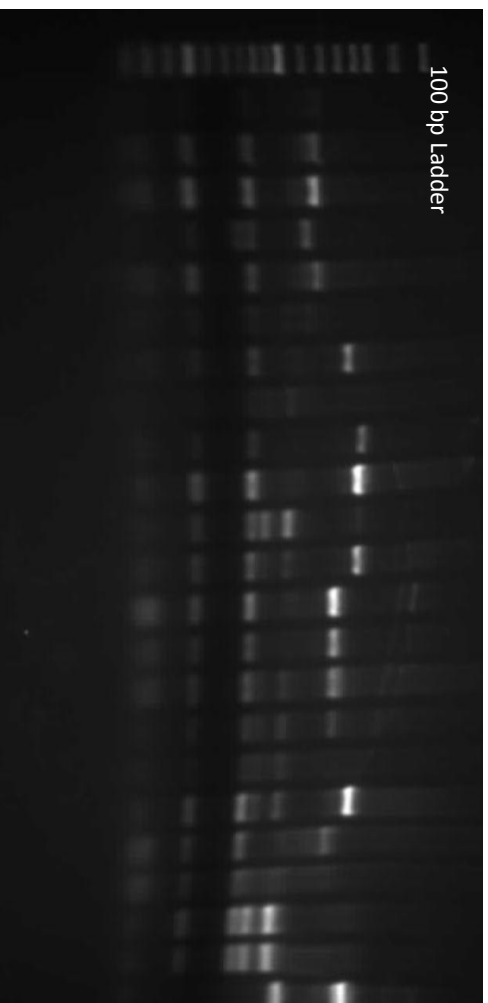
(iii)



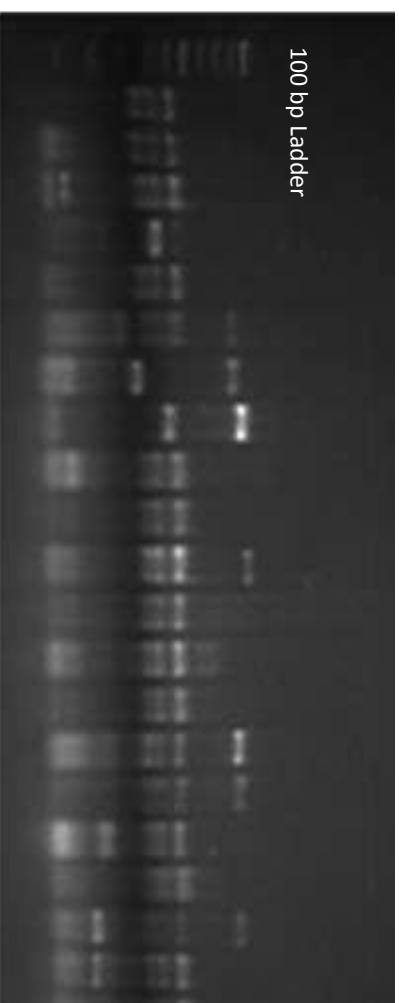
i)	A1 Ladder; A2 DD(1); A3 DD(2); A4 DD(3); A5 DD(4); A6 DD(5); A7 DD(6); A8 DD(7); A9 JD(1); A10 JD(2); A11 JD(3); A12 ND(1); A13 ND(2); A14 ND(3); A15 ND(4); A16 SD(1); A17 SD(2); A18 SD(3); A19 SD(4); A20 SD(5); A21 SD(6); A22 TD(1)	B1 Ladder; B2 TD(2); B3 TD(3); B4 TD(4); B5 KD(1); B6 KD(2); B7 KD(3); B8 KyD(1); B9 KyD(2); B10 KyD(3); B11 KyD(4)
ii)	A1 Ladder; A2 DD(1); A3 DD(2); A4 DD(3); A5 DD(4); A6 DD(5); A7 DD(6); A8 DD(7); A9 JD(1); A10 JD(2); A11 JD(3); A12 ND(1); A13 ND(2); A14 ND(3); A15 ND(4); A16 SD(1); A17 SD(2); A18 SD(3); A19 SD(4); A20 SD(5); A21 SD(6); A22 TD(1)	B1 Ladder; B2 TD(2); B3 TD(3); B4 TD(4); B5 KD(1); B6 KD(2); B7 KD(3); B8 KyD(1); B9 KyD(2); B10 KyD(3); B11 KyD(4)
iii)	A1 Ladder; A2 DD(1); A3 DD(2); A4 DD(3); A5 DD(4); A6 DD(5); A7 DD(6); A8 DD(7); A9 JD(1); A10 JD(2); A11 JD(3); A12 ND(1); A13 ND(2); A14 ND(3); A15 ND(4); A16 SD(1); A17 SD(2); A18 SD(3); A19 SD(4); A20 SD(5); A21 SD(6); A22 TD(1)	B1 Ladder; B2 TD(2); B3 TD(3); B4 TD(4); B5 KD(1); B6 KD(2); B7 KD(3); B8 KyD(1); B9 KyD(2); B10 KyD(3); B11 KyD(4)

Plate 3 ARDRA fingerprints of culturable bacterial communities in rhizosphere soil of root rot infected diseased apple orchards, with (i) *HinfI* (ii) *HpaII* (iii) *Taq I* restriction enzymes

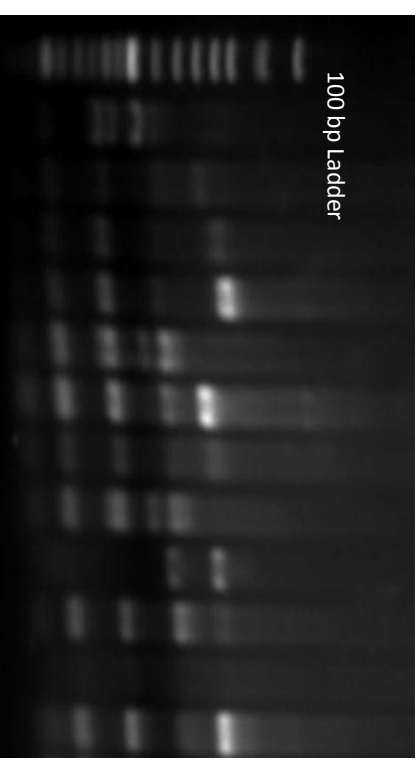
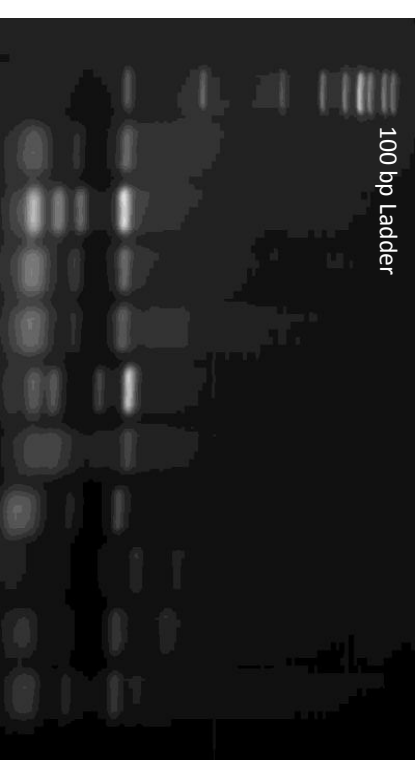
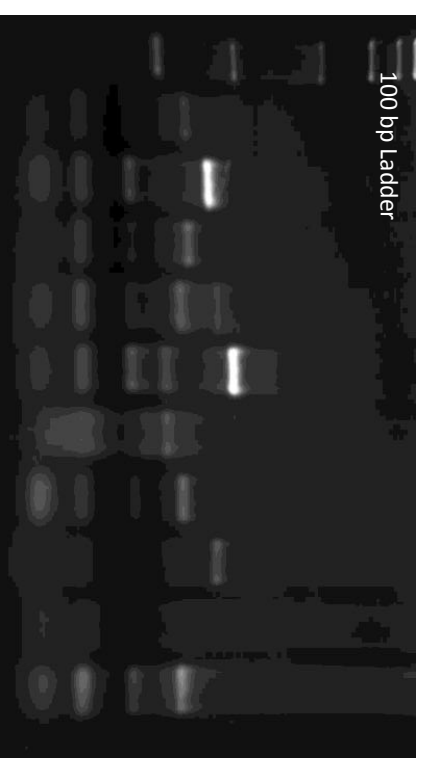
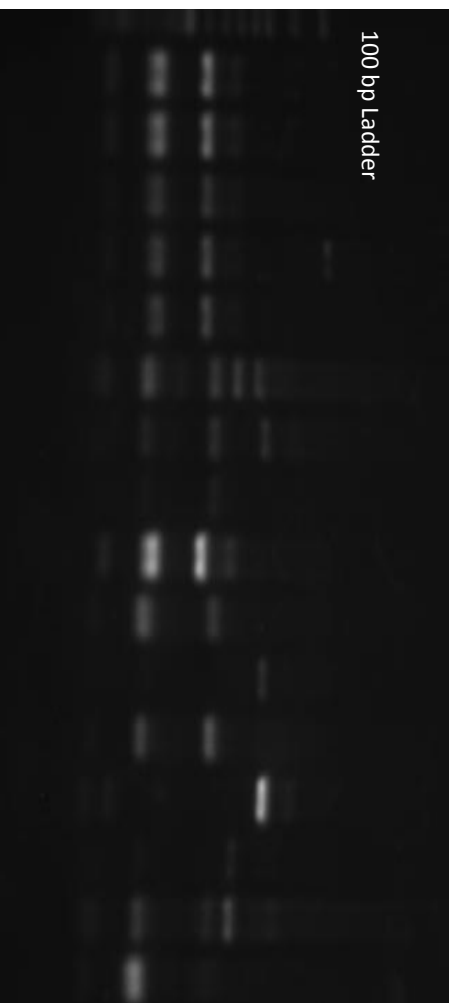
(i)



(ii)



(iii)



Bacillus sonorensis. Strains which clustered closely with genus *Cosenzaea*, *Plesiomonas*, *Providencia* and *Tatumella* were identified as *Cosenzaea myxofaciens*, *Plesiomonas shigelloides*, *Providencia rettgeri* and *Tatumella citrea* respectively.

Table 6. Phylogenetic relatedness of bacterial isolates from healthy apple orchards based on BLASTn analysis

Phylotype	Isolates	Taxonomic Classification	Closest Relative	Per cent similarity BLASTn	Accession number
1	<i>Cosenzaea myxofaciens</i> strain M(1)	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; <i>Cosenzaea</i>	<i>Cosenzaea myxofaciens</i> NR_043999.1	94	KU879059
2	<i>Bacillus subtilis</i> strain N(3)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> strain SBMP4 NR_118383	89	KU879060
3	<i>Bacillus aerius</i> strain Cj(4)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus aerius</i> NR_042338	90	KU879061
4	<i>Bacillus soli</i> strain R(1)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus soli</i> NR_114095	85	KU879062
5	<i>Bacillus subtilis</i> strain J(6)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> strain NCDO 1769NR_118972	99	KU879063
6	<i>Bacillus aerius</i> strain J(1)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus aerius</i> NR_118439	89	KU879064
7	<i>Bacillus aerius</i> strain C(6)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus aerius</i> NR_042338	89	KU879065
8	<i>Plesiomonas shigelloides</i> strain Kot(8)	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; <i>Plesiomonas</i>	<i>Plesiomonas shigelloides</i> NR_044827	89	KU879066
9	<i>Bacillus anthracis</i> strain R(2)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i> <i>Bacillus cereus</i> group.	<i>Bacillus anthracis</i> NR_118536	87	KU879067
10	<i>Bacillus subtilis</i> strain M(8)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> strain SBMP4 NR_118383	95	KU879068
11	<i>Bacillus sonorensis</i> strain K(2)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus sonorensis</i> strain NRRL B-23154NR_025130	99	KU879069
12	<i>Providencia rettgeri</i> strain Cj(3)	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; <i>Providencia</i>	<i>Providencia rettgeri</i> NR_042413	90	KU879070
13	<i>Bacillus subtilis</i> strain T3	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> NR_118383	99	KU879071
14.	<i>Bacillus subtilis</i> strain R(5)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> strain SBMP4NR_118383	99	KU879072
15.	<i>Tatumella citrea</i> strain J(5)	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; <i>Tatumella</i>	<i>Tatumella citrea</i> NR_116799	97	KU879073

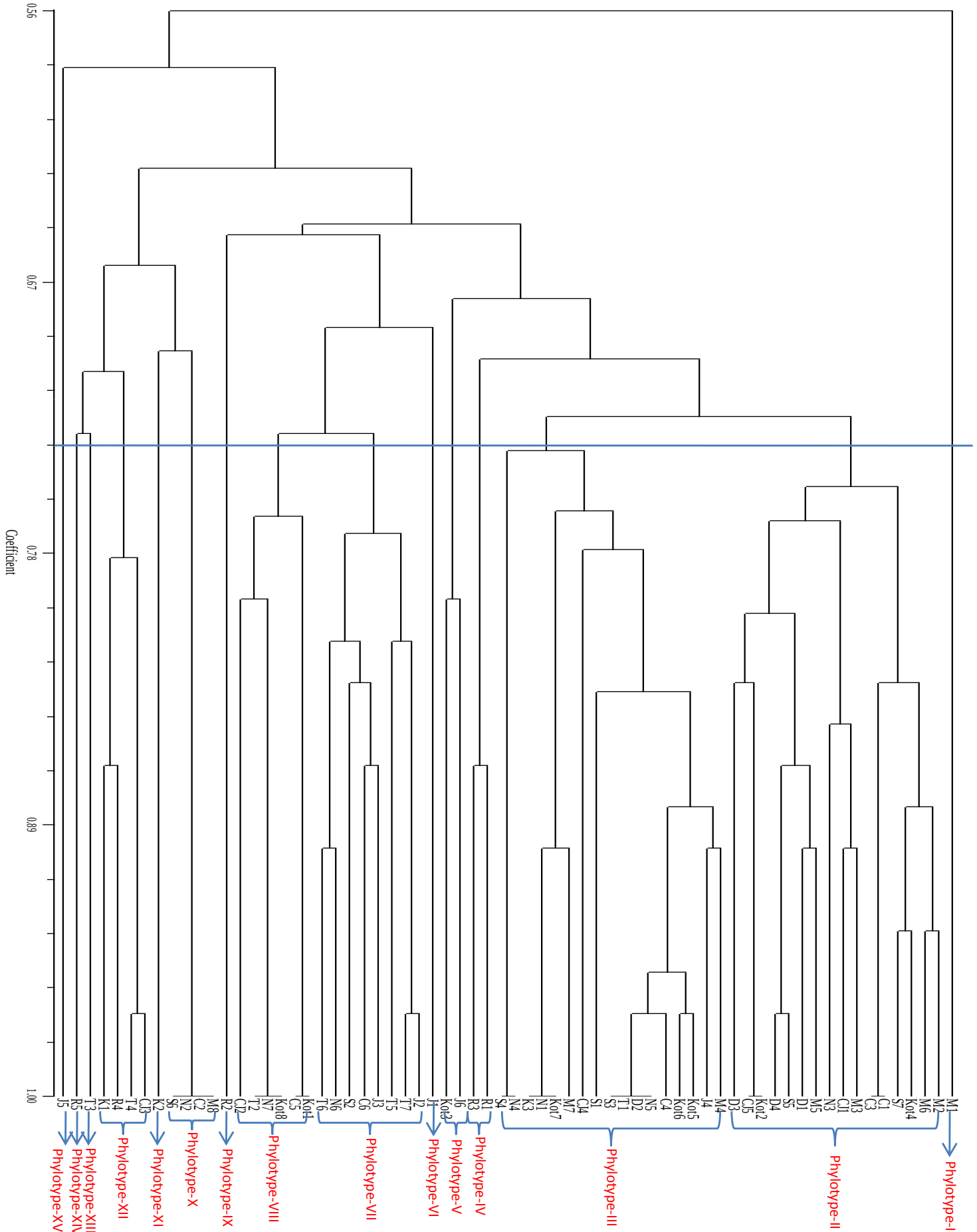
Table 7. Phylogenetic relatedness of bacterial isolates from root rot infected diseased apple orchards based on BLASTn analysis

Phylotype	Isolates	Taxonomic Classification	Closest Relative	Per cent similarity BLASTn	Accssion number
1	<i>Bacillus subtilis</i> strain KD(3)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> NR_118383	97	KU878080
2	<i>Pantoea ananatis</i> strain ND(4)	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; <i>Pantoea</i>	<i>Pantoea ananatis</i> LMG 20103 NR_103927	98	KU878081
3	<i>Bacillus stratosphericus</i> strain KND(3)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus stratosphericus</i> NR_042336	99	KU878082
4	<i>Bacillus subtilis</i> strain KotD(4)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> NR_118383	99	KU878083
5.	<i>Leminorella grimontii</i> strain JD(3)	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; <i>Leminorella</i>	<i>Leminorella grimontii</i> NR_025333.1	98	KU878084
6.	<i>Morganella psychrotolerans</i> strain TD(1)	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; <i>Morganella</i>	<i>Morganella psychrotolerans</i> NR_043750.1	93	KU878085
7.	<i>Bacillus axarquiensis</i> strain DD(7)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus axarquiensis</i> NR_115283	98	KU878086
8.	<i>Bacillus axarquiensis</i> strain ND(3)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus axarquiensis</i> NR_115283	99	KU878087

Phylogenetic studies confirmed the affiliation of the representative bacterial isolates from root rot infected diseased apple orchards into *Bacillus*, *Pantoea*, *Leminorella* and *Morganella* revealed by clustering of each genus to its corresponding group. Phylogenetic tree revealed that out of total eight, five strains were clustered closely with different species of genus *Bacillus* with high boot strap values. The identified bacteria were *Bacillus subtilis*, *Bacillus stratosphericus* and *Bacillus axarquiensis*. Strains which clustered closely with genus *Pantoea*, *Leminorella* and *Morganella* were identified as *Pantoea ananatis*, *Leminorella grimontii* and *Morganella psychrotolerans* respectively. Based on their PGP traits listed in table (2, 3) these novel isolates might be the potential inoculants as a plant growth promoter.

The results indicated that the bacteria belonging to family *Bacillaceae* were amongst the most abundant in the rhizosphere of the apple trees at healthy as well as at root rot

Figure 12. Dendrogram showing ARDRA based genetic diversity analysis of rhizobacterial population isolated from healthy apple orchards



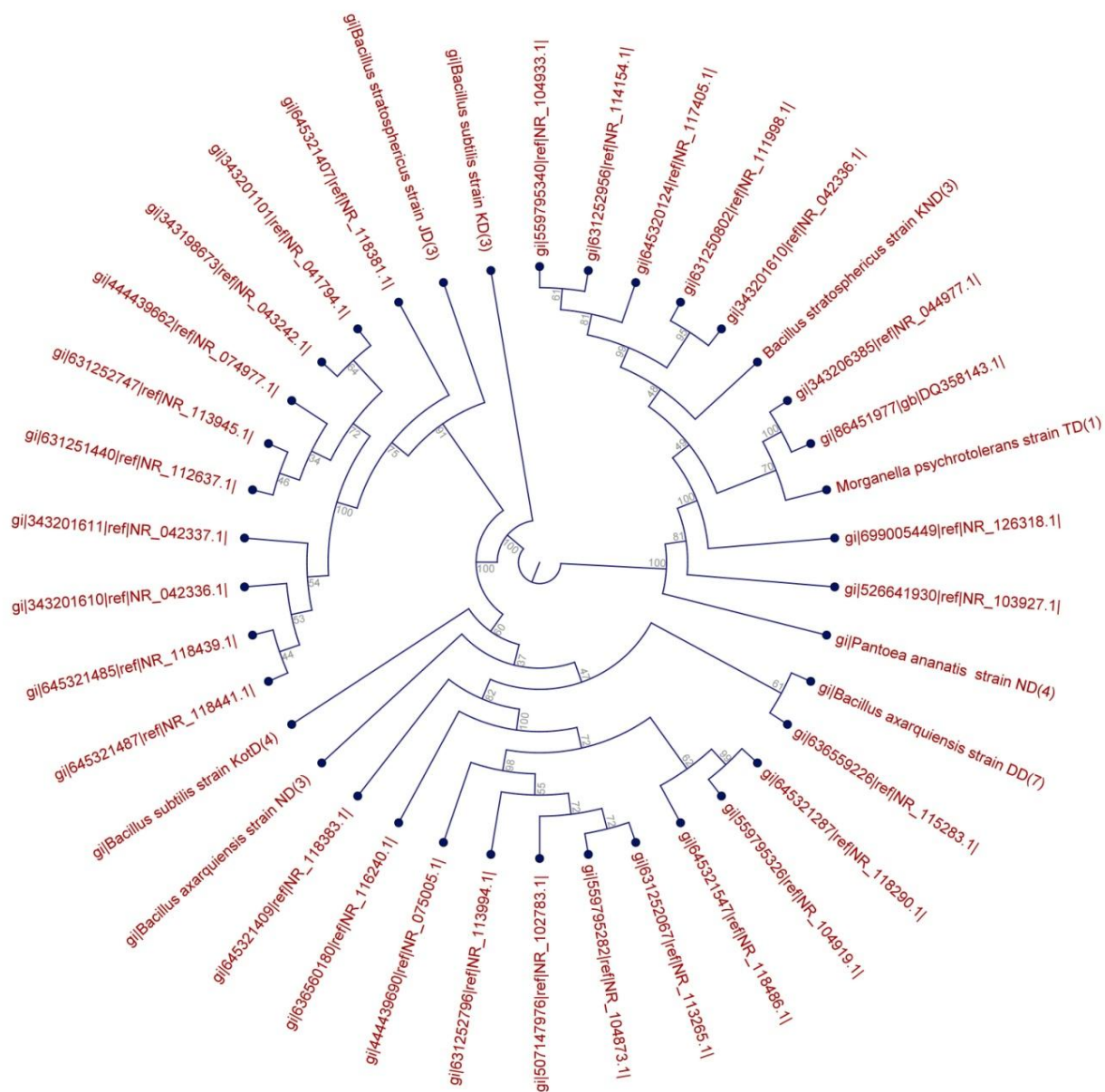


Figure 15 Circular phylogram depicting phylogenetic relatedness of 16S rRNA gene sequences of bacterial isolates from root rot infected diseased orchards

infected diseased apple orchards followed by the bacteria belonging to family Enterobacteriaceae. The dominance of endospore forming bacilli in the rhizosphere of apple trees with high plant growth promoting traits (Table 2, 3) confirms their potential as PGPR. The dominance of genus *Bacillus* corroborates with previous study regarding the bacterial isolates dominating the rhizosphere of apple trees (Guleria *et al.* 2014; Mehta *et al.* 2015). Bacteria belonging to the Enterobacteriaceae family were the second abundant in association with the roots of apple trees. Strains belonging to the Enterobacteriaceae family have also been found to be associated with rhizosphere of a large number of plant species, such as citrus (Araujo *et al.*, 2001), maize (Hinton and Bacon, 1995; McInroy and Kloepper, 1995), rice (Engelhard *et al.* 2000; Gyaneshwar *et al.* 2001; Rosenblueth *et al.* 2004; Verma *et al.* 2004), sweet potato (Asis and Adachi, 2003; Reiter *et al.* 2003), soybean (Kuklinsky *et al.* 2004), and wheat (Iniguez *et al.* 2005). Based on the results of this study, a distinct variation in microbial activity in terms of P-solubilization, siderophore production, IAA production and high antagonistic activity against fungal pathogens was observed in healthy and root rot infected diseased apple soil. It is likely that much greater sampling efforts will be needed at different intervals over a period of time to further explore the ongoing community shift.

4.5 CULTURABLE RHIZOBACTERIAL POPULATION ASSOCIATED WITH APPLE TREES GROWN AT REPLANT AND NEW PLANTATION SITES OF HIMACHAL PRADESH

Exploring microbial and plant diversity from unusual and previously unexplored habitats offers new prospects for bio prospecting which might be exploited for biotechnological purposes (Tamilarasi *et al.* 2008). The apple replant problem has been attributed to various abiotic factors, including pH highs and lows, phytotoxins, poor soil nutrition/structure, heavy metal contamination and cold or drought stress, however, the majority of evidence indicates that the disease is primarily a result of biotic factors (Mazzola, 1998). Studies using soil fumigation (Browne *et al.* 2006; Mai and Abawi, 1981; Spath *et al.* 2015; Yim *et al.* 2013), soil pasteurization and soil sterilization (Jaffee, 1982; Manici *et al.* 2013; Yim *et al.* 2013) showed significantly higher growth of apple trees in fumigated and pasteurized soils when compared to untreated soils.

The plant microbiome can be considered an extension of the host genome, as almost all tissues in plants host diverse microbial communities (Turner *et al.* 2013). As such, the plant microbiome serves as a key determinant of plant health and productivity, and manipulation of the microbiome has the potential to reduce the incidence of plant disease,

thus resulting in higher yields. Recent studies also highlight the ability of plant associated microbiomes to influence plant traits including disease resistance, growth and abiotic stress tolerance (Mendes *et al.* 2011; Panke-Buisse *et al.* 2015). Despite its complexity and dynamism, the plant microbiome should be considered increasingly more in the future, while interpreting the experimental data (Turner *et al.* 2013).

4.5.1 Culturable rhizospheric population associated with replant sites of apple at different locations

Study on culturable communities investigates a partial collection of rhizosphere, which is consequently an appropriate approach to study the whole system and plate count populations in order to provide quantitative yields and specific information on diversity (Torsvik *et al.* 1990; Palomino *et al.* 2005).

The population level of total culturable bacteria, per cent P-solubilizers, per cent siderophore producers, per cent population growing on N-free medium obtained from rhizospheric soil collected from replant sites of apple at different sampling sites is presented in Fig 16(A), 17(A), 18(A) and 19(A), respectively. Rhizospheric soil of replant sites of apple accounted for total of 32.65 per cent P-solubilizers, 9.39 per cent siderophore producers and 26.38 per cent of the population was able to grow on nitrogen free medium.

The results revealed that the total viable population from soil samples of location RHRS, Mashobra was detected by using serial dilution method of isolation on nutrient agar medium that accounted for 70.57×10^5 cfu/g of culturable bacteria. Culturable bacterial population harbouring P-solubilizing bacteria accounted for 28.63 per cent on Pikovskaya's medium and 14.31 per cent siderophore producers on CAS medium and 34.71 per cent culturable bacterial population was able to grow on nitrogen free medium.

Total culturable bacterial population present in the apple rhizosphere soil sample of location Jatog comprised of 77.43×10^5 cfu g⁻¹ on the nutrient agar medium. Of the total population obtained, 35.55 per cent were P-solubilizing, 7.87 per cent were siderophore producers and only 19.15 per cent was able to grow on nitrogen free medium.

Total number of culturable bacteria recovered from rhizospheric soil of location Dhanta, resulted in 82.57×10^5 cfu g⁻¹, out of which 29.18 per cent were P-solubilizers, 6.78

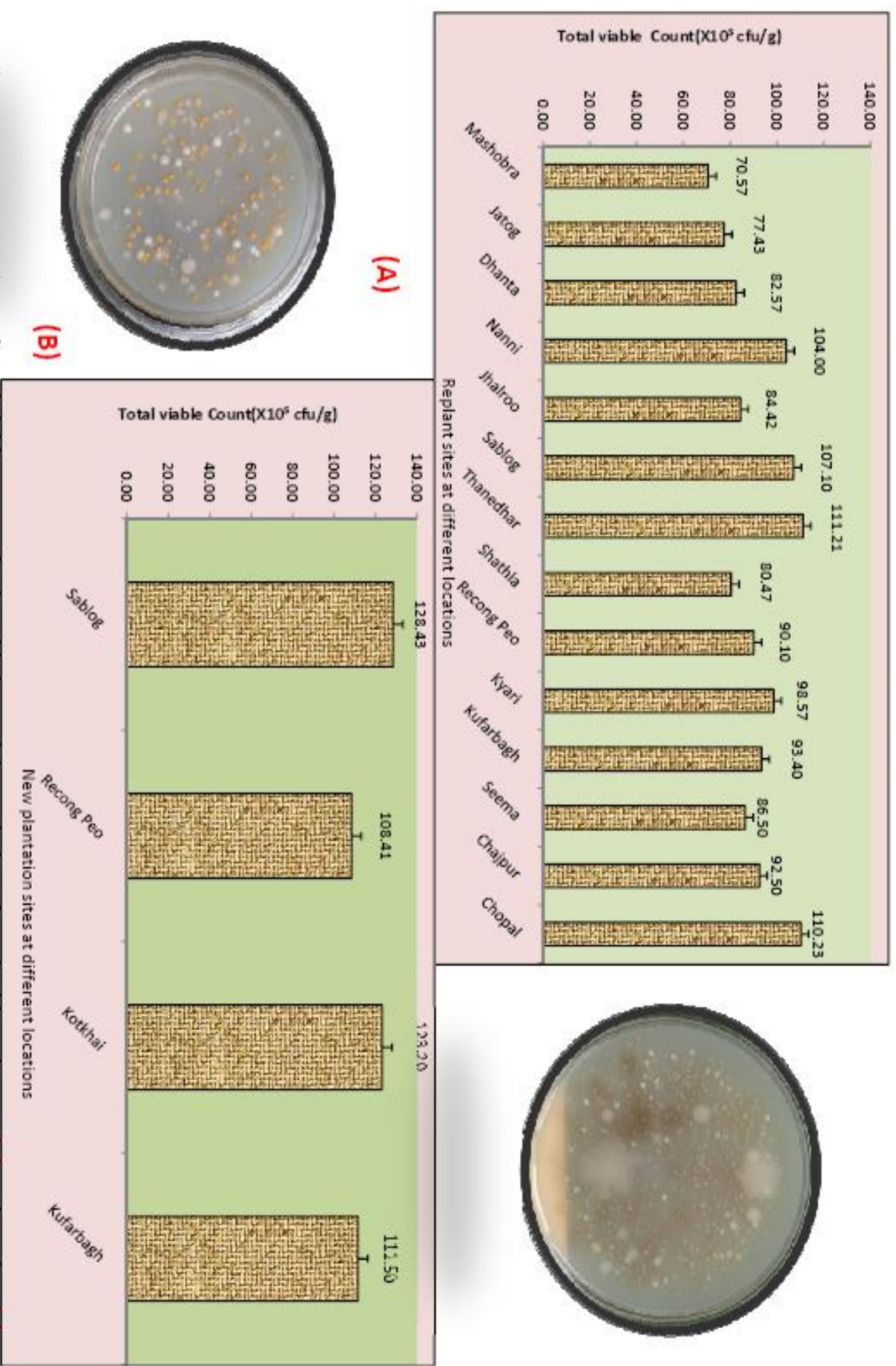
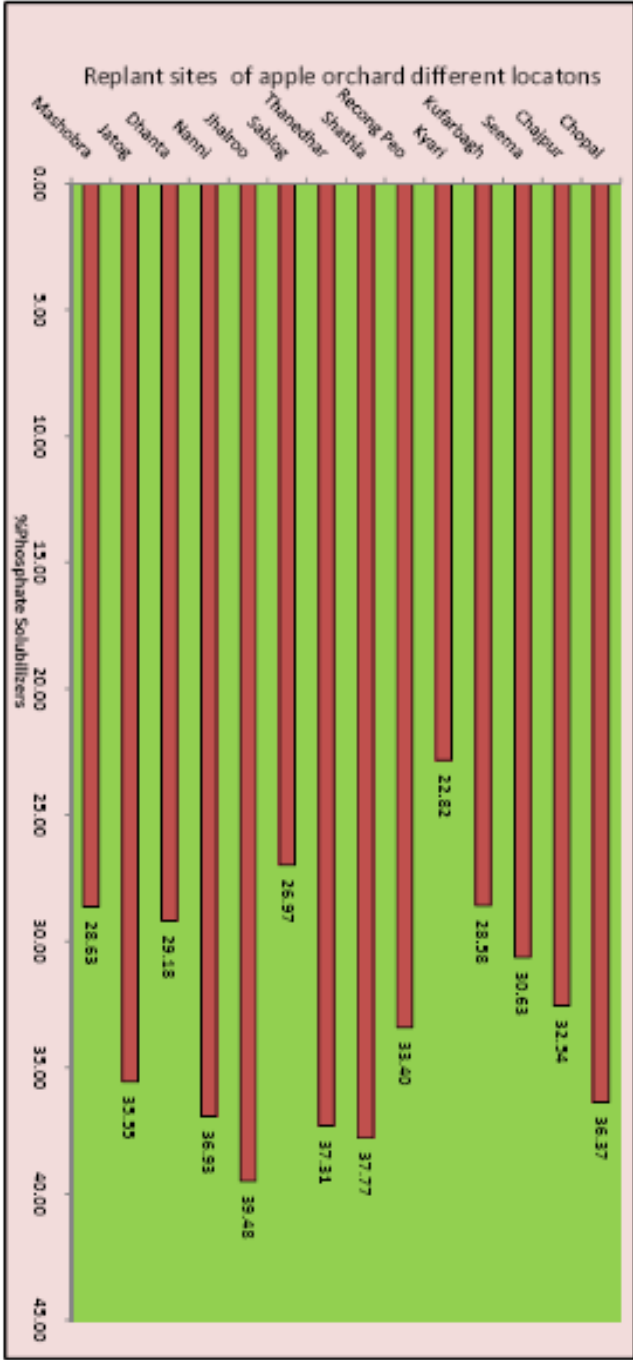


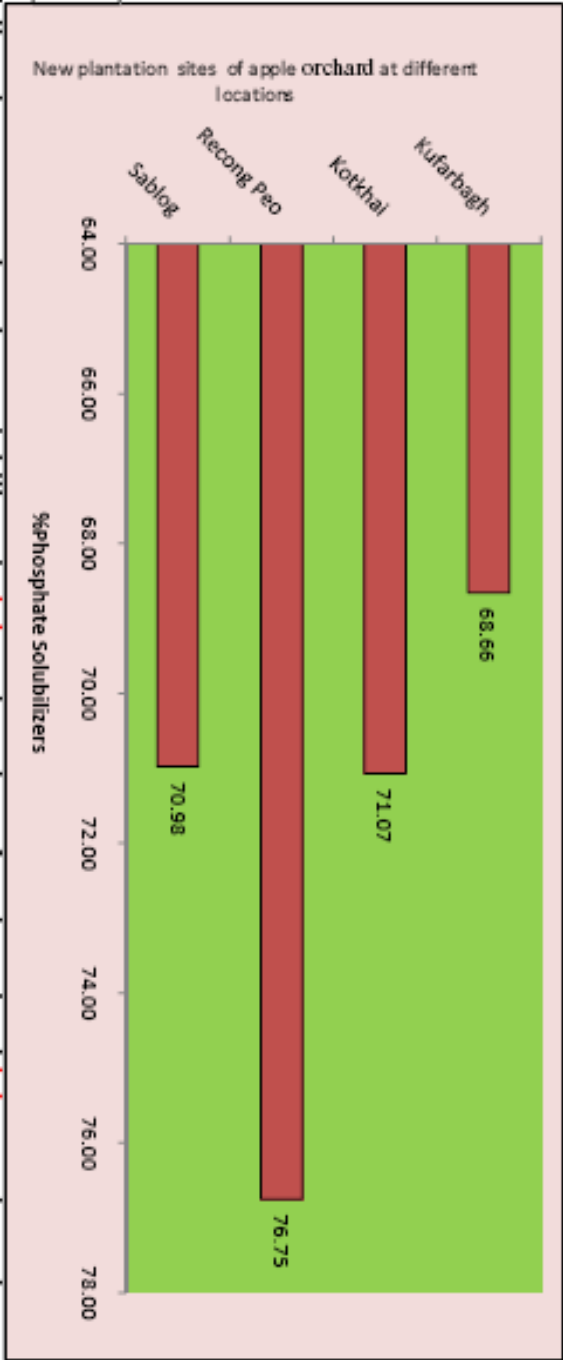
Figure 16. A comparison of total viable count of culturable bacterial population of apple rhizosphere at (A) replant sites (B) new plantation sites of apple orchard at different locations



Average P- solubilizers at replant location =32.65 per cent

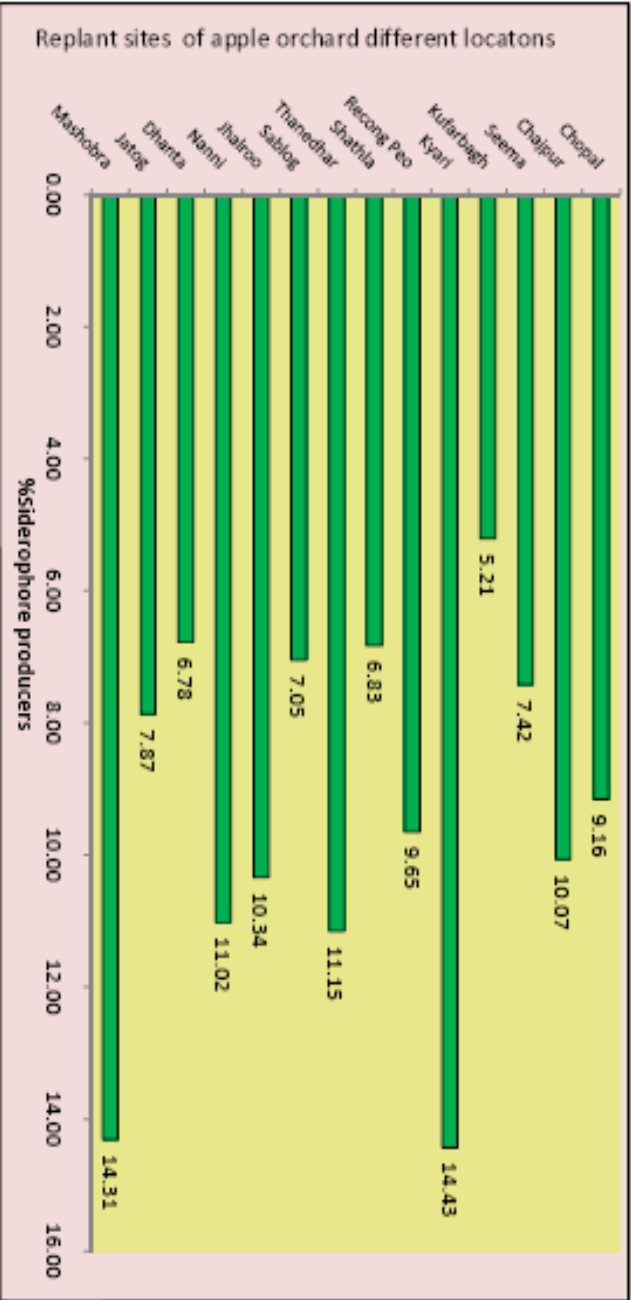


(B)



Average P- solubilizers at New plantation location =72.43 per cent

Figure 17. A comparison of diversity among phosphate solubilizers in (A) replant sites of apple orchard (B) new plantation apple orchard at different locations



Average Siderophore producers at replant =9.39 per cent

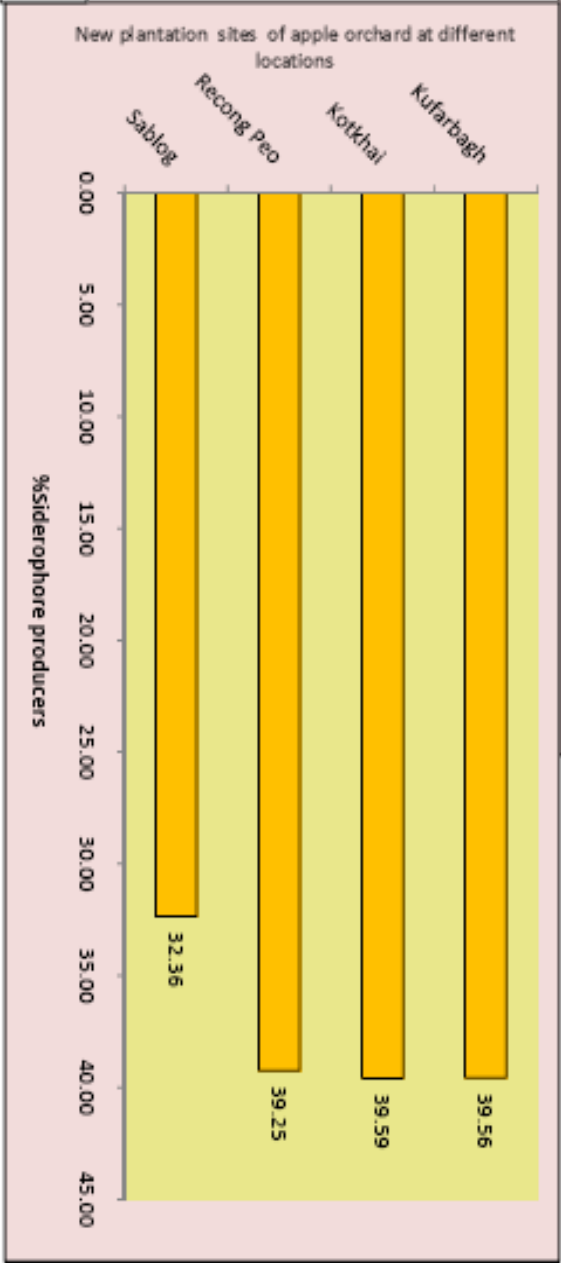
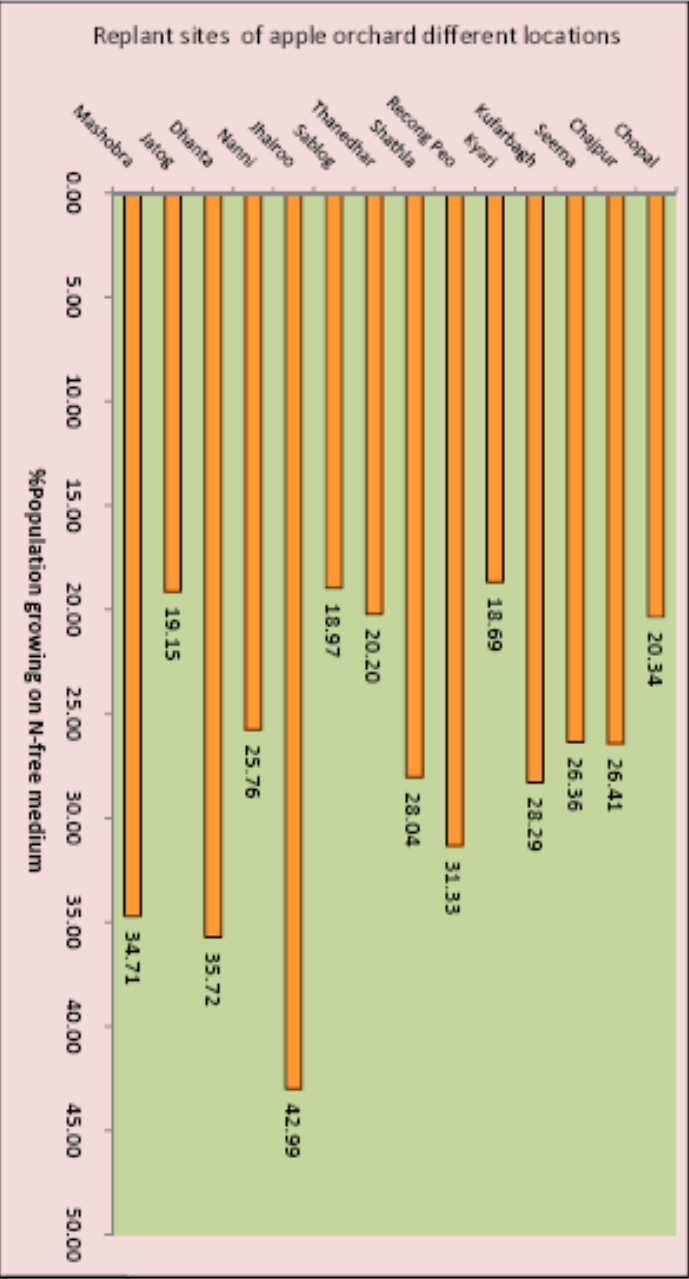
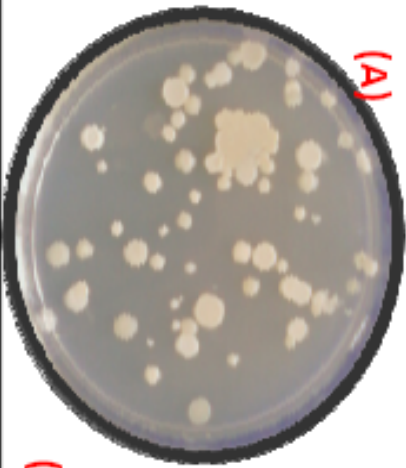


Figure 18. A comparison of diversity among siderophore producers in (A) replant sites of apple orchard (B) new plantation apple orchard at different locations

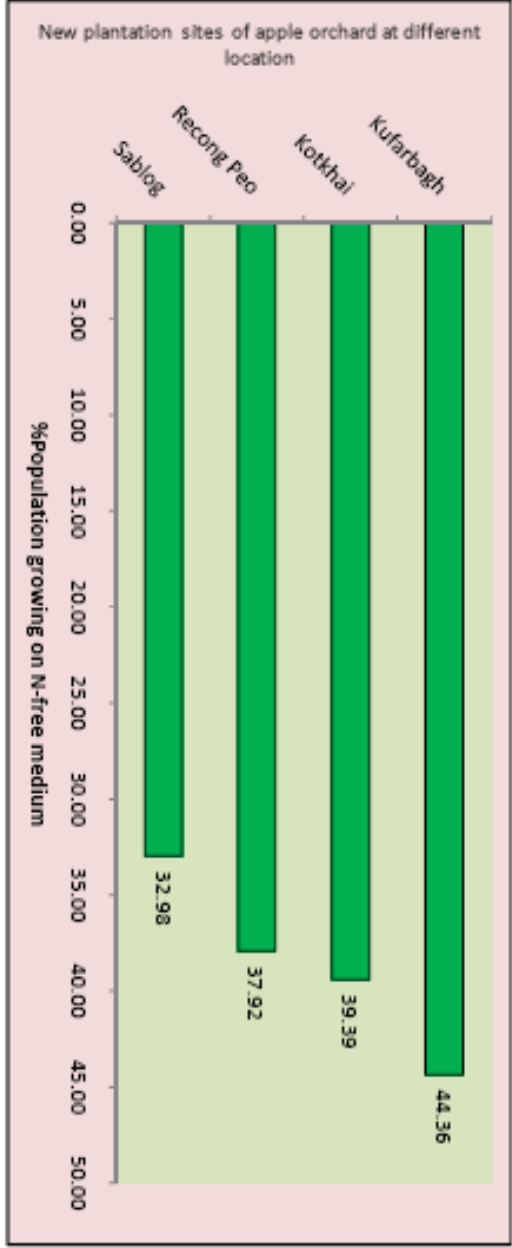


Average Population growing on N-free medium at replant location =26.38 per cent



(A)

Average Population growing at N-free medium at New plantation Location location = 38.48 per cent



(B)

Figure 19. A comparison of diversity among per cent population growing on nitrogen free medium in (A) replant sites of apple orchard (B) new plantation apple orchard at different locations

per cent were siderophore producers and 35.12 per cent of the population was able to grow on nitrogen free medium.

The total viable bacterial population recovered from rhizosphere soil of location Nanni resulted in 104.00×10^5 cfu g⁻¹. The soil samples showed the presence of 36.93 per cent P-solubilizers, 11.02 per cent siderophore producers and 25.76 per cent bacterial population was able to grow on nitrogen free medium.

Location Jhalroo accounted for the total of 84.42×10^5 cfu g⁻¹ of culturable bacteria, out of which 39.48 per cent were P-solubilizers, 10.34 per cent were siderophore producers and 42.99 per cent of the population was able to grow on nitrogen free medium.

Total number of culturable bacteria recovered from rhizospheric soil of Sablog, resulted in 107.10 cfu g⁻¹, out of which 26.97 per cent were P-solubilizers, 7.05 per cent were siderophore producers and 18.97 per cent of the population were able to grow on nitrogen free medium.

The total viable bacterial population recovered from rhizosphere soil of Thanedhar resulted in 111.21 cfu g⁻¹. The soil samples showed the presence of 37.31 per cent P-solubilizers, 11.15 per cent siderophore producers and 20.20 per cent bacteria able to grow on N free medium.

Total culturable bacterial population present in the apple rhizosphere soil sample of Shathla contain 80.47×10^5 cfu g⁻¹ of soil on the nutrient agar medium. Of the total population obtained, 37.77 per cent were P-solubilizers, 6.83 per cent were siderophore producers and 6.83 per cent were able to grow on nitrogen free medium.

Total number of culturable bacteria recovered from rhizospheric soil of Recong Peo, resulted in 90.10×10^5 cfu g⁻¹, out of which 33.40 per cent were P-solubilizers, 9.65 per cent were siderophore producers and 31.33 per cent of the population was able to grow on nitrogen free medium.

The total viable bacterial population recovered from rhizosphere soil of Kyari resulted in 98.57 cfu g⁻¹. The soil samples showed the presence of 22.87 per cent P-solubilizers, 14.43 per cent siderophore producers and 18.43 per cent bacteria was able to grow on nitrogen free medium.

Total culturable bacterial population present in the apple rhizosphere soil sample of Kufarbagh contain 93.40×10^5 cfu g⁻¹ on the nutrient agar medium. Of the total population obtained, 28.58 per cent were P-solubilizing, 5.21 per cent were siderophore producers and 28.29 per cent were able to grow on nitrogen free medium.

Total number of culturable bacteria recovered from rhizospheric soil of Rohru, resulted in 86.50×10^5 cfu g⁻¹, out of which 30.63 per cent were P-solubilizers, 7.42 per cent were siderophore producers and 26.36 per cent of the population was able to grow on nitrogen free medium.

Total culturable bacterial population present in the apple rhizosphere soil sample of Chajpur contain 92.50×10^5 cfu g⁻¹ on the nutrient agar medium. Of the total population obtained, 32.54 per cent were P-solubilizing, 10.07 per cent were siderophore producers and 26.41 per cent was able to grow on nitrogen free medium.

Total number of culturable bacteria recovered from rhizospheric soil of Chopal, resulted in 110.23 cfu g⁻¹, out of which 36.37 per cent were P-solubilizers, 9.16 per cent were siderophore producers and 20.34 per cent of the population was able to grow on nitrogen free medium.

It has been observed that in most of the apple orchards with replant sites the population of P-solubilizers were remarkably higher as compare to siderophore producers, the findings is in agreement with the study of P-solubilizers from conventional and organic orchards (Wieland *et al.* 2001). The higher population of P-solubilizers might be due to the reason that physico-chemical properties of soil of conventional orchards directly affected by management practice adopted by orchardist.

4.5.2 Culturable rhizospheric population associated with at new planting sites of apple at different locations

The population level of total culturable bacteria, per cent P-solubilizers, per cent siderophore producers and per cent population growing on N-free medium obtained from rhizospheric soil collected from replant sites at different location is presented in Fig 16(B), 17(B), 18(B) and 19(B), respectively.

The results revealed that the total viable bacterial population from soil samples of location Sablog was detected on nutrient agar medium that accounted for 86.13×10^5 cfu/g of

culturable bacteria. Culturable bacterial population harbouring 70.98 per cent of P-solubilizers and 32.36 per cent siderophore producers on CAS medium and 32.98 per cent culturable bacterial population was able to grow on nitrogen free medium.

Total culturable bacterial population dominating the apple rhizosphere soil sample of location Recong Peo harboured of 81.6×10^5 cfu g⁻¹ of soil on the nutrient agar medium. Of the total population obtained, 76.75 per cent were P-solubilizing, 39.25 per cent were siderophore producers on CAS medium and 37.92 per cent population was able to grow on nitrogen free medium.

Total number of culturable bacteria recovered from rhizospheric soil collected from apple orchard at location Kyari, resulted in 78.54×10^5 cfu g⁻¹ of culturable bacteria on nutrient agar, out of which 71.07 per cent were P-solubilizers, 39.59 per cent were siderophore producers and 39.39 per cent of the population was able to grow on nitrogen free medium.

The total viable bacterial population recovered from rhizosphere soil of Kufarbagh resulted in 77.53×10^5 cfu g⁻¹. The soil samples showed the presence of 68.66 per cent were P-solubilizers, 39.56 per cent were siderophore producers and 44.36 per cent were able to grow on nitrogen free medium.

Similarly Guo *et al.* 2014 reported higher population of rhizobacteria in case of 14 year old trees of different locations. The quantities of nitrogen fixing bacteria, phosphate solubilizing bacteria in the rhizosphere of 14 year old apple trees were higher than those of 3 year old apple trees under continuous cropping.

Microbial community in the plant rhizosphere soil is highly dynamic. Plant rhizosphere relies on relative abundance of the microbial population that are dependent on the plant species, soil types and environmental conditions. Among several bacteria which are prevalent in the rhizosphere region of plants, plant growth promoting rhizobacteria are most common soil bacteria that can be easily cultured from most of the agricultural soils and rhizosphere. They have been extensively used in plant growth promotion by either directly stimulating the plant growth via nutrient acquisition or by suppressing the soil-borne pathogens due to the antifungal metabolite production.

Plant root exudates play key roles in the mediation of plant-microbe interactions in the rhizosphere (Badri *et al.* 2008, 2009; Chaparro *et al.* 2013). The composition of plant root exudates is determined by plant species, cultivar, developmental stage, and numerous environmental factors including soil type, temperature, humidity, pH, and nutrient availability (Rovira, 1965; Brimecombe *et al.* 2001; Bais *et al.* 2006; Nicholas, 2007). Root exudates are also responsible for biological control by eliciting microbial biofilm formation on the root surfaces which is now widely recognized as a form of biological control of plant pathogens by weakening pathogens competition for nutrients and space (Bais *et al.* 2004; Chen *et al.* 2012).

The presence of large population of bacterial isolates in all the rhizospheric samples may be due to positive influence exerted by root exudates in microbial colonization. Overall the results revealed that more rhizospheric bacterial population was obtained from soil samples associated apple trees in healthy and new plantation sites. Similar to the findings presented in here, Passos *et al.* (2014) also carried out similar studies on plant growth promoting rhizobacteria associated with apple trees cultivated under different crop management system. They observed that more phosphate solubilizers were conventional soil than organic soil. The native field condition presented a reduced number of phosphate solubilizers but larger number of siderophore producers. This may be because bacterial communities residing in the rhizosphere respond, in particular, with respect to density, composition, and activity, to the plethora and diversity of organic root exudates, resulting in plant species-specific microflora which may eventually vary in stage of plant growth (Wieland *et al.* 2001).

4.5.2 A comparative overview of culturable rhizobacterial population associated with apple trees in new plantation and replant sites of apple orchards

A comparison of functional traits among rhizobacterial populations from new plantation and replant sites of apple orchards revealed significant variation, as is evident from the data appended in figures 17, 18 and 19. Phosphate solublizers at both new plantation and replant sites of apple orchards accounted for maximum contribution towards the total rhizobacterial population, which was closely followed by putative N-fixers and siderophore producers. However, detailed comparison revealed that among the P-solublizing rhizobacteria, at new plantation sites of apple orchards, an overall average of 72.43 per cent were P-solubilizers, whereas only 32.65 per cent were observed at replant sites of apple

orchards. A similar trend was observed in case of siderophore producers and putative N-fixers. Siderophore producers at new plantation sites accounted for an average of 37.54 per cent, whereas in replant sites their average contribution was 9.39 per cent. An average contribution of rhizobacterial population growing on nitrogen free medium at new plantation sites was 38.48 per cent, whereas an average of 26.38 per cent were observed at replant sites of apple orchards.

Soil-borne microorganisms are considered to be an important component of soil function, being involved in the release of soil mineral nutrients and plant disease resistance (Zhang *et al.* 2013). Fungi of the genera *Cylindrocarpon* and *Rhizoctonia*, as well as the oomycetes *Phytophthora* and *Pythium* are considered to be essential in the etiology of apple replant disease, and these pathogens have been found frequently at different replant disease sites (Tewoldemedhin *et al.* 2011; Kelderer *et al.* 2012). Several *Fusarium* species have also been reported to be associated with ARD, although the role of the genus as a pathogen is controversial (Tewoldemedhin *et al.* 2011).

The role of bacteria in ARD has been considerably less investigated than the role of fungi. Nonetheless, bacteria belonging to the Actinomycetes, as well as to the genera *Bacillus* and *Pseudomonas* have been implicated (Mazzola *et al.* 2002; Mazzola and Manici, 2012; Utkhede *et al.* 1992). Certain species of *Streptomyces*, members of the Actinobacteria, have been reported to alleviate *Rhizoctonia* root infections in apple (Zhao *et al.* 2009). Similarly, different *Pseudomonas* species have been shown to be important in both causing and suppressing ARD (Mazzola *et al.* 2002). According to numerous authors however, the role of bacteria in ARD is debatable (Dullahide *et al.* 1994; Mazzola 1998; Tewoldemedhin *et al.* 2011).

The results obtained in the present work, especially the findings relating to lower population of P-solubilizers, putative nitrogen fixers and siderophore producers could be associated to the fact that in the soil of apple orchard with replant sites, there is low amount of available nutrients and organic matter, resulting in a less favorable environment to the growth of plants and for many species of microorganisms. Compared with new planting sites, where the soil is rich in nutrients the possibility of obtaining higher nutrient mobilizes in rhizosphere of new planted apple plants is high, as is also evident from the findings in the present study. Soil microorganisms are recognized as the key factor associated with apple

replant sites, but it is a challenging task to fully characterize soil microbial communities (Sun *et al.* 2014). The findings in the present study are therefore of much significance as they provide a comparative overview of the functional diversity of culturable rhizobacteria isolated from new plantation and replant sites.

4.6 SELECTION AND SCREENING OF REPRESENTATIVE RHIZOBACTERIA FROM REPLANT AND NEW PLANTATION SITES OF APPLE ORCHARDS FOR MULTIFARIOUS PLANT GROWTH PROMOTING TRAITS

A total of 104 and 16 bacterial isolates were obtained from replant and new plantation sites of apple at different location respectively, with atleast one of the plant growth promoting traits i.e. phosphate solubilization, siderophore production and N- fixation were selected and screened for other multifarious plant growth promoting activities (Table 8 and 10; Plate 1). All the bacterial isolates exhibited variation in production of different plant growth promoting traits viz. IAA production, HCN production, P-solubilization, and antifungal activity against *Phytophthora* sp., *Pythium aphanidermatum*, *Dematophora necatrix* and *Fusarium oxysporum* and *Sclerotium rolfsii* (Table 10 and 11; Plate 1).

4.6.1 Plant growth promoting traits of culturable bacterial isolates from replant and new plantation sites of apple orchards at different locations

One hundred four and sixteen rhizospheric isolates were obtained from replant site and new plantation sites of apple orchards respectively, at different locations of Himachal Pradesh. They were screened for multifarious plant growth promoting traits and the results revealed that all the culturable bacterial isolates showed variation in their ability to produce IAA, HCN, siderophore, solubilize phosphate and antifungal activity against different fungal pathogens (Table 8 and 9).

4.6.1.1 Tricalcium phosphate solubilization by representative bacterial isolates from replant and new plantation sites of apple orchards in Pikovskaya's broth medium

The phosphate solubilizing activity of the selected bacterial isolates was compared on the basis of their P- solubilization ($\mu\text{g/ml}$) in PVK broth. The results of P-solubilization for isolates obtained from replant sites and new plantation sites of apple orchards showed significant variation as revealed by quantitative estimation methods (Table 8 and 9).

4.6.1.1.1 Tricalcium phosphate solubilization by representative bacterial isolates from replant sites of apple orchards

Introduction of plant growth promoting rhizobacteria (PGPR) including phosphate solubilizing bacteria as biofertilizer is suggested as a sustainable option for the improvement of nutrient availability, plant growth and yield (Vessey, 2003). The search for diverse PGPR is gaining serious attention and therefore, efforts are being made to exploit the biofertilizers for various economically important crops. In the present study, among the different locations, selection of bacteria was made based upon their PGP traits. One of the most important criteria for selection of isolates, was their ability to solubilise tricalcium phosphate.

Among the nine isolates of location RHRS, Mashobra, maximum P- solubilization was observed for isolate MR(8) (34.60 µg/ml) followed by MR(9) (21.31 µg/ml). However, no P-solubilization was observed for isolate MR(2), MR(3) and MR(5).

Of the eight isolates obtained from location Dhanta, maximum P- solubilization was observed for isolate DR(4) (67.00 µg/ml). The isolate DR(8) solubilised minimum TCP with release of 23.80 µg/ml phosphorous. However, no P-solubilization was observed for isolate DR(1).

Among the twelve isolates of the location Jatog, maximum P- solubilization (48.50 µg/ml) was observed for isolate JatR(4) followed by S(4) (48.00 µg/ml). The isolate JatR(12) solubilised minimum TCP with release of 11.00 µg/ml phosphorous. Isolate JatR(6) accounted for no P-solubilization.

Location Jhalroo resulted in selection of six isolates and maximum P-solubilization (26.31 µg/ml) was observed for isolate JR(4) followed by JR(1) (21.00 µg/ml) and minimum TCP solubilization (11.50 µg/ml) was observed for isolate JR(6).

Of the eight isolates obtained from location Nanni, maximum P- solubilization was observed for isolate NR(7) (70.30 µg/ml) followed by NR(5) (66.70 µg/ml). However, no P-solubilization was observed for isolate NR(1).

Among the eight isolates of the location Sablog, maximum P- solubilization (68.32 µg/ml) was observed for isolate SR(2) followed by SR(5) (52.57 µg/ml). The isolate SR(6) solubilised minimum TCP with release of 8.80 µg/ml phosphorous.

Table 8: Screening of culturable bacterial isolates from replant sites of apple orchards for multifarious plant growth promoting activities

Location	Isolates	Quantitative P-estimation (µg/ml)****	Quantitative siderophore estimation (Per cent siderophore unit)**	Growth on Nitrogen free medium	HCN Production ^y	IAA Production (µg/ml)
RHRS, Mashobra	MR(1)	13.57	0.00	-	+	6.00
	MR(2)	0.00	0.00	+	-	8.00
	MR(3)	0.00	0.00	+	-	11.00
	MR(4)	9.20	18.80	-	-	9.00
	MR(5)	0.00	0.00	+	+	17.00
	MR(6)	19.10	14.50	+	-	3.00
	MR(7)	2.30	33.00	+	-	24.00
	MR(8)	34.60	0.00	-	-	0.00
	MR(9)	21.31	19.73	+	-	0.80
Dhanta	DR(1)	0.00	0.00	-	+	11.00
	DR(2)	29.00	4.58	-	+	19.30
	DR(3)	58.40	0.00	+	-	0.00
	DR(4)	67.00	40.50	+	-	23.40
	DR(5)	53.00	0.00	-	+	27.00
	DR(6)	34.50	21.00	+	-	29.00
	DR(7)	31.50	0.00	+	+	33.00
	DR(8)	23.80	33.47	-	-	24.00
Jatog	JatR(1)	24.50	33.47	-	+	8.00
	JatR(2)	21.50	0.00	-	-	7.50
	JatR(3)	18.97	55.20	-	+	0.00
	JatR(4)	48.50	0.00	+	+	23.00
	JatR(5)	13.80	41.20	+	-	0.00
	JatR(6)	0.00	0.00	+	+	4.00
	JatR(7)	40.70	33.47	-	+	18.00
	JatR(8)	20.00	41.84	-	-	7.00
	JatR(9)	16.00	0.00	-	-	13.00
	JatR(10)	16.70	0.00	+	-	9.00
	JatR(11)	20.00	10.46	+	-	0.00
	JatR(12)	11.00	9.23	-	-	14.00
Jhalroo	JR(1)	21.00	43.00	-	+	5.00
	JR(2)	18.80	28.00	-	+	11.00
	JR(3)	12.50	0.00	+	-	7.00
	JR(4)	26.31	0.00	+	-	13.00
	JR(5)	18.00	26.50	+	-	4.00
	JR(6)	11.50	21.00	-	-	0.00
Nanni	NR(1)	0.00	0.00	-	-	14.00
	NR(2)	42.43	0.00	-	+	8.50
	NR(3)	56.41	0.00	-	-	11.30
	NR(4)	40.50	47.50	+	-	27.00
	NR(5)	66.70	53.80	+	+	0.00
	NR(6)	24.30	49.00	-	+	20.50
	NR(7)	70.30	66.60	-	+	12.00
	NR(8)	40.00	63.00	-	-	10.53
Sablog	SR(1)	21.57	54.39	-	-	4.00
	SR(2)	68.32	0.00	+	-	11.00
	SR(3)	14.87	29.28	-	-	0.00
	SR(4)	26.60	25.10	-	+	17.00
	SR(5)	52.57	43.30	+	+	20.00
	SR(6)	8.80	22.45	-	+	6.00
	SR(7)	33.97	0.00	-	-	9.30
	SR(8)	22.41	18.65	-	+	0.00
Thanedhar	TR(1)	27.00	33.47	-	-	0.00
	TR(2)	29.50	0.00	-	-	19.00
	TR(3)	31.00	37.66	+	-	21.00
	TR(4)	18.80	54.39	+	+	9.00
	TR(5)	43.00	29.50	+	-	7.00
	TR(6)	51.00	33.44	-	+	5.30
	TR(7)	36.00	30.20	-	+	6.00

Location	Isolates	Quantitative P-estimation (µg/ml)****	Quantitative estimation siderophore (Per cent siderophore unit)**	Growth on Nitrogen free medium	HCN Production ^y	IAA Production (µg/ml)
Shathla	ShR(1)	56.00	15.73	+	-	20.00
	ShR(2)	43.00	74.00	+	-	14.00
	ShR(3)	15.00	0.00	-	+	0.00
	ShR(4)	14.00	14.30	-	+	0.00
	ShR(5)	11.57	0.00	-	-	6.70
	ShR(6)	8.70	18.00	-	-	12.50
	ShR(7)	14.50	42.37	+	-	11.00
	ShR(8)	45.30	38.90	+	-	10.00
	ShR(9)	25.73	24.00	-	-	21.00
Reckong Peo	KR(1)	13.80	0.00	+	+	6.00
	KR(2)	5.30	14.00	-	-	8.00
	KR(3)	21.50	0.00	-	-	9.00
	KR(4)	16.00	0.00	-	-	0.00
	KR(5)	9.20	0.00	+	+	4.00
	KR(6)	4.60	29.00	-	-	3.00
	KR(7)	20.70	33.47	-	-	0.00
	KR(8)	36.50	22.56	+	+	15.50
Kyari	KyR(1)	18.50	0.00	-	-	16.00
	KyR(2)	26.60	16.00	+	-	3.00
	KyR(3)	41.20	38.50	+	-	0.00
	KyR(4)	48.87	0.00	+	+	23.00
Kufarbagh	KuR(1)	22.45	55.43	+	+	4.00
	KuR(2)	44.30	14.71	-	-	14.50
	KuR(3)	36.50	0.00	-	-	7.00
	KuR(4)	44.00	0.00	+	-	19.30
Seema	RR(1)	4.60	54.40	+	-	22.00
	RR(2)	30.00	29.20	-	-	18.50
	RR(3)	9.20	58.50	+	-	16.00
	RR(4)	36.00	4.10	-	+	5.00
	RR(5)	40.70	0.00	-	+	0.00
	RR(6)	18.50	11.20	-	+	6.00
	RR(7)	21.50	0.00	+	-	3.00
	RR(8)	47.60	9.00	-	-	9.50
Chajpur	CjR(1)	0.00	0.00	+	-	0.00
	CjR(2)	28.80	25.10	-	+	8.50
	CjR(3)	44.50	0.00	-	-	23.00
	CjR(4)	63.50	50.20	-	+	19.00
	CjR(5)	40.00	20.90	+	+	34.00
Chopal	CR(1)	40.70	44.50	+	+	8.00
	CR(2)	30.00	41.00	+	-	0.00
	CR(3)	54.50	0.00	-	-	19.30
	CR(4)	67.60	0.00	-	-	0.00
	CR(5)	8.30	55.00	-	+	0.00
	CR(6)	13.80	29.00	+	-	10.00
	CR(7)	16.00	0.00	+	-	20.50
	CR(8)	5.30	0.00	-	+	0.00

%Siderophore Unit = Where, Ar=Absorbance of Reference at 630 nm As=Absorbance of test at 630 nm * P- solubilized = T-C; Where, T: P-solubilized in test, C: P-solubilized in control ; ^yHCN : - No colour change of the filter paper from yellow to brown; + change in colour of filter paper from yellow to orange brown

In liquid PVK medium, out of seven isolates of location Thanedhar, maximum P-solubilization was observed for bacterial isolate TR(6) (51.00 µg/ml) followed by isolate TR(5) (43.00 µg/ml) and minimum TCP solubilization (27.00 µg/ml) was observed for isolate TR(1).

In liquid PVK medium, maximum P-solubilization was observed for bacterial isolate ShR(1) (56.00 µg/ml) followed by isolate ShR(8) (45.30µg/ml) of location Shathla. The isolate ShR(6) solubilised minimum TCP with release of 8.70 µg/ml phosphorous.

Location Reckong Peo resulted in selection of eight isolates and maximum P-solubilization (36.50 µg/ml) was observed for isolate KR(8) and isolate KR(6) solubilised minimum TCP with release of 4.60 µg/ml phosphorous

Of the four isolates obtained from location Kyari, maximum P- solubilization was observed for isolate KyR(4) (48.87 µg/ml) followed by KyR(3) (41.20 µg/ml).

Location Kufarbagh resulted in selection of four isolates and maximum P-solubilization (44.30 µg/ml) was observed for isolate KuR(2) and minimum TCP solubilization ((22.45 µg/ml) was observed for isolate KuR(1).

In liquid PVK medium,out of eight isolates maximum P-solubilization was observed for bacterial isolate RR(8) (47.60 µg/ml) followed by isolate RR(5) (40.70µg/ml) of location Seema. The isolate RR(3) solubilised minimum TCP with release of 9.20 µg/ml phosphorous.

Among the five isolates of the location Chajpur, maximum P- solubilization (63.50µg/ml) was observed for isolate CjR(4). However, no P-solubilization was observed for isolate CjR(1).

Location Chopal resulted in selection of eight isolates and maximum P-solubilization (67.60 µg/ml) was observed for isolate CR(4) followed by CR(3) (54.50 µg/ml) and minimum TCP solubilization (5.300 µg/ml) was observed for isolate CR(8).

P-solubilization was observed in almost all the isolates of replant sites of apple orchards at different locations. It is estimated that phosphate-solubilizing micro-organisms constitute 20–40% of the culturable population of soil micro-organisms of which significant proportion of these bacteria can be isolated from rhizosphere soil (Chabot *et al.* 1993). Most phosphate solubilizers isolated from the rhizosphere of various plants are known to be metabolically more active than those isolated from sources other than rhizosphere, as has also been observed in P-solubilizers isolated from rhizosphere of apple trees at replant sites.

4.6.1.1.2 Tricalcium phosphate solubilization by representative bacterial isolates from new plantation sites of apple orchards

Location Sablog resulted in selection of four isolates and maximum P-solubilization (65.00µg/ml) was observed for isolate SF(3) and minimum TCP solubilization (28.00 µg/ml) was observed for isolate SF(1).

Of the two isolates obtained from location Reckong Peo, maximum P- solubilization was observed for isolate KF(1) (67.50 µg/ml).

Table 9: Screening of culturable bacterial isolates from fresh plantation sites in apple orchards for multifarious plant growth promoting activities

Location	Isolates	Quantitative P-estimation (µg/ml)****	Quantitative estimation siderophore (Per cent siderophore unit)**	Growth on Nitrogen free medium	HCN Production ‡	IAA Production (µg/ml)
Sablog	SF(1)	28.00	37.65	+	-	19.00
	SF(2)	30.00	25.10	+	++	40.00
	SF(3)	65.00	0.00	-	-	21.00
	SF(4)	63.80	54.60	-	-	16.00
Reckong Peo	KF(1)	67.50	18.80	+	++	33.00
	KF(2)	43.20	48.40	-	-	17.00
Kyari	KyF(1)	61.50	48.50	-	+	31.00
	KyF(2)	50.00	CI	+	++	23.00
	KyF(3)	77.60	0.00	+	-	18.00
	KyF(4)	67.00	0.00	-	-	14.00
	KyF(5)	67.70	50.25	-	+	41.00
	KyF(6)	58.40	0.00	+	+	34.00
Kufarbagh	KuF(1)	27.00	21.80	-	++	0.00
	KuF(2)	80.00	93.00	-	++	26.50
	KuF(3)	89.00	0.00	+	-	31.00
	KuF(4)	68.00	47.00	+	+	19.00

%Siderophore Unit = $\frac{A_r}{A_t} \times 100$ Where, A_r =Absorbance of Reference at 630 nm A_t =Absorbance of test at 630 nm * P- solubilized = T-C; Where, T: P-solubilized in test, C: P-solubilized in control ; ‡HCN : - No colour change of the filter paper from yellow to brown; + change in colour of filter paper from yellow to orange brown

Among the six isolates of the location Kyari, maximum P- solubilization (77.60 µg/ml) was observed for isolate KyF(3) followed by KyF(5) (67.70 µg/ml). The isolate KyF(2) solubilised minimum TCP with release of 50.00 µg/ml phosphorous.

In liquid PVK medium, out of four isolates of location Kufarbagh, maximum P- solubilization was observed for bacterial isolate KuF(3) (89.00 µg/ml) and minimum TCP solubilization (27.00 µg/ml) was observed for isolate KuF(1).

Phosphate tends to react with calcium (Ca), iron (Fe), or aluminum (Al) leading to its precipitation making it unavailable for plant uptake. Inorganic phosphate in acidic soils is associated with Al and Fe compounds, whereas calcium phosphates are found in calcareous

soils in the form of inorganic phosphates. Organic phosphate makes up a large fraction of soluble phosphate which is about 50% in soils with high organic matter (Barber, 1995). Hexaphosphate salt of inositol, so-called phytate, is the major form of phosphate in organic form which constitute up to 80% of the total organic phosphate (Alexander, 1977). Although micro-organisms are known to produce phytases that can hydrolyze phytate, however phytate tends to form insoluble complexes with Fe, Al, and Ca and accumulates in soils (Alexander, 1977; Jha *et al.* 2012). The finding in the present study, highlight the fact that efficient population of phosphate solubilizers were present at new plantation sites. Occurrence of much efficient P-solubilizers thereby indicate that rhizobacteria in the presence of root exudates from newly planted apple trees were able to efficiently mobilize the insoluble phosphorus.

4.6.1.2 Quantitative estimation of siderophore production by selected bacterial isolates from replant site and new plantation sites of apple orchards at different location

Another important trait of PGPR is the production of siderophore. Siderophore is one of the biocontrol mechanisms belonging to PGPR groups, under iron limiting condition. PGPR produces a range of siderophore which have a very high affinity for iron. Therefore, the low availability of iron in the environment would suppress the growth of pathogenic organisms including plant pathogenic fungi (Whipps, 2000). The siderophore production of bacterial isolates were compared on the basis of percent siderophore unit (%SU) on nutrient broth amended with CAS dye (Table 8 and 9; Plate 1). Large variation was observed during the quantitative estimation for siderophore production for isolates obtained from replant and new plantation sites of apple orchards.

4.6.1.2.1 Quantitative estimation of siderophore production representative bacterial isolates from replant sites of apple orchards

Among the nine isolates obtained from replant sites of apple orchards at location RHRS, Mashobra, maximum %SU (33.00%) was obtained for isolate MR(7) followed by MR(9) (19.73%) and minimum (14.50%) for isolate MR(5). However, no siderophore production was observed for isolate MR(1), MR(3), MR(2), MR(5) and M(8).

Location Dhanta resulted in selection of eight isolates and maximum % SU (40.50%) was observed for isolate DR(4), minimum % SU (4.58%) for isolate DR(2). Isolates DR(1), DR(3), DR(5) and DR(7) were not able to produce siderophore.

Among the twelve isolates of the location Jatog, maximum %SU (55.20%) was observed for isolate JatR(3) followed by JatR(8) (41.84%). The isolate JatR(12) produces minimum siderophore with %SU of 9.23% and isolates JatR(2), JatR(4), JatR(6), JatR(9) and JatR(10) were not able to produce siderophore.

Location Jhalroo resulted in selection of total six isolates, out of which isolate JR(1) resulted in maximum %SU (43.00%) followed by JR(2) (28.00%) and minimum %SU (21.00%) was observed for isolate JR(6). However, two isolates JR(3) and JR(4) were not able to produce siderophore.

Of the eight isolates obtained from location Nanni, maximum %SU was observed for isolate NR(7) (66.60 %) followed by NR(8) (63.00%). However, no siderophore production was observed for three isolate NR(1), NR(2) and NR(3).

Among the eight isolates of the location Sablog, maximum %SU (54.39%) was observed for isolate SR(1) followed by SR(5) (43.30%). The isolate SR(8) produced minimum siderophore with %SU of 18.65% and isolate sSR(2) and SR(7) were not able to produce siderophore.

Of the seven isolates obtained from location Thanedhar, maximum % SU (54.39%) was observed for TR(4) and minimum % SU(29.50%) for isolate TR(5). Isolate T(2) was not able to produce siderophore.

Among the nine isolates of the location Shathla, maximum % SU (74.00%) was observed for isolate ShR(2) followed by ShR(7) (42.37%). The isolate ShR(4) produced minimum Siderophore with %SU of 14.30 % and no siderophore production was observed in two isolates ShR(3) and ShR(5).

Location Reckong Peo resulted in selection of eight isolates and maximum % SU (33.47%) was observed for isolate KR(7). However, four isolates KR(1), KR(3), KR(4) and KR(5) were not able to produce siderophore.

Of the four isolates obtained from location Kyari, maximum % SU was observed for isolate KyR(3) (38.50%). However, two isolates KyR(1), and KyR(4) were not able to produce siderophore.

Among the four isolates of the location Kufarbagh, maximum % SU (55.43%) was observed for isolate KuR(1) and no siderophore production was observed in isolates KuR(3) and KuR(4) respectively.

In liquid nutrient broth medium, out of eight isolates of location Seema, maximum siderophore production was observed for bacterial isolate RR(3) (58.50%) followed by isolate RR(1) (54.40%). Two isolates RR(5) and RR(7) resulted in no siderophore production.

Among the five isolates of the location Chajpur, maximum % SU (50.20%) was observed for isolate CjR(4) followed by CjR(2) (25.10%). and no siderophore production was observed in isolate CjR(1) and CjR(3).

Location Chopal resulted in selection of eight isolates and maximum % SU (55.00%) was observed for isolate CR(5) and minimum % SU (29.00%) was observed for isolate CR(6). However four isolates CR(3), CR(4), CR(7) and C(8) were not able to produce siderophore.

Rhizosphere bacteria release siderophore to increase their competitive potential, since these substances have an antagonistic activity and improve iron nutrition for the plant (Glick, 1995). Siderophore-producing rhizobacteria improve plant health at various levels: they improve iron nutrition, inhibit the growth of other micro-organisms with the release of their antibiotic molecule, and hinder the growth of pathogens by limiting the iron available for the pathogen, generally fungi, which are unable to absorb the iron–siderophore complex (Kang *et al.* 2013). Findings in the present study, indicate significantly lower population of siderophore producers at replant sites which may be attributed to the presence of several fungal pathogens as has been reported by researchers while reporting the etiology of ARD (Kelderer *et al.* 2012 ;Tewoldemedhin *et al.* 2011).

4.6.1.2.2 Quantitative estimation of siderophore production representative bacterial isolates from new plantation sites of apple orchards

Of the four isolates obtained from location Sablog, maximum %SU was observed for isolate SF(4) (54.60%). However, no siderophore production was observed for isolate SF(3).

Among the two isolates of the location Recong Peo, maximum %SU (48.40%) was observed for isolate KF(2).

Of the six isolates obtained from location Kyari maximum % SU (50.25%) was observed for KyF(5). Three isolates KyF(3), KyF(4) and KyF(6) were not able to produce Siderophore.

Location Kufarbagh resulted in selection of four isolates and maximum % SU (93.00%) was observed for isolate KuF(2) and isolate KuF(4) produces minimum siderophore with %SU of 47.00%.

Passos *et al.* (2014) reported that in the soil of the conventional orchard there are low amount of available nutrients and organic matter resulting in a less favorable environment to the growth of many plants and for many species of microorganisms. This situation could favor the selection of microorganisms more adapted to this limiting condition, such as, for example, those able to produce siderophores, which bind to the available form of iron (Fe^{3+}) in the rhizosphere, thus making it unavailable to the phytopathogens and protecting plant health. Finding in the present work are in concomitance to the above cited results of Passos *et al.* (2014) as efficient siderophore producers were observed at new plantation sites where the nutrients in the soil are in unavailable form because of the lack of any earlier plantation and thereby an absence of rhizospheric effect.

4.6.1.3 IAA production by representative bacterial isolates from replantation sites and new plantation sites of apple

Biosynthesis of IAA is not limited to higher plants. Organisms such as bacteria are able to make physiologically active IAA that may have pronounced effects on plant growth and development. The selected bacterial isolates showed variation in production of IAA.

Like plants, L-tryptophan is also considered as the IAA precursor in bacteria, because its addition to IAA producing bacterial cultures promotes and increases IAA synthesis (Tsavkelova *et al.* 2007). Root exudates are natural source of L-tryptophan for rhizosphere microflora, which may enhance auxin biosynthesis in the rhizosphere. Production of IAA in the presence of a suitable precursor such as tryptophan has been reported for several PGPR belonging to the genera *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Pantoea*, *Pseudomonas*, and *Serratia*. 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 (Bhatt and Vyas, 2014).

Salkowski reaction of culture supernatants of selected bacterial isolates grown in Luria bertani broth showed that 79.80 per cent isolates from replantation site of apple orchards were observed as their ability to produce IAA while 20.19 per cent lack the ability of IAA production from tryptophan. The amount of IAA produced by large number of isolates corresponds to earlier reports on higher IAA production (Fischer *et al.* 2007; Tahir *et al.* 2015).

The maximum IAA production (34.00 µg/ml) was observed in case of isolate CjR(5) of healthy apple of Chajpur followed by DR(6) (29.00 µg/ml) from location Dhanta. No IAA production was observed in twenty one isolates from replant sites of apple at different location viz MR(8) from RHRS, Mashobra, DR(3) from Dhanta, JatR(3), JatR(5), JatR(11) from Jatog, JR(6) from Jhalroo, NR(5) from Nanni, NR(4) from Nanni, SR(3) and SR(8) from Sablog, TR(1) from Thanedhar, ShR(3) and ShR(4) from Shathla, KR(4) and KR(7) from Recong Peo, KyR(3) from Kyari, RR(5) from Rohru, CjR(1) from Chajpur, CR(2), CR(4), CR(5) and CR(8) from Chopal respectively (Table 8 and 9). IAA production by these isolates was under *in vitro* conditions. It has been suggested that IAA production under invitro conditions depends on the particular bacterial strain or the condition of culture such as oxygen and pH (Radwan *et al.* 2013).

All the collected samples of soil from new plantation sites of apple were evaluated for the status of IAA producing bacterial population. The variation among PGPR to produce IAA found in the present study had also been reported earlier (Zahir *et al.* 2000, Abbassi *et al.* 2011). There was large variation in IAA producing bacterial population among all the locations. Salkowski reaction of culture supernatants of selected bacterial isolates grown in Luria bertani broth showed that 93.97 per cent isolates from new plantation site of apple orchards were observed as their ability to produce IAA while 6.25 per cent lack the ability of IAA production from tryptophan. The highest IAA production by the isolates was observed in rhizosphere soil of Sablog SF(2) (40.00 µg/ml) followed by isolate of Kyari KyF(6) (34.00 µg/ml). No IAA production was observed in isolate KuF(1) of fresh plantation site of apple at location Kufarbagh. This variation is attributed to the various biosynthetic pathways, location of gene involved, regulator sequences and the presence of enzyme to convert active free IAA into conjugated forms (Patten and Glick, 1996). Higher level of IAA has been recorded for different genera like *Bacillus*, *Pseudomonas* etc. (Ahmad *et al.* 2005; Verma *et al.* 2015).

4.6.1.4 Antifungal activity of representative culturable bacterial isolates against different fungal pathogens from replant and new plantation sites of apple orchards

Table 10 and 11 revealed that antifungal activity of selected culturable bacterial isolates from replantation sites and new plantation sites of apple against fungal pathogen viz. *Phytophthora sp.*, *Fusarium oxysporum*, *Dematophora necatrix*, *Sclerotium rolfsii*, *Pythium aphanidermatum*. Selected bacterial isolates exhibited variation in antifungal activity against all the fungal pathogens (Plate 1).

Among the one hundred four isolates of replantation sites of apple at different location (Table 10), thirty five (33.65%) of them were able to inhibit the growth of *D. necatrix*, nine isolates (8.65%) were able to inhibit *Phytophthora sp.*, twenty nine (27.88%) isolates showed inhibition against *F.oxysporum*, twenty seven (25.96%) of them were able to inhibit the growth of *S. rolfsii* and twenty eight (26.92%) showed antagonistic activity against *P.aphanidermatum*. Maximum percent growth inhibition was exhibited by isolate ShR(8) (51.25%) of Shathla and RR(3)(51.25%) of Seema, CjR(1) (48.75%) of Chajpur, TR(3) (51.25%) of Thanedhar, ShR(8) (48.50%) of Shathla and TR(1) (48.50%) of Thanedhar against *D.necatrix*, *F. oxysporum*, *S. rolfsii*, *P. aphanidermatum*, respectively.

Of the sixteen isolates obtained from new plantation sites of apple (Table 11), nine isolates (56.25%) showed inhibition against *D.necatrix*, two isolates (12.50%) were able to inhibit the growth of *Phytophthora sp.*, ten (62.50%) isolates against *F.oxysporum*, eight isolates (50.00%) against *S.rolfsii*, twelve isolates (75.00%) against *P.aphanidermatum*. Maximum percent growth inhibition was exhibited by isolates KuF(2) (68.50%), KyF(2) (51.00%), KyF(3) (48.75%) against *D.necatrix*, *S.rolfsii*, and *F.oxysporum* respectively.

Soil bacteria living in the rhizosphere can enhance plant growth by several mechanisms like antagonism against plant pathogens, solubilization of phosphates (De Freitas *et al.* 1997). These results are in agreement with earlier report that plant growth promoting rhizobacteria produce antifungal substances with activity against a number of mycelia fungi (Sultana *et al.* 2010; Cazorla *et al.* 2007). This effect is caused by different antifungal metabolites including siderophores, organic acid, IAA and antifungal antibiotics in the cell pellets (Sadfi *et al.* 2002).

Table 10: Screening of culturable bacterial isolates from replant sites of apple orchard for antagonistic activity against different fungal pathogens

Location	Isolates	Antagonistic activity against fungal pathogens (% Growth inhibition)**				
		<i>Dematophora necatrix</i>	<i>Phytophthora sp.</i>	<i>Fusarium oxysporum</i>	<i>Sclerotium rolfsii</i>	<i>Pythium aphanidermatum</i>
RHRS, Mashobra	MR(1)	CI	-	CI	46.00	-
	MR(2)	-	-	-	-	-
	MR(3)	-	-	-	-	-
	MR(4)	-	-	-	-	-
	MR(5)	CI	-	-	CI	CI
	MR(6)	45.00	CI	CI	-	-
	MR(7)	-	-	-	-	-
	MR(8)	-	-	-	-	-
	MR(9)	-	CI	CI	-	CI
Dhanta	DR(1)	CI	-	-	CI	CI
	DR(2)	-	-	CI	-	-
	DR(3)	-	-	-	CI	-
	DR(4)	-	-	-	48.50	CI
	DR(5)	47.50	-	48.00	-	-
	DR(6)	-	-	-	-	-
	DR(7)	-	-	-	-	-
	DR(8)	-	-	-	-	-
Jatog	JatR(1)	-	-	-	-	-
	JatR(2)	-	-	-	-	-
	JatR(3)	CI	-	CI	-	-
	JatR(4)	-	-	CI	-	CI
	JatR(5)	-	-	48.70	-	CI
	JatR(6)	47.00	-	-	-	-
	JatR(7)	-	-	-	CI	-
	JatR(8)	CI	CI	-	-	-
	JatR(9)	CI	-	-	-	-
	JatR(10)	-	-	CI	CI	CI
	JatR(11)	-	-	-	-	-
	JatR(12)	-	-	-	-	-
Jhalroo	JR(1)	-	-	-	-	-
	JR(2)	-	-	-	CI	-
	JR(3)	-	-	-	-	-
	JR(4)	-	-	CI	-	CI
	JR(5)	-	-	-	-	-
	JR(6)	CI	-	-	-	-
Nanni	NR(1)	-	-	-	-	-
	NR(2)	-	-	-	-	CI
	NR(3)	CI	-	48.75	46.00	-
	NR(4)	CI	CI	-	-	-
	NR(5)	-	-	-	-	-
	NR(6)	-	-	CI	-	-
	NR(7)	46.00	-	CI	-	-
	NR(8)	CI	-	-	CI	47.50
Sablog	SR(1)	-	-	-	-	-
	SR(2)	-	-	CI	-	-
	SR(3)	CI	-	-	CI	-
	SR(4)	CI	-	-	-	-
	SR(5)	CI	-	CI	-	CI
	SR(6)	-	-	-	CI	-
	SR(7)	-	-	-	CI	-
	SR(8)	-	-	-	-	48.50
Thanedhar	TR(1)	-	-	-	-	-
	TR(2)	-	-	CI	-	48.50
	TR(3)	-	-	-	51.25	-
	TR(4)	CI	-	-	-	-
	TR(5)	-	-	48.50	-	CI
	TR(6)	-	-	-	-	-
	TR(7)	47.50	CI	-	-	-

Location	Isolates	Antagonistic activity against fungal pathogens (% Growth inhibition)**				
		<i>Dematophora necatrix</i>	<i>Phytophthora sp.</i>	<i>Fusarium oxysporum</i>	<i>Sclerotium rolfsii</i>	<i>Pythium aphanidermatum</i>
Shathla	ShR(1)	-	-	-	CI	-
	ShR(2)	-	-	CI	CI	-
	ShR(3)	-	-	CI	-	-
	ShR(4)	CI	-	CI	-	-
	ShR(5)	CI	-	-	-	CI
	ShR(6)	-	-	-	-	-
	ShR(7)	-	-	-	CI	CI
	ShR(8)	51.25	-	CI	CI	CI
	ShR(9)	-	-	CI	-	CI
Reckong Peo	KR(1)	CI	-	CI	-	47.50
	KR(2)	CI	-	46.50	-	CI
	KR(3)	-	-	-	-	-
	KR(4)	-	-	-	-	-
	KR(5)	-	-	CI	CI	-
	KR(6)	CI	-	-	-	CI
	KR(7)	46.00	-	CI	CI	-
	KR(8)	-	CI	CI	-	CI
Kyari	KyR(1)	51.20	-	CI	-	-
	KyR(2)	-	-	-	-	-
	KyR(3)	-	-	-	-	-
	KyR(4)	-	CI	CI	-	-
Kufarbagh	KuR(1)	-	-	CI	-	CI
	KuR(2)	-	-	-	-	47.50
	KuR(3)	-	-	-	-	-
	KuR(4)	-	-	-	-	-
Seema	RR(1)	-	-	-	CI	-
	RR(2)	-	-	-	CI	-
	RR(3)	51.25	CI	-	-	CI
	RR(4)	-	-	CI	-	-
	RR(5)	-	-	CI	-	-
	RR(6)	-	-	-	-	-
	RR(7)	CI	-	-	-	CI
	RR(8)	-	-	-	47.00	-
Chajpur	CjR(1)	CI	CI	48.75	CI	-
	CjR(2)	-	-	-	-	CI
	CjR(3)	-	-	-	-	-
	CjR(4)	CI	-	-	CI	-
	CjR(5)	48.30	-	47.50	-	-
Chopal	CR(1)	-	-	-	-	-
	CR(2)	-	-	49.50	52.10	-
	CR(3)	CI	-	-	-	-
	CR(4)	CI	-	-	-	-
	CR(5)	-	-	-	45.00	-
	CR(6)	-	-	-	-	CI
	CR(7)	48.50	-	CI	-	-
	CR(8)	-	-	-	-	-

**% Growth inhibition = $\frac{C-T}{T} \times 100$ Where, C: growth of fungus in control; T: Growth of fungus in test

CI- Contact Inhibition

Table 11: Screening of culturable bacterial isolates from new plantation sites in apple orchards for antagonistic activity against fungal pathogens

Location	Isolates	Antagonistic activity against fungal pathogens (% Growth inhibition)**				
		<i>Dematophora necatrix</i>	<i>Phytophthora sp.</i>	<i>Fusarium oxysporum</i>	<i>Sclerotium rolfsii</i>	<i>Pythium aphanidermatum</i>
Sablog	SF(1)	-	-	CI	-	CI
	SF(2)	CI	-	CI	48.00	-
	SF(3)	-	-	-	CI	49.00
	SF(4)	CI	-	-	50.00	CI
Recong Peo	KF(1)	CI	-	CI	-	CI
	K(2)	CI	CI	-	CI	CI
Kyari	KyF(1)	48.50	-	CI	-	-
	KyF(2)	CI	-	-	50.20	48.70
	KyF(3)	-	-	48.75	-	CI
	KyF(4)	-	-	47.00	-	CI
	KyF(5)	50.25	-	-	CI	-
	KyF(6)	-	-	-	-	-
Kufarbagh	KuF(1)	-	-	CI	-	CI
	KuF(2)	68.50	CI	CI	CI	47.50
	KuF(3)	CI	-	48.00	-	CI
	KuF(4)	-	-	CI	CI	CI

**% Growth inhibition = $\frac{C-T}{C} \times 100$ Where, C: growth of fungus in control; T: Growth of fungus in test

4.7 PRINCIPAL COMPONENT ANALYSIS: APPLE REPLANT SITES AND NEW PLANTATION SITES OF APPLE ORCHARDS

Principal Component Analysis (PCA) was carried to evaluate correlations between apple associated rhizobacterial populations and different plant growth promoting traits in apple replant sites and new plantation sites of apple orchards. The variables were normalized by division of their standard deviation, and the Eigen values and Eigenvectors of the correlation matrix were calculated, as well as the percentages of variance for each component.

PCA plots showing relationship between apple rhizobacterial isolates (from (a) new plantation sites of apple and (b) replant apple) and plant growth promoting traits viz. phosphate solubilization, siderophore production, IAA production are presented in Fig.20. Among the rhizobacterial population at new plantation sites of apple, maximum variance (42.08%) was observed among phosphate solublizers followed by siderophore producers (34.03%) and IAA producers (23.88%). A similar trend was observed among rhizobacterial isolates from replant sites. Results further revealed that principal component 1 (phosphate solubilization) and principal component 2 (siderophore production) at both the locations accounted for more than 70% of the total variance.

A comparative analysis of per cent variance as a result of component 1 (phosphate solubilization) revealed that, a statistically non-significant difference in variance was observed between phosphate solubilizers at new plantation (42.08%) and at replant sites (42.96%). The results therefore, suggest that rhizobacterial mediated P-mobilization is not a limiting factor that determines the establishment of apple plants whether at new plantation sites or at replant sites. However, comparative analysis of the variance as a result of component 2 (siderophore production) did not result in similar conclusions. Higher variance (34.03%) was observed at new plantation sites, while a lower variance of 33.06% was observed in replant apple, which correlated lower populations of siderophore producers in replant apple. Lower populations variance of siderophore producers at replant apple locations; suggest that the lower iron chelating properties of rhizobacteria, allowed the pathogens to survive at replant apple sites, which can be attributed as the plausibly reason for poor establishment of apple seedlings at replant sites. Thus, the findings indicate that populations of siderophore producers is critical when comparing relationship between apple rhizobacterial isolates (isolated from new plantation sites and replant sites apple) and plant growth promoting traits.

PCA plots showing relationship between apple rhizobacterial isolates (isolated from (a) new plantation sites and (b) replant site apple) and antagonistic abilities against different fungal pathogens are presented in Fig.21. At new plantation sites, maximum variance was exhibited by rhizobacterial isolates antagonistic to *Dematophora necatrix* (41.40%), which was followed by *Phytophthora* sp. (24.73%). Similarly, at replant sites maximum variance was exhibited by rhizobacterial isolates antagonistic to *Dematophora necatrix* (28.68%), which was followed by *Phytophthora* sp. (21.04%). Results further revealed that principal component 1 (rhizobacterial populations exhibiting antagonism to *Dematophora necatrix*) and principal component 2 (rhizobacterial populations exhibiting antagonism to *Phytophthora* sp.) at new plantation sites accounted for more than 66.13% of the total variance, contrary to 49.72% of total variance observed at replant sites. The results thus, highlight the fact that among the different fungal pathogens (*Dematophora necatrix*, *Phytophthora* sp., *Fusarium oxysporum*, *Sclerotium rolfsii* and *Pythium aphanidermatum*) evaluated in the present study, while comparing new plantation and replant sites, the antagonism of rhizobacterial populations against *Dematophora necatrix* and *Phytophthora* sp. is of critical importance.

A comparative analysis of per cent variance as a result of component 1 (rhizobacterial populations exhibiting antagonism to *Dematophora necatrix*) revealed that, a significantly higher variance (41.40%) in new plantations was observed. While only 28.68% variance was observed in replant site apple. The results suggest that lower population variance of rhizobacterial antagonists to *Dematophora necatrix* are present at the replant sites, thus indicating the fact that, the fungal pathogen *Dematophora necatrix* significantly contributes to the poor establishment of new apple seedlings at replant sites. Further analysis of the data appended in Fig 21 reveals that per cent variance as a result of component 2 (rhizobacterial populations exhibiting antagonism to *Phytophthora* sp.) and component 3 (rhizobacterial populations exhibiting antagonism to *Fusarium oxysporum*) followed a trend similar to that as observed with component 1. A variance of 24.73% and 20.39% was observed in new plantation sites as compared to 21.04% and 19.97% variance in rhizobacterial populations exhibiting antagonism to *Phytophthora* sp. and *Fusarium oxysporum* respectively, in replant apple. However, an opposite trend was observed with components 4 (rhizobacterial populations exhibiting antagonism to *Sclerotium rolfsii*) and component 5 (rhizobacterial populations exhibiting antagonism to and *Pythium aphanidermatum*). The findings thus highlight the fact, that when comparing relationship between apple rhizobacterial isolates (isolated from new plantation and replant sites of apple orchards) and antagonistic abilities against different fungal pathogens, the most critical factor that plausibly determines the establishment and survival of fresh apple seedlings is the population of rhizobacteria that inhibit *Dematophora necatrix* and *Phytophthora* sp.

Soil-borne microorganisms are considered to be an important component of soil function, being involved in the release of soil mineral nutrients, plant disease resistance, and plant diseases (Zhang *et al.* 2013). Fungi of the genera *Cylindrocarpon* and *Rhizoctonia*, as well as the oomycetes *Phytophthora* and *Pythium* are considered to be essential in the etiology of ARD, and these pathogens have been found frequently at different replant disease sites (Kelderer *et al.* 2012; Tewoldemedhin *et al.* 2011). Several *Fusarium* species have also been reported to be associated with ARD, although the role of the genus as a pathogen is controversial (Tewoldemedhin *et al.* 2011). Findings in the present work are in concomitance with the work carried out by researchers as cited above, however present study shed further light on the etiology of ARD by citing involvement of *D. necatrix* and *Phytophthora* sp. antagonists rhizobacterial population at replant sites of apple orchards.

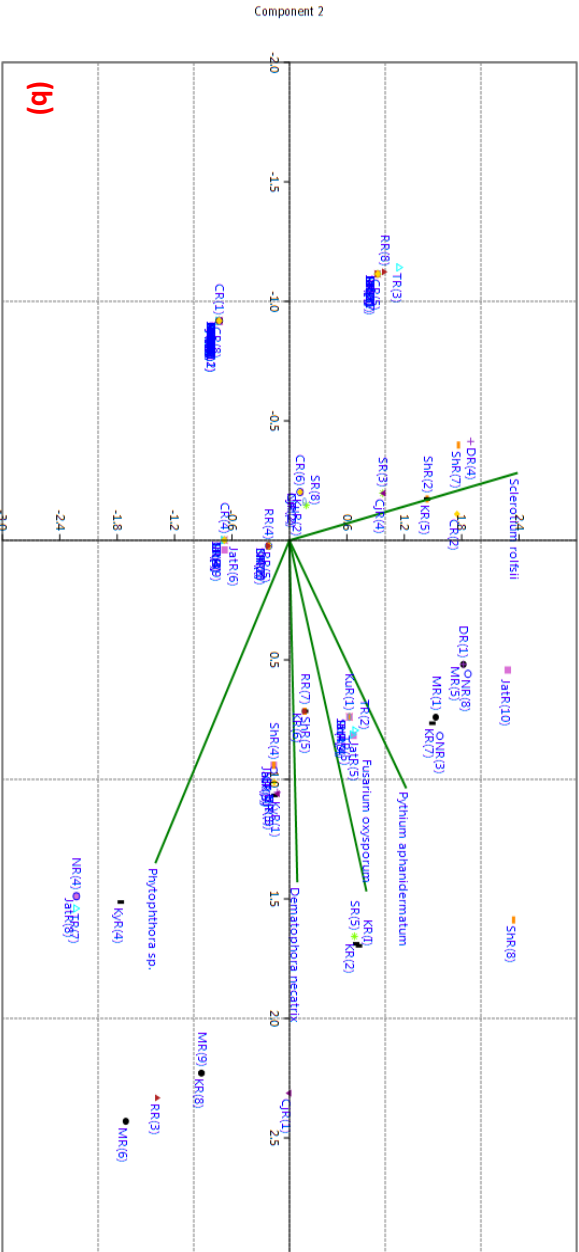
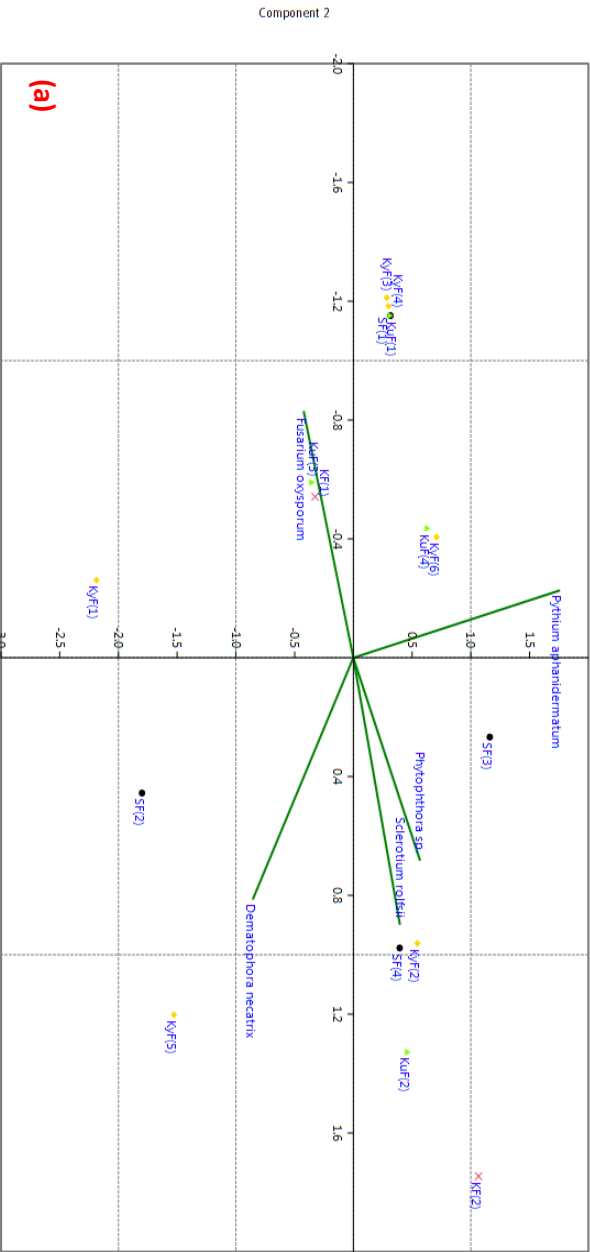


Figure 21. PCA plots showing relationship between apple rhizobacterial isolates (isolated from **(a)** new plantation apple orchards and **(b)** replant apple orchards) and antagonistic abilities against different fungal pathogens

Principal Components		New plantation apple orchards	Replant apple orchards
Component 1	41.40 (2.07)	28.68 (1.43)	
Component 2	24.73 (1.24)	21.04 (1.05)	
Component 3	20.39 (1.02)	19.97 (1.00)	
Component 4	8.38 (0.42)	15.53 (0.78)	
Component 5	5.10 (0.26)	14.79 (0.74)	

Components 1 and 2 account for 66.13 % variance

Components 1 and 2 accounts for 49.72 % variance

Figure in parenthesis indicate Eigen values

Component 1: *Dematophora necatrix*

Component 2: *Phytophthora* sp.

Component 3: *Fusarium oxysporum*

Component 4: *Sclerotium rolfsii*

Component 5: *Pythium aphanidermatum*

4.8 GENETIC DIVERSITY OF REPRESENTATIVE PLANT GROWTH PROMOTING RHIZOBACTERIA FROM REPLANT AND NEW PLANTATION SITES OF APPLE ORCHARDS USING AMPLIFIED RIBOSOMAL DNA RESTRICTION ANALYSIS

PCR amplification of 16S rRNA gene followed by ARDRA with three restriction endonucleases was carried out to evaluate species variation among the morphotype selected from the replantation and new plantation sites at different locations. The 16S rRNA amplicons were digested with three restriction enzymes, which generated profiles having three to eleven fragments ranging in size from 100-1000 base pairs (Plate 4, 5). 16S rRNA sequence analysis indicate that all isolates were diverse in terms of species. ARDRA based genetic diversity analysis using tetra cutter restriction endonuclease namely; *HinfI*, *HpaII* and *Taq I* have been reported previously as a highly informative molecular tool by Upadhyay *et al.* (2009) and Aranda *et al.* (2011). Verma *et al.* (2015) performed ARDRA analysis using three restriction endonucleases *AluI*, *HaeIII* and *MspI* to study the diversity of culturable bacilli associated with the wheat rhizosphere from six diverse agro-ecological zones of India.

The dendrogram of genetic similarity using Dice similarity coefficient index, revealed the similarity among 104 isolates from replant sites and 16 isolates from new plantation sites of apple orchards at 75% similarity level (Fig. 22 and 24). Analysis of the dendrograms for replant sites (Fig.23) revealed that the isolates were grouped into 23 different phylotypes, whereas 8 phylotypes were observed in case of dendrogram drawn for new plant sites (Fig.25).

4.8.1 Phylogenetic analysis of representative isolates from replant and new plantation sites of apple orchards

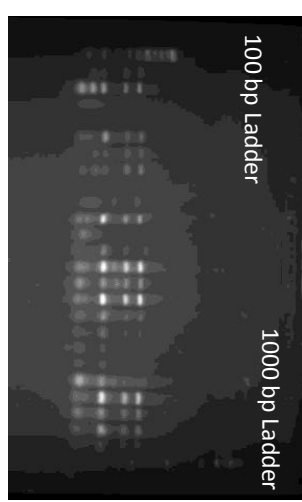
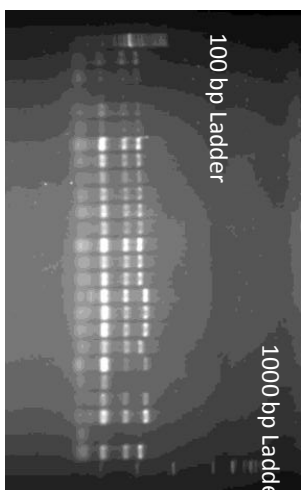
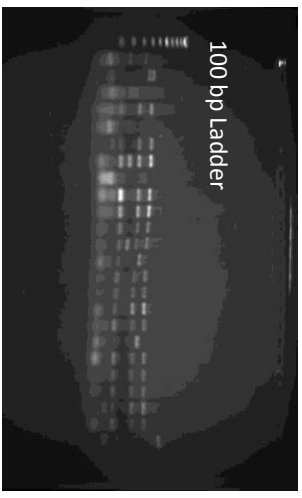
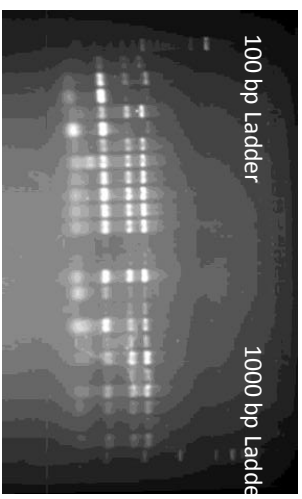
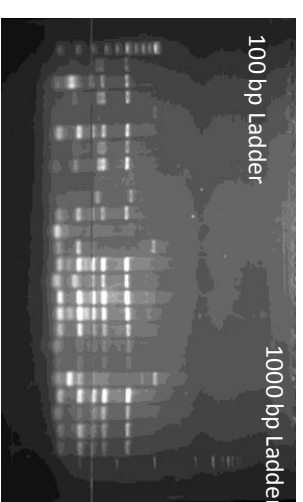
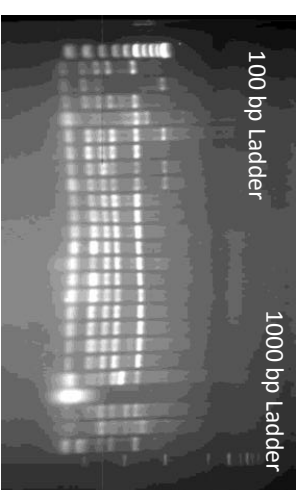
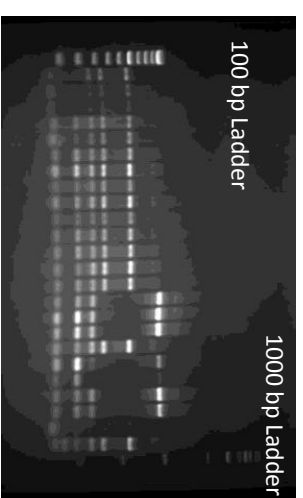
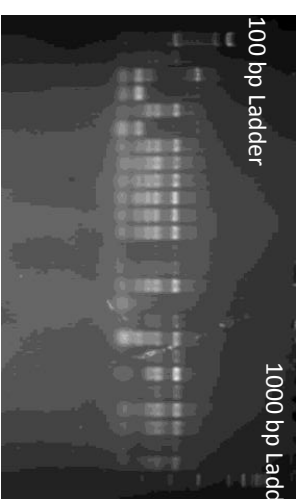
Representative isolates, showing high growth promoting potential, belonging to different phylotypes as derived from ARDRA analysis, were selected for the 16S rRNA gene based identification. The sequenced data was analyzed using BLASTn analysis, which revealed the dominance of genus *Bacillus* at replant sites of apple, showing 94-99% similarity with their nearest relatives in the NCBI GenBank database (Table 12). Whereas, at new plantation sites bacteria belonging to genus *Bacillus*, *Pantoea* and *Pluralibacter* were observed, showing 95-100% similarity with the nearest relatives in the NCBI GenBank database (Table 13).

Table 12. Phylogenetic relatedness of bacterial isolates from replant sites in apple orchards based on BLASTn analysis

Phylotype	Isolates	Taxonomic Classification	Closest Relative	Per cent similarity BLASTn	Accession number
1	<i>Bacillus amyloliquefaciens</i> strain MR1	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus amyloliquefaciens</i> NR_113993	95	KU879074
2	<i>Bacillus subtilis</i> strain NR(7)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> subsp. subtilis NR_114998	99	KU879075
	<i>Bacillus enclensis</i> strain MR(8)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus enclensis</i> NR_133700	96	KU879076
4	<i>Bacillus subtilis</i> strain Kuf R(4)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> NR_118383	99	KU879077
5	<i>Bacillus subtilis</i> strain MR(7)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> NR_118383	99	KU879078
6	<i>Bacillus subtilis</i> strain JR(3)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> NR_118383	99	KU879079
7	<i>Bacillus enclensis</i> strain TR(3)	Bacteria Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus enclensis</i> NR_133700	98	KU879080
8	<i>Bacillus axarquiensis</i> strain JatR(4)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus axarquiensis</i> NR_115283	99	KU879081
9	<i>Bacillus axarquiensis</i> strain CR(2)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus axarquiensis</i> NR_115283	98	KU879082
10	<i>Bacillus subtilis</i> strain JatR(7)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> NR_118383	99	KU879083
11	<i>Bacillus axarquiensis</i> strain KR(8)	Bacteria Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus axarquiensis</i> NR_115283	99	KU879084
12	<i>Bacillus safensis</i> strain CjR(1)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus safensis</i> NR_041794	94	KU879085
13	<i>Bacillus methylophilus</i> strain NR(5)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus methylophilus</i> NR_116240	97	KU879086
14	<i>Bacillus subtilis</i> strain KR(6)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> NR_118383	99	KU879087
15	<i>Bacillus subtilis</i> strain DR(8)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> NR_118383	99	KU879088
16	<i>Bacillus subtilis</i> strain KR(6)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> NR_118383	99	KU879089
17	<i>Bacillus enclensis</i> strain JatR(9)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus enclensis</i> NR_133700	98	KU879090
18	<i>Bacillus aerius</i> strain CR(5)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus aerius</i> NR_042338	98	KU879091
19	<i>Bacillus subtilis</i> strain ShR(4)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> strain SBMP4 NR_118383	99	KU879092
20	<i>Bacillus subtilis</i> strain MR(3)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> subsp. subtilis NR_115001	99	KU879093
21	<i>Bacillus safensis</i> strain ShR(8)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus safensis</i> NR_041794	97	KU879094
22	<i>Bacillus subtilis</i> strain KyR(4)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> strain SBMP4NR_118383	99	KU879095
23	<i>Bacillus malacitensis</i> strain Jat R(1)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus malacitensis</i> NR_042336	93	KU879096

i)	A1 Ladder; A2 MR(1); A3 MR(2); A4 MR(3); A5 MR(4); A6 MR(5); A7 MR(6); A8 MR(7); A9 MR(8); A10 MR(9); A11 DR(1); A12 DR(2); A13 DR(3); A14 DR(4); A15 DR(5); A16 DR(6); A17 DR(7); A18 DR(8); A19 JatR(1); A20 JatR(2); A21 JatR(3); A22 JatR(4); A23 JatR(5); A24 JatR(6); A25 JatR(7); A26 Ladder	B1 Ladder; B2 JatR(8); B3 JatR(9); B4 JatR(10); B5 JatR(11); B6 JatR(12); B7 JR(1); B8 JR(2); B9 JR(3); B10 JR(4); B11 JR(5); B12 JR(6); B13 NR(1); B14 NR(2); B15 NR(3); B16 NR(4); B17 NR(5); B18 NR(6); B19 NR(7); B20 NR(8); B21 SR(1); B22 SR(2); B23 SR(3); B24 SR(4); B25 SR(5); B26 Ladder	C1 Ladder; C2 SR(6); C3 SR(7); C4 SR(8); C5 TR(1); C6 TR(2); C7 TR(3); C8 TR(4); C9 TR(5); C10 TR(6); C11 TR(7); C12 ShR(1); C13 ShR(2); C14 ShR(3); C15 ShR(4); C16 ShR(5); C17 ShR(6); C18 ShR(7); C19 ShR(8); C20 ShR(9); C21 KR(0); C22 KR(2); C23 KR(3); C24 KR(4); C25 KR(5); C26 Ladder	D1 Ladder; D2 KR(6); D3 KR(7); D4 KR(8); D5 KyR(1); D6 KyR(2); D7 KyR(3); D8 KyR(4); D9 KuR(1); D10 KuR(2); D11 KuR(3); D12 KuR(4); D13 RR(1); D14 RR(2); D15 RR(3); D16 RR(4); D17 RR(5); D18 RR(6); D19 RR(7); D20 RR(8); D21 CjR(1); D22 CjR(2); D23 CjR(3); D24 CjR(4); D25 CjR(5); D26 Ladder
ii)	A1 Ladder; A2 MR(1); A3 MR(2); A4 MR(3); A5 MR(4); A6 MR(5); A7 MR(6); A8 MR(7); A9 MR(8); A10 MR(9); A11 DR(1); A12 DR(2); A13 DR(3); A14 DR(4); A15 DR(5); A16 DR(6); A17 DR(7); A18 DR(8); A19 JatR(1); A20 JatR(2); A21 JatR(3); A22 JatR(4); A23 JatR(5); A24 JatR(6); A25 JatR(7); A26 Ladder	B1 Ladder; B2 JatR(8); B3 JatR(9); B4 JatR(10); B5 JatR(11); B6 JatR(12); B7 JR(1); B8 JR(2); B9 JR(3); B10 JR(4); B11 JR(5); B12 JR(6); B13 NR(1); B14 NR(2); B15 NR(3); B16 NR(4); B17 NR(5); B18 NR(6); B19 NR(7); B20 NR(8); B21 SR(1); B22 SR(2); B23 SR(3); B24 SR(4); B25 SR(5); B26 Ladder	C1 Ladder; C2 SR(6); C3 SR(7); C4 SR(8); C5 TR(1); C6 TR(2); C7 TR(3); C8 TR(4); C9 TR(5); C10 TR(6); C11 TR(7); C12 ShR(1); C13 ShR(2); C14 ShR(3); C15 ShR(4); C16 ShR(5); C17 ShR(6); C18 ShR(7); C19 ShR(8); C20 ShR(9); C21 KR(0); C22 KR(2); C23 KR(3); C24 KR(4); C25 KR(5); C26 Ladder	D1 Ladder; D2 KR(6); D3 KR(7); D4 KR(8); D5 KyR(1); D6 KyR(2); D7 KyR(3); D8 KyR(4); D9 KuR(1); D10 KuR(2); D11 KuR(3); D12 KuR(4); D13 RR(1); D14 RR(2); D15 RR(3); D16 RR(4); D17 RR(5); D18 RR(6); D19 RR(7); D20 RR(8); D21 CjR(1); D22 CjR(2); D23 CjR(3); D24 CjR(4); D25 CjR(5); D26 Ladder
iii)	A1 Ladder; A2 MR(1); A3 MR(2); A4 MR(3); A5 MR(4); A6 MR(5); A7 MR(6); A8 MR(7); A9 MR(8); A10 MR(9); A11 DR(1); A12 DR(2); A13 DR(3); A14 DR(4); A15 DR(5); A16 DR(6); A17 DR(7); A18 DR(8); A19 JatR(1); A20 JatR(2); A21 JatR(3); A22 JatR(4); A23 JatR(5); A24 JatR(6); A25 JatR(7); A26 Ladder	B1 Ladder; B2 JatR(8); B3 JatR(9); B4 JatR(10); B5 JatR(11); B6 JatR(12); B7 JR(1); B8 JR(2); B9 JR(3); B10 JR(4); B11 JR(5); B12 JR(6); B13 NR(1); B14 NR(2); B15 NR(3); B16 NR(4); B17 NR(5); B18 NR(6); B19 NR(7); B20 NR(8); B21 SR(1); B22 SR(2); B23 SR(3); B24 SR(4); B25 SR(5); B26 Ladder	C1 Ladder; C2 SR(6); C3 SR(7); C4 SR(8); C5 TR(1); C6 TR(2); C7 TR(3); C8 TR(4); C9 TR(5); C10 TR(6); C11 TR(7); C12 ShR(1); C13 ShR(2); C14 ShR(3); C15 ShR(4); C16 ShR(5); C17 ShR(6); C18 ShR(7); C19 ShR(8); C20 ShR(9); C21 KR(0); C22 KR(2); C23 KR(3); C24 KR(4); C25 KR(5); C26 Ladder	D1 Ladder; D2 KR(6); D3 KR(7); D4 KR(8); D5 KyR(1); D6 KyR(2); D7 KyR(3); D8 KyR(4); D9 KuR(1); D10 KuR(2); D11 KuR(3); D12 KuR(4); D13 RR(1); D14 RR(2); D15 RR(3); D16 RR(4); D17 RR(5); D18 RR(6); D19 RR(7); D20 RR(8); D21 CjR(1); D22 CjR(2); D23 CjR(3); D24 CjR(4); D25 CjR(5); D26 Ladder

Plate 4 ARDRA fingerprints of culturable bacterial communities in rhizosphere soil of replant sites of apple orchards, with (i) *HinfI* (ii) *HpaII* (iii) *Taq I* restriction enzymes



(䷋)

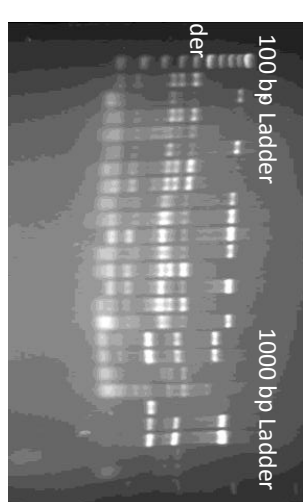
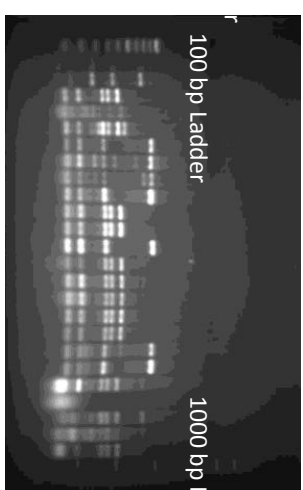
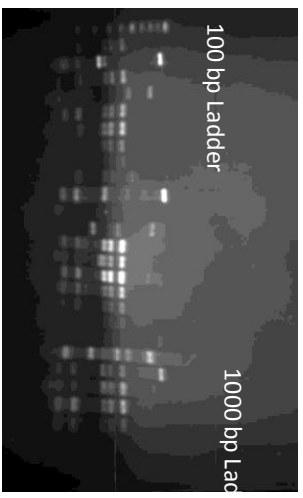
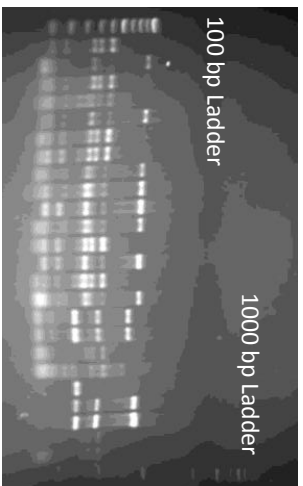


Table 13 Phylogenetic relatedness of bacterial isolates from new plantation sites in apple orchards at different location based on BLASTn analysis

Phylotype	Isolates	Taxonomic Classification	Closest Relative	Per cent similarity BLASTn	Accession Number
1	<i>Pluralibacter gergoviae</i> strain KF(1)	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; <i>Pluralibacter</i>	<i>Pluralibacter gergoviae</i> NR_024641	95	KU878623
2	<i>Bacillus subtilis</i> strain KUF(2)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> strain SBMP4NR_118383	99	KU878624
3	<i>Bacillus axarquiensis</i> strain KyF(1)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus axarquiensis</i> NR_115283	100	KU878625
4	<i>Bacillus subtilis</i> strain SF(3)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> strain SBMP4NR_118383	99	KU878626
5	<i>Bacillus subtilis</i> strain SF(4)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus subtilis</i> NR_118383	99	KU878627
6.	<i>Pantoea ananatis</i> strain KyF(2)	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; <i>Pantoea</i>	<i>Pantoea ananatis</i> NR_103927.1	97	KU878628
7.	<i>Bacillus enclensis</i> strain KyF(5)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus enclensis</i>	97	KU878629
8.	<i>Bacillus axarquiensis</i> strain KyF(6)	Bacteria; Firmicutes; Bacilli; Bacillales; Bacillaceae; <i>Bacillus</i>	<i>Bacillus axarquiensis</i> NR_115283	100	KU878630

Figure 23 and 25 represent the circular phylogram of apple rhizobacterial isolates from replant sites and new plant sites of apple, which were constructed using neighbor joining method with 100 bootstrap samplings. Phylogenetic studies confirmed the affiliation of the selected isolates from replant sites of apple with genus *Bacillus*, as is revealed by clustering pattern in the phylogenetic tree. All the twenty three isolates from replant sites were clustered closely with different species of genus *Bacillus* with high boot strap values. The identified *Bacillus* species were *Bacillus subtilis*, *Bacillus enclensis*, *Bacillus axarquiensis*, *Bacillus aerius*, *Bacillus safensis*, *Bacillus malacitensis*, *Bacillus amyloliquefaciens* and *Bacillus methylotropicus*. *Bacillus* is frequently isolated from the rhizosphere because of predominance due to their spore forming ability and also they are common plant endophytes.

Phylogenetic analysis of representative rhizobacteria from new plantation sites of apple confirmed their affiliation to genus *Bacillus*, *Pantoea* and *Pluralibacter* as revealed by clustering patterns (Fig. 25). Phylogenetic tree revealed that out of total eight rhizobacteria,

six strains were clustered closely with different species of genus *Bacillus* with high boot strap values. The identified *Bacillus* species were *Bacillus subtilis*, *Bacillus enclensis* and *Bacillus axarquiensis*. Rhizobacteria, which clustered closely with genus *Pantoea* and *Pluralibacter* were identified as *Pantoea ananatis* and *Pluralibacter gergoviae*, respectively.

Thus, the phylogenetic analysis confirms the predominance of Gram's positive bacteria i.e. *Bacillus* under the phylum Firmicutes in the rhizospheric soil of both replant as well as new plant sites of apple. *Bacillus* has been reported largely in the literature as a plant growth promoter (Verma *et al.* 2014; Mehta *et al.* 2013, 2015; Sharma *et al.* 2013; Chauhan *et al.* 2014) and active in the biocontrol of pathogenic bacteria (Mehta *et al.* 2014; Sharma *et al.* 2015). Replant and new plant soil were not found to differ significantly from each other at phylum and genus level, indicating that the microbial composition did not largely vary between replant and fresh plant sites of apple at different location. These findings are in concomitance with those observed for root endophytic fungal communities in new and replant soils whereby most species were detected in both soil types, although differences in species abundance existed (Manici *et al.* 2013).

4.9 BIOCHEMICAL AND 16S rDNA BASED PHYLOGENETIC CHARACTERIZATION OF PLANT GROWTH PROMOTING RHIZOBACTERIA ISOLATED FROM APPLE

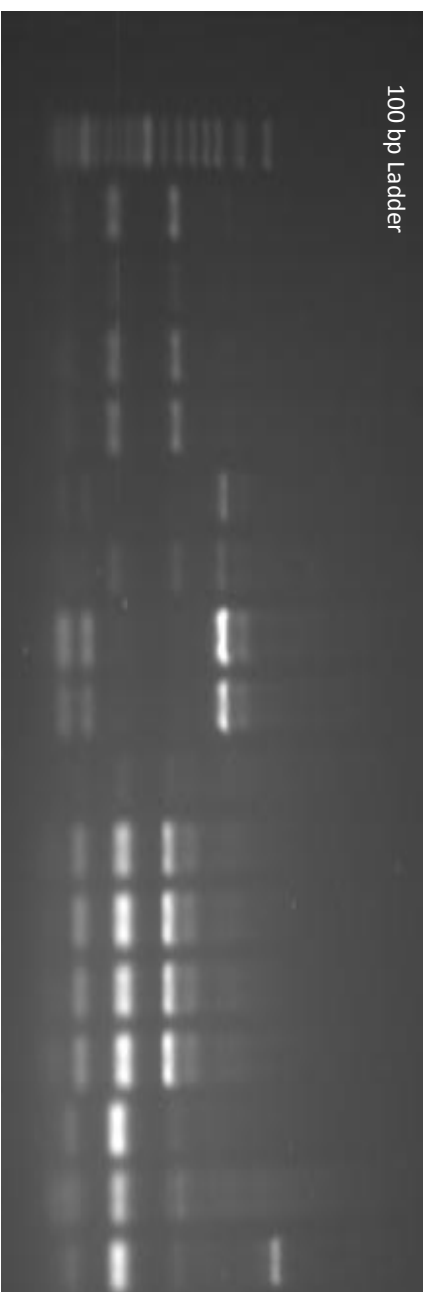
Bacterial endophytic (*B.licheniformis* strain CKA1 and *B.methylotropicus* strain CKAM) and rhizospheric (*S.plymuthica* strain G4(3) and *B.methylotropicus* strain RG1(3)) isolated from apple (*Malus domestica* Borkh.) trees were selected in the present study based on their ability of exhibiting multifarious plant growth promoting (PGP) traits (Table14; Plate1) viz. nutrient mobilization (P-solubilization, ability to fix atmospheric nitrogen, iron chelating ability); phyto-hormone production (indole-3-acetic acid production); bio-protecting agent (siderophore production, chitinase and protease enzyme production, and antifungal activity against soil borne fungal pathogens such as *Phytophthora sp.*, *Fusarium oxysporum*, *Pythium aphanidermatum*, *Dematophora necatrix* and *Corticium salmonicolor*).

Plant growth promoting rhizobacteria (PGPR) have been reported to influence plant growth, yield and nutrient uptake by a diverse array of mechanisms, the specific traits by which PGPR promote growth have been limited to the expression of one or more of the traits expressed at a given site of plant/microbe interaction. The potential benefit in the application

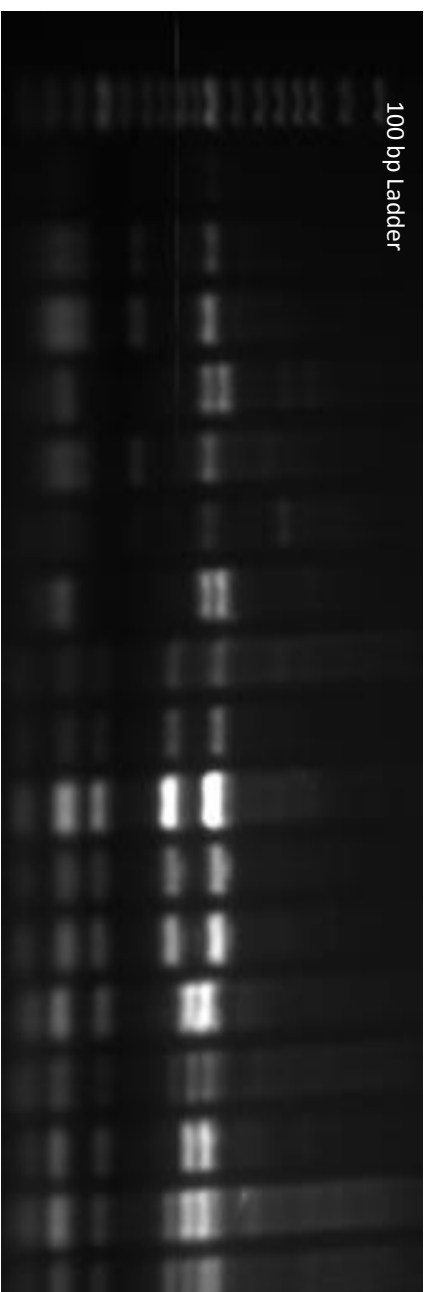
i)	A1 Ladder; A2 SF(1); A3 SF(2); A4 SF(3); A5 SF(4); A6 KF(1); A7 KF(2); A8 KyF(1); A9 KyF(2); A10 KyF(3); A11 KyF(4); A12 KyF(5); A13 KyF(6); A14 KuF(1); A15 KuF(2); A16 KuF(3); A17 KuF(4)
ii)	A1 Ladder; A2 SF(1); A3 SF(2); A4 SF(3); A5 SF(4); A6 KF(1); A7 KF(2); A8 KyF(1); A9 KyF(2); A10 KyF(3); A11 KyF(4); A12 KyF(5); A13 KyF(6); A14 KuF(1); A15 KuF(2); A16 KuF(3); A17 KuF(4)
iii)	A1 Ladder; A2 SF(1); A3 SF(2); A4 SF(3); A5 SF(4); A6 KF(1); A7 KF(2); A8 KyF(1); A9 KyF(2); A10 KyF(3); A11 KyF(4); A12 KyF(5); A13 KyF(6); A14 KuF(1); A15 KuF(2); A16 KuF(3); A17 KuF(4)

Plate 5 ARDRA fingerprints of culturable bacterial communities in rhizosphere soil of new plantation sites of apple orchards, with (i) *HinfI* (ii) *HpaII* (iii) *Taq I* restriction enzymes

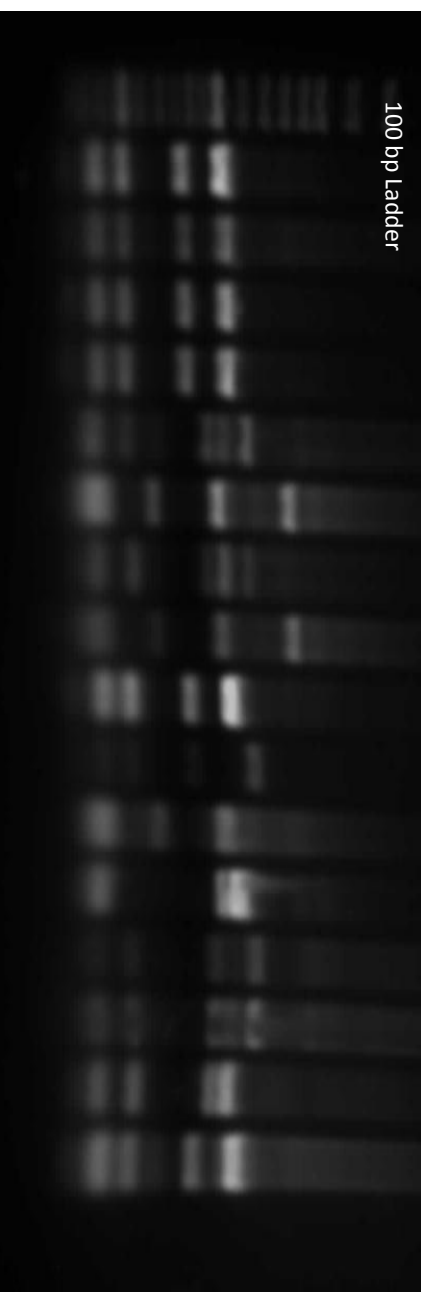
(i)



(ii)



(iii)



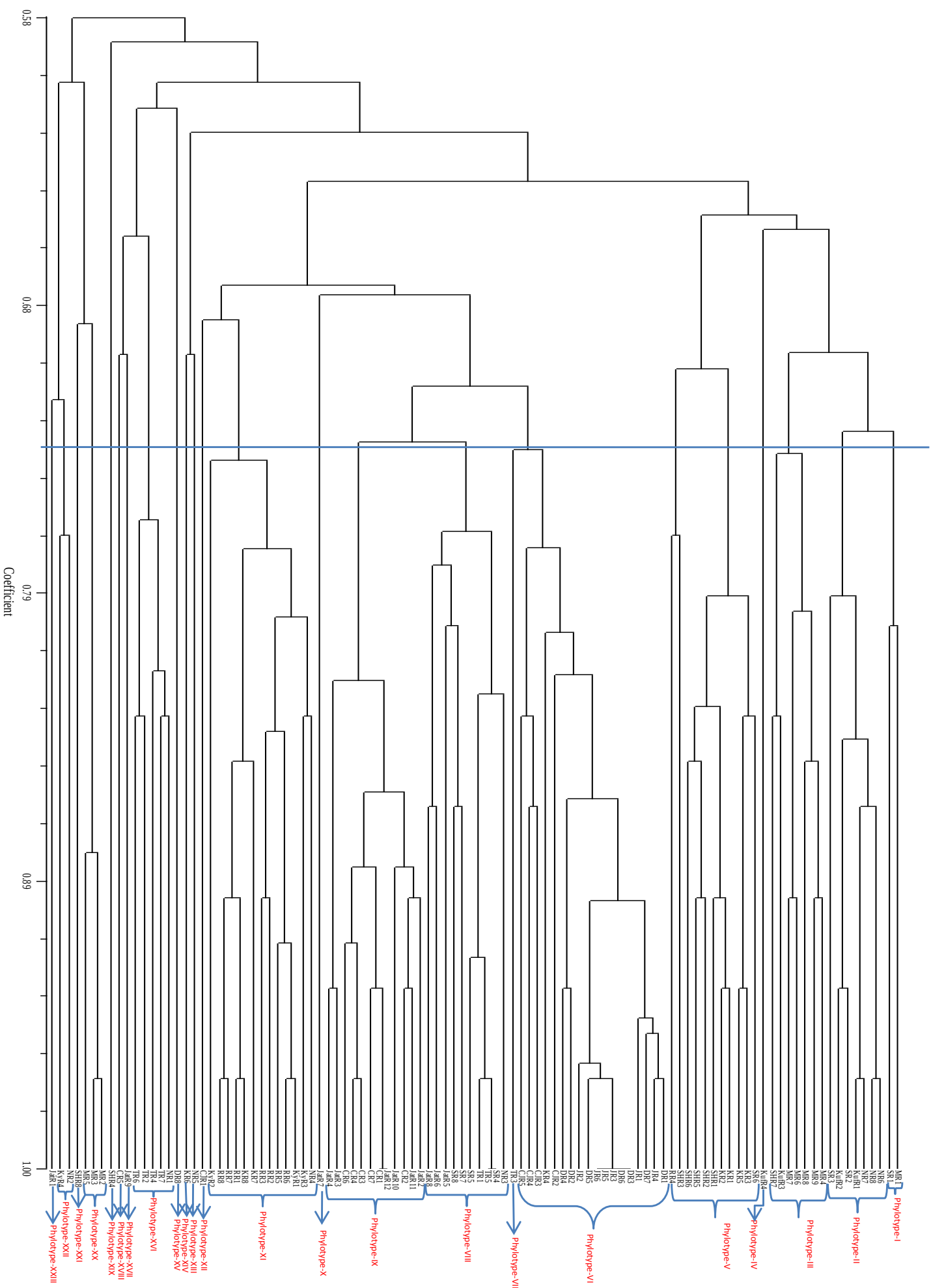


Figure 22. Dendrogram showing ARDRA based genetic diversity analysis of rhizobacterial population isolated from apple replant sites.

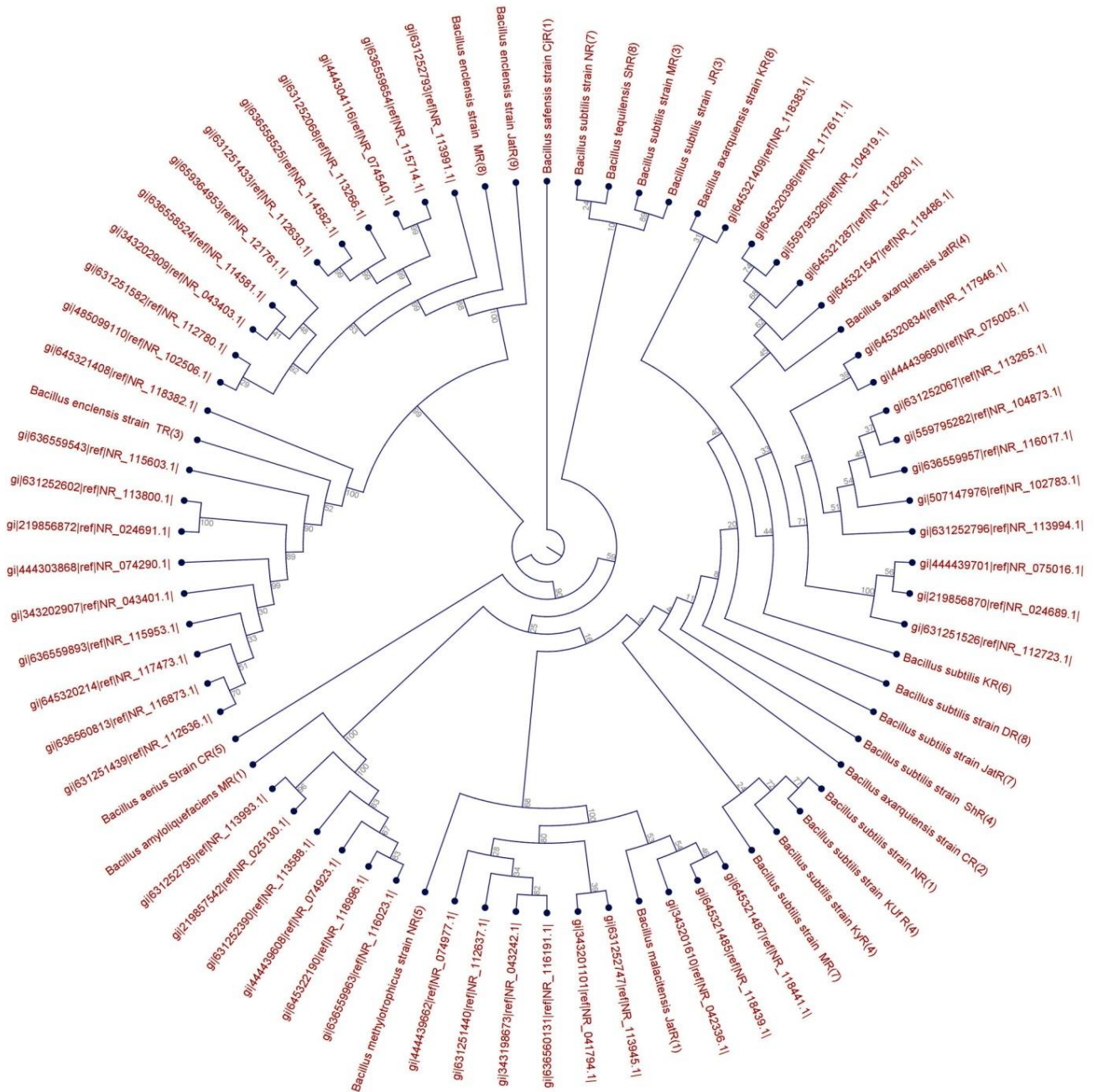


Figure 23. Circular phylogram depicting phylogenetic relatedness of 16S rRNA gene sequences of bacterial isolates from replant sites of apple orchards

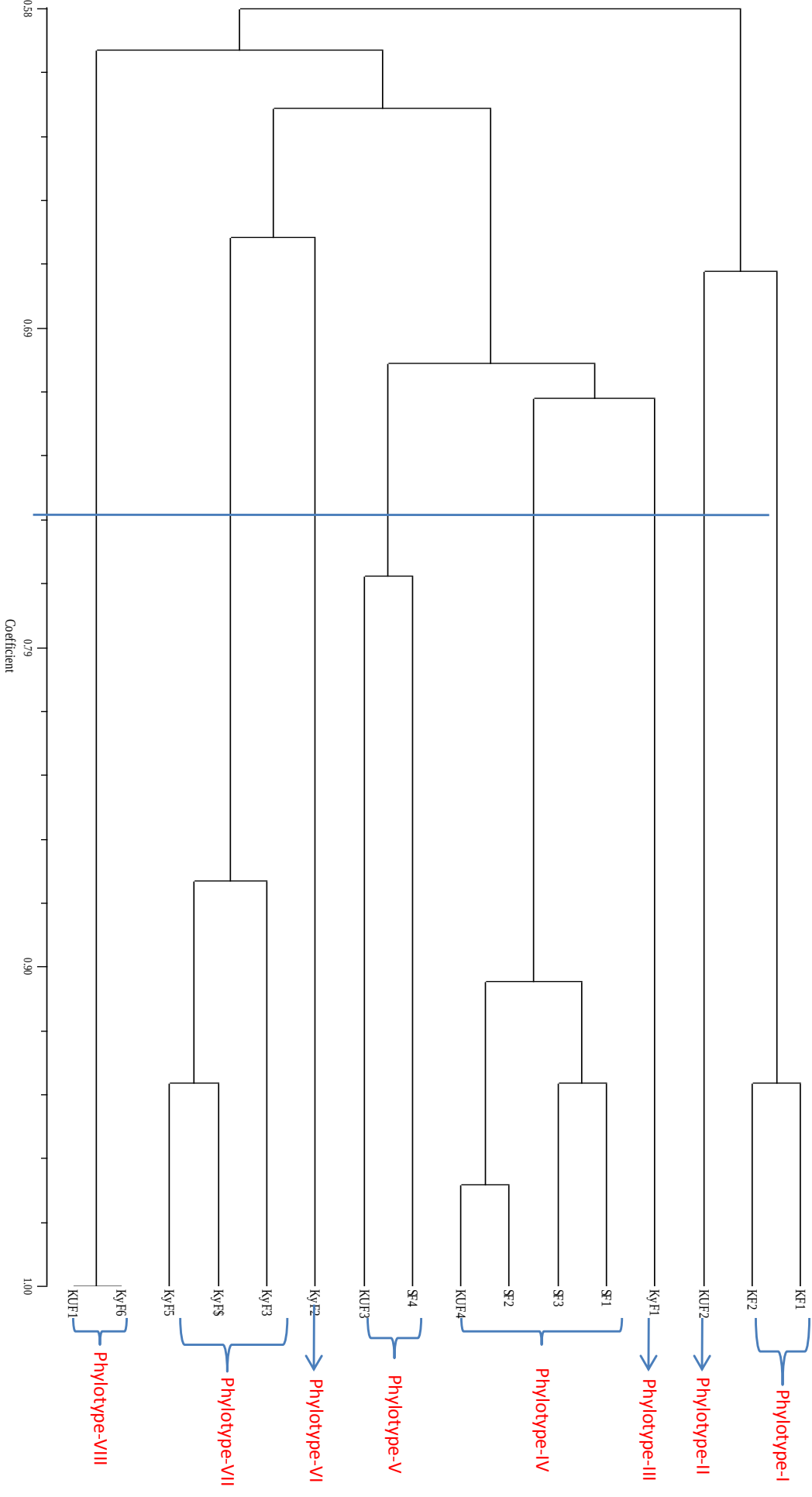


Figure 24. Dendrogram showing ARDRA based genetic diversity analysis of rhizobacterial population isolated from new plantation sites of apple orchards

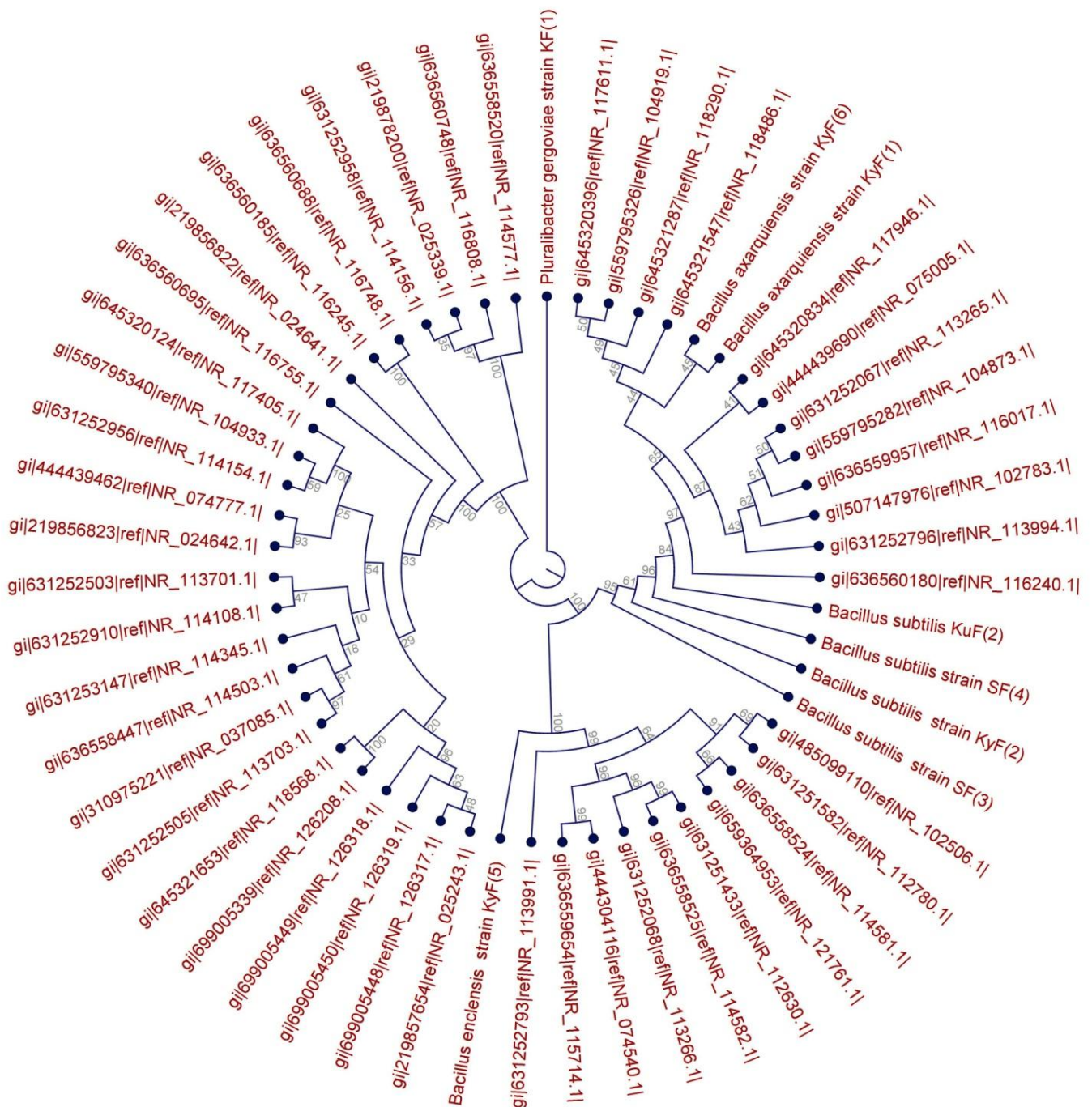


Figure 25. Circular phylogram depicting phylogenetic relatedness of 16S rDNA gene sequences of bacterial isolates from new plantation of apple orchards

of PGPR formulation (single strain/ consortium PGPR) has been evaluated in the present study.

Table 14: Characterization of most efficient already/previously isolated strain of apple for plant growth promoting traits

	CKA1 <i>B.licheniformis</i>	G4(3) <i>S.plymuthica</i>	RG1(3) <i>B.methylotropicus</i>	CKAM <i>B.methylotropicus</i>
Bacterial strains ability of nutrient mobilization				
TCP solubilization (µg/ml)	84.60	50.30	35.00	687
Ability to fix atmospheric nitrogen	-	-	+	+++
Iron chelating ability(%SU)	138.00	100.40	54.39	96.40
Bacterial strains ability of phyto-hormone production				
Indole-3-acetic acid production (µg/ml)	27.00	13.00	160.00	34.00
Bacterial strains ability to act as bio-protecting agent				
Chitinase enzyme activity (EI)	4.85	-	4.14	8.75
Protease enzyme activity(EI)	1.59	-	2.12	3.40
Antifungal activity against soil borne fungal pathogens	Per cent Growth Inhibition (Dual culture plate assay)			
• <i>Phytophthora spp.</i>	Contact Inhibition	46.20%	45.00%	70.00
• <i>Fusarium oxysporum</i>	Contact Inhibition	Contact Inhibition	57.70%	88.22
• <i>Pythium aphanidermatum</i>	47.00%	Contact Inhibition	48.57%	62.56
• <i>Dematophora necatrix</i>	73.00%	Contact Inhibition	55.00%	61.73
• <i>Sclerotium rolfsii</i>	Contact Inhibition	-	46.10%	44.54
• <i>Corticium Salmonicolor</i>	56.87	46.00	Contact Inhibition	50.58

The reported inconsistent performances and comparatively reduced beneficial effects in use might be primarily due to the expression of fewer plant growth promoting traits (Larcher *et al.* 2003; Cakmakc *et al.* 2007).

Multiple strain mixtures of PGPR, each with a specific capability and function, may have certain advantages: broad spectrum activity; enhanced efficacy; and reliability of the biocontrol agent, thereby allowing the combination of various traits without the use of genetic engineering. The concept of using PGPR mixtures for disease control and plant growth promotion has already been well demonstrated in several crops (Glick, 1995; Khalid *et al.* 2004). However, in some instances root colonization of one member of a mixture can be severely affected. PGPR root colonization is an essential process in plant microbe interactions determining the optimum population level of the inoculated strain. All PGPR strains of a particular genus and species may not have identical metabolic capabilities and interactions with plants. Compatibility of individual strains in mixture among themselves, and with commonly used agrochemicals is also some of the constraints in the use of multiple strain mixtures of PGPR. Isolation and selection of a PGPR strain with multiple plant growth promoting activities might help to address these problems.

Therefore, selection of endophytic and rhizospheric strains as a potential PGPR strain in the present study was based on the objective of developing a single strain PGPR formulation with multifarious PGP traits. In the present study, evaluation of *B.licheniformis* strain CKA1, *B.methylotropicus* strain CKAM, *S.plymuthica* strain G4(3) and *B.methylotropicus* strain RG1(3) and their consortium was evaluated on plant growth promotion of apple.

4.9.1 Phenotypic and biochemical characterization of strains selected for *in vitro* and *in vivo* screening

Strains positive for Gram's reaction were metabolically characterized using HiCarbohydrateTM Kit (HiMedia, Mumbai) where the strains were tested for utilization of thirty five carbohydrates, based on the principle of pH change and substrate utilization Table 15).

Colony morphology of strain CKA1 on nutrient agar medium after 48h of incubation was whitish coloured (96h to 120h colony became brown), opaque with rough surface, convex elevation and margins varied from undulate to fimbriate. The isolated colonies in nitrogen free glucose medium after 48h of incubation were larger in size (4-5mm) as compared to colony size (2-3mm) on nutrient agar. The colonies were opaque with rough surface and convex elevation. Microscopic examination showed the strain was rods shaped, arranged singly, Gram positive and formed oval shaped endospores positioned centrally.

Physiological tests revealed the ability of strain CKA1 to grow over a wide range of temperature (10°C to 45°C) with an optimum at 37±2°C; pH tolerance over a range of 5.0 to 11.0, with optimum at 7.0±0.2 and could tolerate up to 8.5% NaCl concentration (w/v). In nutrient broth under unshaken conditions, growth of strain CKA1 (48h to 72h) was uniformly turbid.

Results appended in table 15 reveal that strain CKA1 gave positive reaction for maltose, fructose, dextrose, galactose, trehalose, sucrose, mannose, insulin, glycerol, salicin, inositol, sorbitol, mannitol, α -methyl-d-glucoside, ortho nitro phenyl β -D galactopyranoside, esculin hydrolysis and citrate utilization..

Strain CKAM was Gram positive, motile rods, round colony configuration having rough surface, convex elevation and wavy margin and creamish white in colour. The isolate produced a yellow zone of clearance in siderophore specific medium (CAS agar medium). The strain was able to grow up to 8.5% NaCl between 20 and 40°C at pH 5.5 -9.5. The isolate was positive for catalase, Voges-Proskauer's test, citrate utilization, ortho nitro phenyl β -D galactopyranoside, nitrate reduction, gelatin hydrolysis, starch hydrolysis and could utilize dextrose, fructose, insulin, xylose, glycerol and salicin as a sole carbon source (Table 15).

The isolated colonies of strain RG1 (3) on nutrient agar medium after 48 h of incubation were creamish colored, irregular colony configuration having rough surface, flat elevation and undulate margin. The isolate produced yellowish halozone on the PVK medium. Pellet formation and slight turbidity was observed when the isolate was grown in nutrient broth under unshaken conditions. The isolate produced a yellow zone in siderophore specific medium (CAS agar medium). The morphological and biochemical characteristics of the isolate are as follows: cells were Gram positive, aerobic, rods present singly, endospore former and was positive for catalase, Voges Proskauer's reaction, citrate utilization, ONPG, nitrate reduction, arginine dehydrolase, gelatin hydrolysis, starch hydrolysis, esculin hydrolysis and could utilize dextrose, fructose, inulin, glycerol, salicin as sole carbon source (Table 15).

Results based on morphological, physiological and biochemical tests as per Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994) suggested that the strain CKA1, strain CKAM, strain RG1(3) belongs to genus *Bacillus*.

This was further confirmed using HiBacillusTM Identification Kit (HiMedia, Mumbai), where the strains were tested based on carbohydrate utilization and other biochemical tests specific for *Bacillus* species as per Bergey's Manual of Determinative Bacteriology (Holt *et al.* 1994).

Dastager *et al.* (2011) also used HiMedia's carbon source utilization kit (Hi 25 Kit) for phenotypic and biochemical characterization of plant growth promoting bacteria associated with black pepper (*Piper nigrum* L.).

HiCarbohydrateTM Kit has been employed by Mehta *et al.* (2013) and Sharma *et al.* (2015) for the phenotypic characterization of bacterial isolates from rhizosphere of apple and tomato respectively.

Table 15. Biochemical characteristics of selected bacterial isolates

(a) Biochemical characterization using HiCarbohydrateTM Kit									
Test	CKA1	CKAM	RG1(3)	G4(3)	Test	CKA1	CKAM	RG1(3)	G4(3)
Lactose	-	-	-	-	Inulin	+	-	-	-
Xylose	-	-	-	+	Sodium glucanate	-	-	-	-
Maltose	+	+	+	-	Glycerol	+	-	-	+
Fructose	+	-	-	-	Salicin	+	-	-	+
Dextrose	+	-	-	+	Dulcitol	-	-	-	-
Galactose	+	+	+	+	Inositol	+	+	+	-
Raffinose	-	+	+	+	Sorbitol	+	+	+	+
Trehalose	+	+	+	+	Mannitol	+	+	+	-
Melibiose	-	-	-	+	Adonitol	-	-	-	+
Sucrose	+	+	+	+	Arabitrol	-	-	-	-
L-Arabinose	-	-	-	+	Erythritol	-	-	-	-
Mannose	+	+	+	+	α -Methyl-D-glucoside	+	+	+	-
Rhamnose	-	-	-	-	α -Methyl-D-mannoside	-	-	-	+
Cellobiose	-	-	-	-	Xylitol	-	+	-	-
Melezilose	-	-	-	-	ONPG	+	+	+	-
D-Arabinose	-	-	-	+	Esculin hydrolysis	+	+	+	+
Citrate utilization	+	+	+	+	Malonate utilization	-	-	-	-
Sorbose	-	+	+	-					
(b) Biochemical characterization									
	CKA1	CKAM	RG1(3)	G4(3)		CKA1	CKAM	RG1(3)	G4(3)
Malonate	-	-	-	+	Arginine	+	+	+	-
Nitrate Reduction	+	+	+	-	Arabinose	-	-	-	+
Mannitol	+	+	+	-	ONPG	+	+	+	-
VogesProskauer's	+	+	+	+	Sucrose	+	+	+	+
Catalase	+	+	+	+	Trehalose	+	+	+	+
Glucose	+	+	+	+	Citrate	+	+	+	+

Another bacterial Strain G4(3) exhibited morphologically and physiologically characters typically different. Cells were Gram negative, cocco bacillus short rods present singly and colonies were having smooth surface, convex elevation and entire margin with a size of colony on nutrient agar medium was 0.2cm to 0.25cm. The isolate produced yellowish halozone on the PVK medium. Turbidity was observed when the isolate was grown in nutrient broth under unshaken or shaken conditions at an optimum temperature 30°C. The isolate produced a yellow zone of clearance in siderophore specific medium (CAS agar medium). The biochemical characteristics of the isolate showed that it was positive for ONPG test, Esculin test, Citrate utilization, Malonate, Catalase, Indole, Voges Proskauer's, α -Methyl-D mannoside Xylose, Dextrose, Galactose, Raffinose, Trehalose, Melibiose, Sucrose, L-Arabinose, Mannose, Glycerol and Salicin (Table 15). The phenotypic and biochemical characteristics suggested that the strain belongs to genus *Serratia*.

In the previous study (Aslantas *et al.* 2007), all the PGPR isolates from apple trees were oxidase, nitrate reduction and catalase positive and belonged to genus *Bacillus* and *Burkholderia*. However, in the present study, one of the isolate G4(3) was negative for nitrate reduction, oxidase and catalase and was identified as *Serratia* sp.

Similar methodology has been employed by Gulati *et al.* (2008) and Chauhan (2014) for the phenotypic characterization of rhizospheric bacterial isolates using HiCarbohydrate™ Kit and the results were represented in the form of a dendrogram based on similarity coefficients.

4.9.2 Genotypic characterization of potential PGPR isolates from rhizospheric soil and root endosphere of apple

All the four strains viz strain CKA1, strain CKAM, strain RG1(3) and strain G4(3) were originally isolated from apple rhizosphere and root endosphere and were genetically characterized by 16S rDNA analysis and gene sequences of these strains were submitted in NCBI having accession number GQ 980017, JQ023625, KP295448 and JX177363, respectively.

4.10 EFFECT OF *Bacillus licheniformis* CKA1, *B. methylotropicus* CKAM, *Serratia plymuthica* G4(3) AND *B. methylotropicus* RG1(3) INOCULATION ON GROWTH OF APPLE SEEDLINGS/PLANTS UNDER NET HOUSE AND NURSERY FIELD CONDITIONS

Biochemical and molecular based characterization of bacterial strains helped in the identification of most potential bacterial strains with multifarious plant growth promoting

traits, involved in the direct mechanism of plant growth promotion viz. nutrient mobilization (P-solubilization, ability to fix atmospheric nitrogen, iron chelating ability); phyto-hormone production (indole-3-acetic acid); efficient rhizosphere colonization ability, these strains were evaluated for their effect on growth of apple seedling/plants.

4.10.1 Effects of inoculations with selected PGPR on apple seed germination and plant growth under net house conditions

The following treatments were assayed for their effect on apple seed germination:

1. Un-inoculated seeds as control
2. Seed treatment with different PGPR:
 - a) *B. licheniformis* strain CKA1,
 - b) *Serratia plymuthica* strain G4(3),
 - c) *B. methylotropicus* strain CKAM,
 - d) *B. methylotropicus* Strain RG1(3),
 - e) Consortium (CKA1+CKAM+G4(3)+RG1(3))

The data pertaining to per cent germination after 45 days of germination in net house (average of data observed in two individual experiments carried out in 2013 and 2014) is presented in figure 26, plate 6. The present work revealed that under net house conditions, seed treatment with PGPR strains improved seed germination, seedling emergence and seedling stand over the control (Table 16).

. Under net house conditions, per cent germination of seeds bacterized with liquid formulation of all bacterial strains were significantly higher than the per cent germination observed for untreated seeds (Table 16).

Maximum germination (75.43%) was observed when apple seeds were bacterized with liquid based culture of *B. licheniformis* strain CKA1 which was significantly higher when compared with per cent germination of other treated and untreated seeds.

Liquid based culture of *B. methylotropicus* RG1(3) accounted for 68.35 per cent germination which was statistically at par with per cent germination of seeds treated with consortium of all the four strains (67.50%). Similar trend was observed for seeds bacterized with *B. methylotropicus* CKAM (60.21%) and with *S. plymuthica* G4(3) (58.40%).

Seed treatment with *B. licheniformis* strain CKA1, *S.plymuthica* strain G4 (3), *B.methylotropicus* strain CKAM, *B.methylotropicus* strain RG1 (3) and consortium accounted for 52.53, 18.09, 21.75, 38.22 and 36.40 per cent increase, respectively, in case of seed germination over uninoculated control (Fig. 26).

The results of the present study are in concomitance with previously reported results of PGPR treated different crops such as *Pseudomonas putida*, *P.fluorescens*, *Pantoea agglomerans* and *Bacillus subtilis* significantly increase seedling emergence of crops such as wheat and soyabean (Luz, 2001; Tripathi *et al.* 2005; Fatima *et al.* 2009). Similar improvement in seed germination parameters with the application of rhizobacteria has been reported for various cereals crops such as sorghum (Raju *et al.* 1999) and pearl millet (Niranjan *et al.* 2004). The positive influence of rhizobacterial application on germination rate of apple seeds under net house might be due to increased synthesis of hormones like gibberlins and auxins etc. which triggered the activity of enzymes required for the germination and early growth promotion (Bharathi *et al.* 2004).

Plant growth promoting bacteria are the natural potential resource which colonize roots of plants and stimulate growth and yield directly and indirectly (Afzal and Bano, 2008). Direct promotion of growth by PGPR including production of metabolites that enhance plant growth such as auxins (Ali *et al.* 2010), cytokinins, gibberellins and through the solubilization of minerals (El- Hadad *et al.* 2010).

Table 16. Effect of selected PGPR inoculations on seed germination and growth promotion of apple seedling under net house conditions

Treatments	Germination (%)	Shoot length (cm)	Shoot diameter (mm)	Root length (cm)	Leaf surface area (cm ²)
Un-inoculated control	49.45	9.01	4.20	3.77	14.23
<i>B. licheniformis</i> CKA1	75.43	15.57	5.43	5.91	17.57
<i>S. plymuthica</i> G4(3)	58.40	10.31	4.83	4.76	15.73
<i>B. methylotropicus</i> CKAM	60.21	11.71	4.90	5.03	16.80
<i>B. methylotropicus</i> RG1(3)	68.35	14.20	5.23	5.37	17.14
Consortium*	67.50	13.05	5.11	5.23	16.87
CD _{0.05}	6.54	3.40	1.08	0.95	2.97

*Consortium: CKA1+G4(3)+CKAM+RG1(3)

Bacterial strains are known with accelerating potential on germination rate, improvement in seedling emergence, and promotion on plant growth establishment under adverse conditions (Chanway, 1997; Wu *et al.* 2005) and enhanced early plant growth (Bent and Chanway, 1998; Aslantas *et al.* 2007). These observations were documented by different researchers suggested towards the hypothesis that bacterization of apple seeds with bacterial strains could have an encouraging impact on apple seed germination (Meshram *et al.* 1994).

4.10.2 Plant growth response of apple seedlings to different bacterial strain inoculation under net house and field nursery conditions

Plant growth response to seed and seedling treatment with liquid based formulation of bacterial strains on various attributes of apple seedlings was studied under net house and field nursery conditions.

4.10.2.1 Effect of seed treatment on the growth of apple seedlings under net house conditions

The following treatments were assayed for their effect on apple seed germination:

1. Un-inoculated seeds as control
2. Seed treatment and drenching with different PGPR:
 - a) *B. licheniformis* strain CKA1,
 - b) *Serratia plymuthica* strain G4 (3),
 - c) *B.methylophilus* strain CKAM,
 - d) *B.methylophilus* Strain RG1 (3)
 - e) Consortium (CKA1+CKAM+G4(3)+RG1(3))

Ten seedlings (six month old) from each treatment selected at random were uprooted and the effect on various shoot and root attributes was observed. The data represented in Table 16 is an average of data observed for two individual experiments carried out in year 2013 and 2014. The result revealed significant increase in various shoot and root parameters when the seeds were treated with liquid based formulation of bacterial strains over uninoculated control (Fig.26 and Plate 6 and 7).

Among the different PGPR treatments, maximum shoot length (15.57 cm) was observed in case of seedlings raised from apple seeds treated with *B. licheniformis* strain CKA1 and minimum was observed in *S.plymuthica* strain G4(3) (10.31cm).



Plate 6. Effect of selected PGPR inoculations on plant growth parameter of apple seedling raised from treated seeds under net house conditions



Plate 7. Comparison of different PGPR inoculations on growth of apple seedling under net house conditions

Shoot length of seedlings raised with *B.methylootropicus* strain RG1(3) (14.20 cm) and with consortium (CKA1+CKAM+G4(3)+RG1(3)) (13.05cm) were statistically at par with *B. licheniformis* strain CKA1. Similar trend was observed in case of seedlings raised with *S.plymuthica* strain G4(3) (10.31cm).

Among all the treatments, maximum shoot diameter (5.43mm) was observed in case of seedlings raised from *B. licheniformis* strain CKA1 seed treatment which was significantly higher than uninoculated control (4.20mm). Shoot diameter of seedlings raised after seed treatment with *B.methylootropicus* strain RG1(3) (5.23mm), consortium (5.11), *B.methylootropicus* strain CKAM (4.90mm) and *S.plymuthica* strain G4(3) (4.83mm) were at par with *B. licheniformis* strain CKA1.

Effect of PGPR inoculations on leaf surface area followed a trend similar to as observed in shoot diameter. Maximum leaf surface area (17.57cm²) was observed with *B. licheniformis* strain CKA1 treatment. *B.methylootropicus* strain RG1 (3), consortium, *B.methylootropicus* strain CKAM and *S.plymuthica* strain G4 (3) accounted for 17.14 cm², 16.87 cm², 16.80 cm², 15.73 cm² leaf surface area, respectively.

Maximum root length (5.91cm) was observed in *B. licheniformis* strain CKA1 treated seedlings. Root length of seedlings after treatment with *B.methylootropicus* strain RG1(3) (5.37cm), consortium (5.23cm), *B.methylootropicus* strain CKAM (5.03cm) were statistically at par with each other and with *B. licheniformis* strain CKA1 and significantly higher than *S.plymuthica* strain G4(3) (4.76cm).

Seed treatment with *B. licheniformis* strain CKA1, *S.plymuthica* strain G4 (3), *B.methylootropicus* strain CKAM, *B.methylootropicus* strain RG1 (3) and consortium accounted for 72.80, 14.42, 29.90, 57.60 and 44.83 per cent increase over uninoculated control, respectively in case of shoot length (Fig. 26); 29.07, 15.00, 16.66, 24.52 and 21.66 per cent increase over un-inoculated control, respectively, in case shoot diameter; 56.76, 26.25, 33.42, 42.44 and 38.42 per cent increase, respectively, over un-inoculated control in case of root length; 23.47, 10.54, 18.06, 20.44, 18.55 per cent increase, respectively, over un-inoculated in case of leaf surface area.

4.10.2.2 Effects of inoculations with selected PGPR on growth of one year old apple seedlings under net house conditions

Effect on one year old apple plants under net house conditions

The results obtained from two independent trials carried out in 2013 and 2014 on one year apple plants under net house conditions showed significant increase in various plant growth parameters (Table 17; Fig. 27 and Plate 8).

The following treatments were assayed in the trial carried out in 2013 and 2014:

1. Un-inoculated apple plants
2. Apple seedlings dipped in liquid formulation of following PGPR (in the month of January; followed by its repeated inoculation through soil drenching, post grafting thrice in the months of April, July and October, 2014)
 - a) *B. licheniformis* strain CKA1,
 - b) *S. plymuthica* strain G4 (3),
 - c) *B. methylotrophicus* strain CKAM,
 - d) *B. methylotrophicus* Strain RG1 (3)
 - e) Consortium (CKA1+CKAM+G4(3)+RG1(3))

Twenty five apple seedlings, from each treatment, selected at random were uprooted and the effect on various shoot and root attributes were observed and the data is presented in Table 17; Figure 27; Plate 8. The result revealed a significant increase in various shoot and root parameters over un-inoculated control.

Shoot parameters

Based on the data obtained from trial carried out in 2013, significant increase in shoot length, shoot diameter and leaf surface area was observed over un-inoculated control (Table 17, Fig. 27).

Seedlings raised with *B. licheniformis* strain CKA1 resulted in maximum shoot length (197.40cm) and shoot diameter (9.42 mm) which was significantly higher than un-inoculated control (156.90cm shoot length and 7.03 mm shoot diameter) and all other PGPR treatments except *B. methylotrophicus* strain RG1(3) accounting for 25.81 and 33.99 per cent increase over uninoculated control in case of shoot length and leaf surface area (Fig. 27).

B.methylootropicus strain RG1(3) treatment resulted in significant increase in shoot length (187.40cm) and shoot diameter (8.56) over un inoculated control and was found at par with *B. licheniformis* strain CKA1.

Similar trend was observed in case of root length and leaf surface area, with a maximum of 51.43cm and 29.50cm² of root length and leaf surface area, respectively with *B. licheniformis* strain CKA1 treatment. Results were significantly higher than un-inoculated control with root length of 42.34cm and leaf surface area of 20.50 cm² and all other PGPR treatments except *B.methylootropicus* strain RG1(3). *B. licheniformis* strain CKA1 treatment accounted for 21.46 per cent increase over un-inoculated control in case root length and 43.90 per cent increase over un-inoculated in case of leaf surface area (Fig. 27).

B.methylootropicus strain RG1(3) treated seedlings resulted in 49.28cm and 28.90 cm² of root length and leaf surface area, respectively which were significantly higher than un-inoculated control and statistically at par with *B. licheniformis* strain CKA1.

Table 17. Effect of selected PGPR inoculation on vegetative growth of one year old apple seedling under net house conditions

Treatments	Shoot length (cm)	Shoot diameter (mm)	Root length (cm)	Leaf surface area (cm ²)
Un-inoculated control	156.90	7.03	42.34	20.50
<i>B.Licheniformis</i> CKA1	197.40	9.42	51.43	29.50
<i>S.plymuthica</i> G4(3)	181.26	7.81	46.57	22.76
<i>B.methylootropicus</i> CKAM	190.95	8.56	49.28	28.90
<i>B.methylootropicus</i> RG1(3)	187.40	7.80	46.90	22.50
Consortium*	184.30	8.05	47.70	26.73
CD _{0.05}	9.34	1.36	4.57	3.70

*Consortium :CKA1+G4(3)+CKAM+RG1(3)

4.10.2.3 Effect of PGPR inoculation on vegetative growth of two year old grafted apple seedlings under net house conditions

After one year, i.e. in 2014 all the seedlings from each treatment were grafted with different cultivars and effect of each treatment on grafted seedlings were observed. Twenty five apple plants from each cultivar i.e. Red Chief, Oregon Spur and Top Red, from each treatment, selected at random were uprooted and the effect on various shoot and root attributes were observed and the data is presented in table 18; fig. 28 (A, B, C). The result

revealed a significant increase in various shoot and root parameters of grafted apple plants over un-inoculated control.

After nine months of grafting, among different PGPR strains and their consortium, maximum shoot length, shoot diameter and leaf surface area were observed in *B. licheniformis* CKA1 treated plants (cv. Red Chief, Top Red and cv. Oregon spur) followed by *B. methylotropicus* RG1(3) treatment. Minimum shoot length (74.57cm, 69.00cm, 65.82cm), shoot diameter (18.95cm, 18.41cm, 18.56cm) and leaf surface area (21.44cm², 17.96 cm², 19.50 cm²) were observed in CKAM treated plants viz. Red Chief, Top red and Oregon Spur respectively (Table 18).

CKA1 treated apple plants (cv. Red Chief) resulted in 79.23cm as compared to 69.90cm of shoot length observed in un-inoculated plants, accounting for maximum 13.34 per cent increase, which is significantly higher than un-inoculated control (Fig. 28(A)).

Table: 18 Effect of PGPR inoculation on vegetative growth of two year old grafted apple seedling under net house conditions

cv. Red chief			
Treatments	Shoot length (cm)	Shoot diameter (cm)	Leaf surface area(cm ²)
Un-inoculated control	69.90	14.77	20.80
<i>B.licheniformis</i> CKA1	79.23	20.90	25.56
<i>S.plymuthica</i> G4(3)	75.44	19.10	22.00
<i>B.methylotropicus</i> RG1(3)	78.60	20.62	24.34
<i>B.methylotropicus</i> CKAM	74.57	16.79	21.44
Consortium*	75.68	19.46	24.30
CD _{0.05}	4.83	3.95	1.46
cv. Oregon Spur			
Un-inoculated control	68.50	13.13	17.43
<i>B.licheniformis</i> CKA1	80.52	20.57	20.41
<i>S.plymuthica</i> G4(3)	74.32	19.34	18.89
<i>B.methylotropicus</i> RG1(3)	78.85	20.50	19.10
<i>B.methylotropicus</i> CKAM	73.17	18.41	18.70
Consortium*	72.37	19.48	17.90
CD _{0.05}	5.95	4.23	1.49
cv. Top Red			
Un-inoculated control	68.66	14.40	18.72
<i>B.licheniformis</i> CKA1	77.32	20.98	21.36
<i>S.plymuthica</i> G4(3)	72.41	19.52	19.79
<i>B.methylotropicus</i> RG1(3)	74.20	20.81	21.00
<i>B.methylotropicus</i> CKAM	70.10	19.40	19.50
Consortium*	70.81	18.01	20.36
CD _{0.05}	4.75	4.10	1.57

*Consortium: CKA1+G4(3)+CKAM+RG1(3)

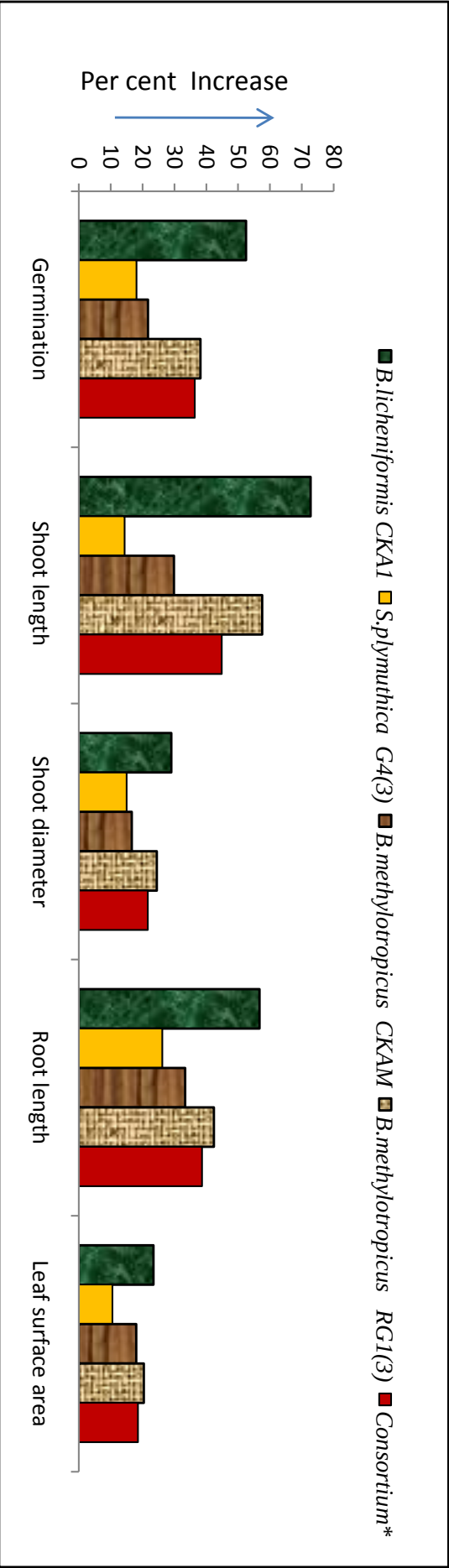


Figure 26. Per cent increase in seed germination and shoot and root parameters of apple seedling over untreated control under net house conditions

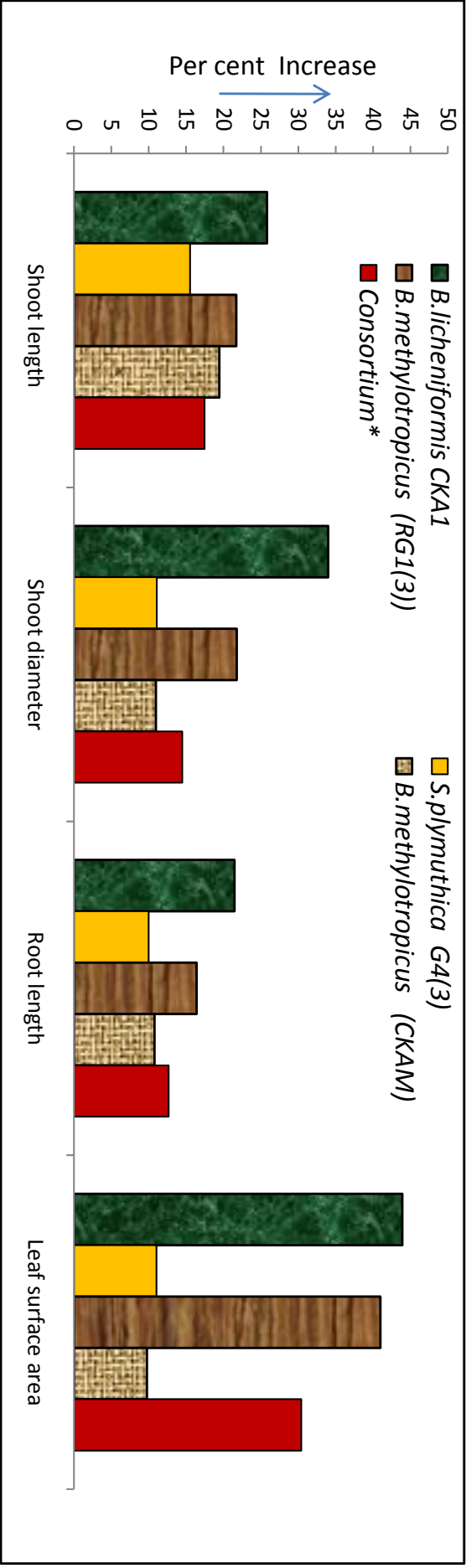


Figure 27. Per cent increase in growth parameters over un-inoculated control in one year old apple seedling under net house conditions



Plate 8. Effect of bacterial strains on growth of apple seedlings (uprooted) under net house conditions

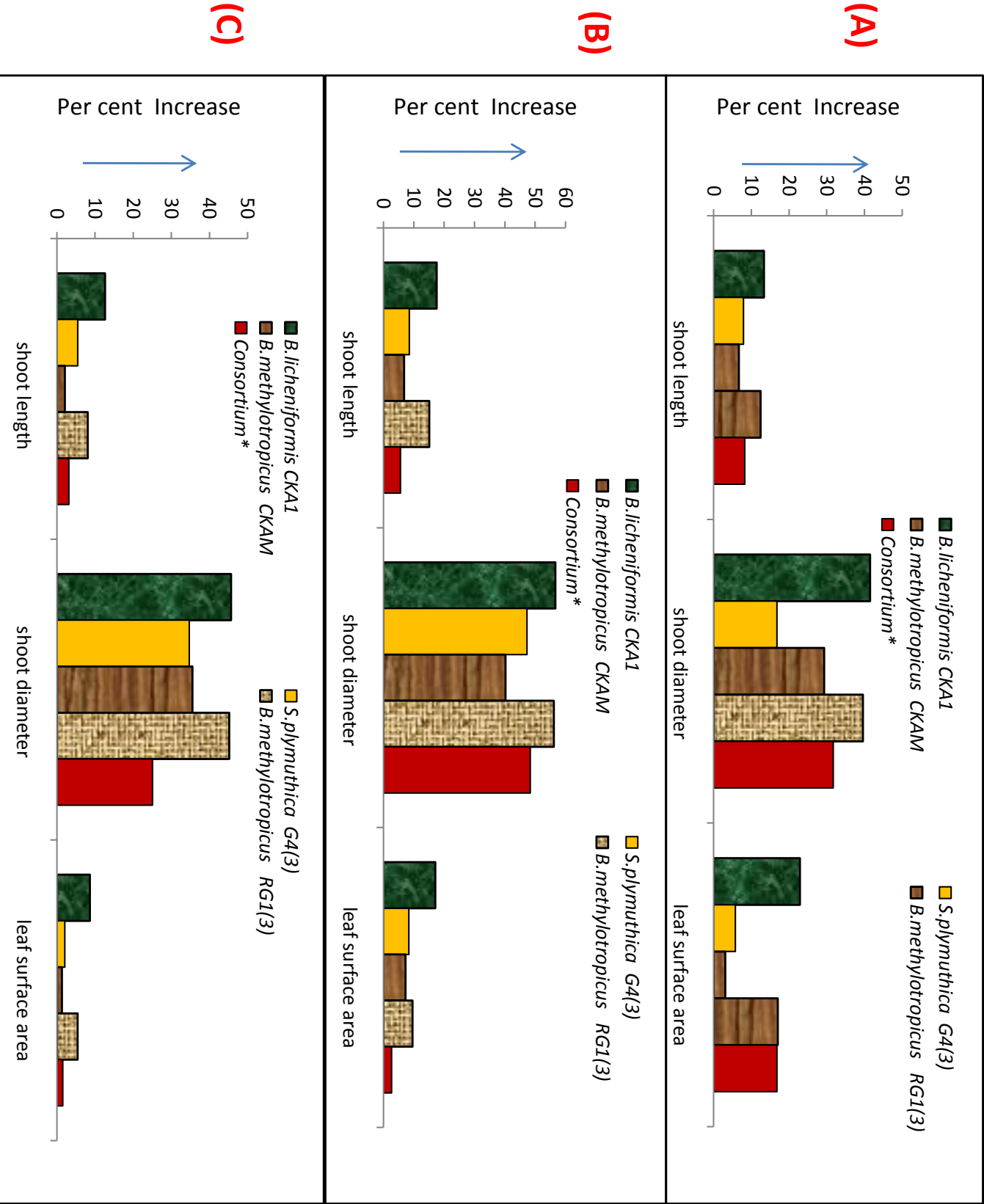


Figure 28. Per cent increase in growth parameters of grafted apple seedling over un-inoculated control under net house conditions (A) Red Chief (B) Oregon Spur (C) Top Red

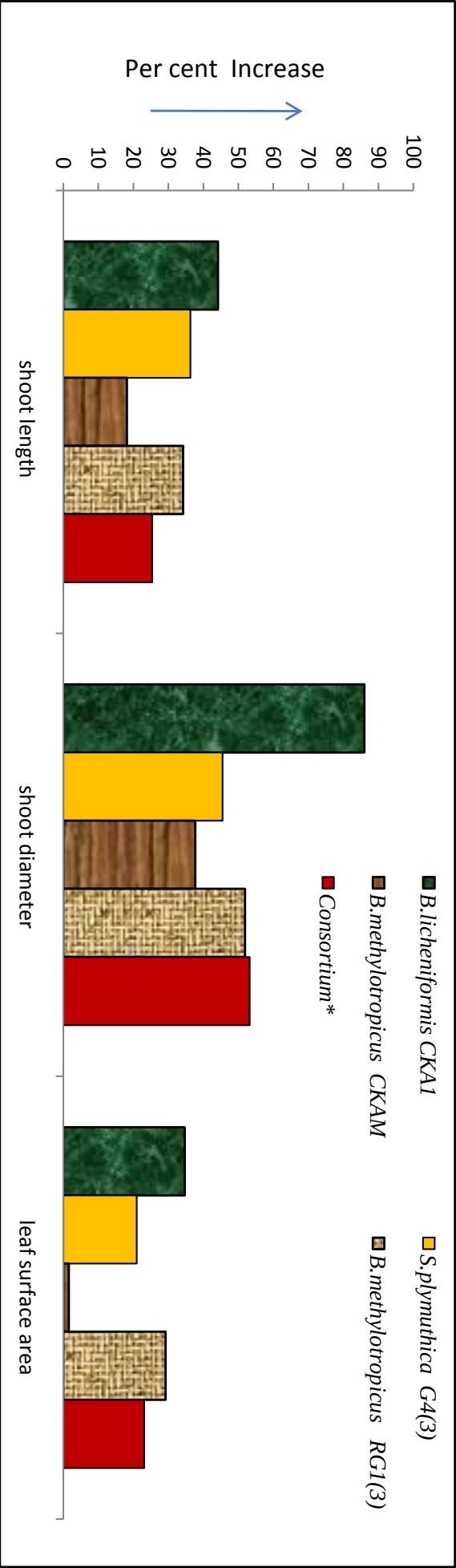


Figure 29. Per cent increase in growth parameters of two months old transplanted apple seedling over un-inoculated control under nursery field



Plate 9. Growth promotion of two months old transplanted apple seedlings under apple field nursery

1993; Kundu *et al.* 2002) including apple (Larcher *et al.* 2003; Shirkot and Sharma, 2005; Cakmakc *et al.* 2007). IAA positively influences root growth and development, thereby, enhancing nutrient uptake (Khalid *et al.* 2004). An increase in root length and the volume of soil foraged by the roots, which leads to an enhanced nutrient uptake, is the most commonly proposed explanation for the beneficial effects of plant beneficial bacteria on plant growth.

Table: 19 Effect of PGPR inoculation on vegetative growth of two month old transplanted apple seedling under nursery field conditions after six months of plantation

Treatments	Shoot length (cm)	Shoot diameter (mm)	Leaf surface area (cm ²)
Un-inoculated control	88.27	3.87	23.91
<i>B.licheniformis</i> CKA1	127.30	7.20	32.21
<i>S.plymuthica</i> G4(3)	120.30	5.63	28.92
<i>B.methylotropicus</i> CKAM	104.32	5.33	24.29
<i>B.methylotropicus</i> RG1(3)	118.50	5.88	30.90
Consortium*	110.70	5.93	29.43
CD _{0.05}	6.44	1.28	4.59

*Consortium: CKA1+G4(3)+CKAM+RG1(3)

The study illustrated the significance of rhizobacteria under *in vitro* conditions for multiple PGPR traits and their evaluation under controlled conditions in a pot trial experiment and uncontrolled conditions under nursery field conditions. Findings in the present study based upon comparison of different PGPR strains and their consortia conclusively indicate that *B.licheniformis* strain CKA1 with multiple PGPR traits proved statistically most effective in improving the plant growth parameters of apple plants and therefore led to its selection for subsequent studies on growth yield of apple under field conditions.

4.10.2.5 Effect of most efficient *B. licheniformis* strain CKA1 on percent nutrient (NPK) in six months old apple seedling under net house and apple nursery field conditions

Effect of seedling treatment with liquid based formulation of *B. licheniformis* strain CKA1 on per cent nitrogen (N), phosphorus (P) and potassium (K) content of whole shoot

system was determined in six months apple seedlings under net house and apple field nursery (Fig. 30). The results revealed significant increase in per cent NPK content in apple seedlings raised from CKA1 treated seeds, over un-inoculated control.

Seedlings raised with *B. licheniformis* strain CKA1 resulted in maximum (2.00) per cent nitrogen under net house and (1.81) per cent nitrogen under apple nursery field and minimum (1.18) per cent nitrogen was observed in un-inoculated seedlings. *B. licheniformis* strain CKA1 treatment resulted in 9.52 per cent increase over un-inoculated control under net house and 53.26 per cent increase over un-inoculated control under nursery field conditions.

Maximum (1.45) per cent phosphorus was also observed in seedlings raised from with *B. licheniformis* strain CKA1 under net house, which accounted for 178.64 per cent increase over un-inoculated control. Seedling treatment with CKA1 resulted in 1.15 per cent phosphorus in apple seedlings under apple nursery field (134.47 per cent increase over un-inoculated control). Minimum (0.95) per cent phosphorus content was observed in un-inoculated seedlings.

A trend similar to that observed in case of per cent nitrogen and phosphorus content in six month apple seedlings was also observed for per cent potassium content. Seedlings raised with *B. licheniformis* strain CKA1 treatment under net house condition resulted in maximum (0.57) per cent potassium (51.84 per cent increase over un-inoculated control) and 0.48 per cent potassium as observed in case of seedlings raised from with liquid formulation under apple nursery field (20.25 per cent increase over un-inoculated control) and minimum (0.21) per cent potassium was observed in seedlings raised from un-inoculated seeds.

The findings in the present study highlight the multifarious plant growth promoting potential of *B. licheniformis* strain CKA1 in effectively mobilizing nutrients in soil and to make it available for plant uptake. Similar studies employing different PGPR inoculation in different cropping system have resulted in mobilizing nutrients from soil to plants (Ashrafuzzaman *et al.* 2009; Mahajan, 2013; Sharma *et al.* 2015).

4.11 EFFECT OF *Bacillus licheniformis* CKA1, *B. methylotrophicus* CKAM, *Serratia plymuthica* G4 (3) AND *B. methylotrophicus* RG1 (3) INOCULATION ON HEALTH OF APPLE SEEDLINGS (WHITE ROOT ROT AND COLLAR ROT DISEASE MANAGEMENT IN APPLE)

Having biochemically and molecularly characterized bacterial strains for the indirect mechanism of plant growth promotion i.e. via production of antifungal antibiotic metabolites

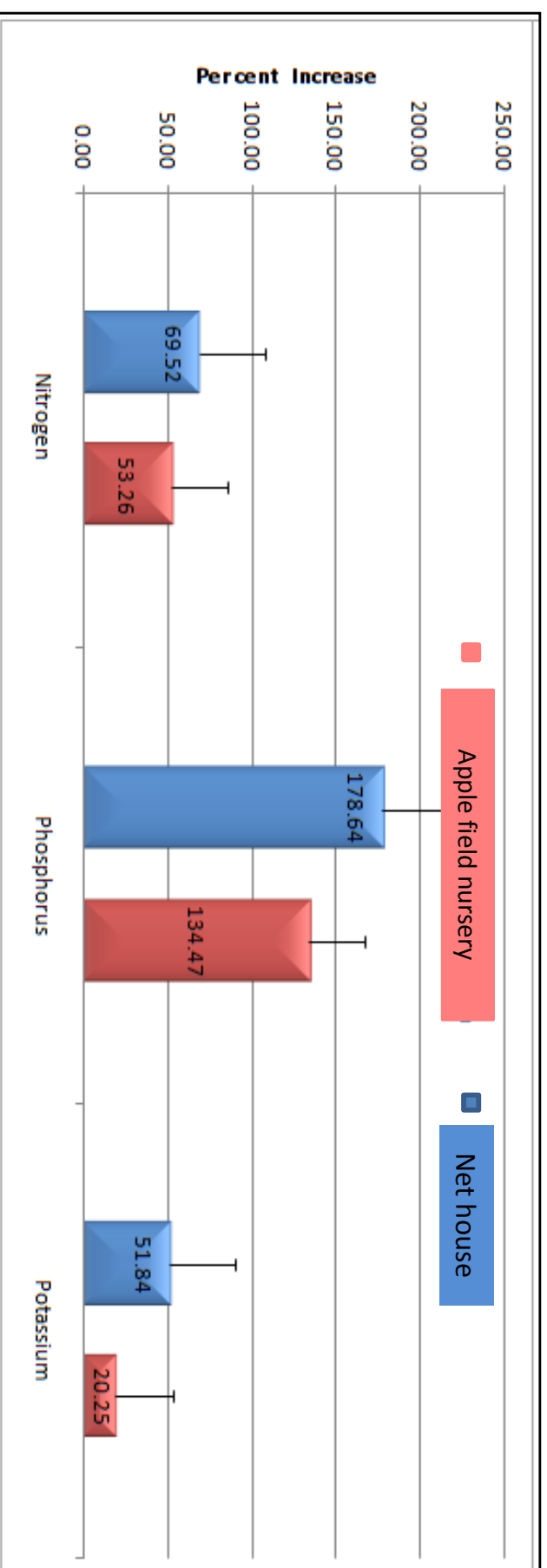
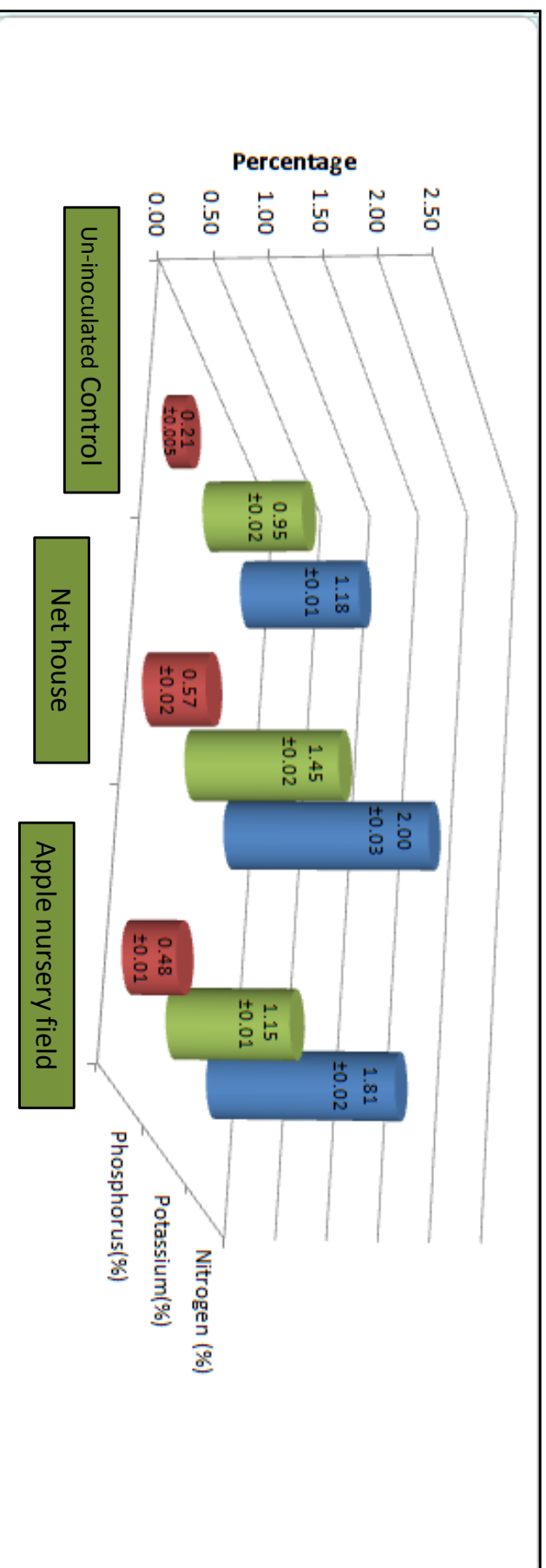


Figure:30 Effect of *B. licheniformis* strain CKA1 on (a) percent nutrient (NPK) in six month old apple seedling under net house and apple nursery field conditions (b) per cent increase over un-inoculated control (data average 2013 and 2014)

against *Dematophora necatrix* and *Phytophthora cactorum*, the strains CKA1 was evaluated for its effect in managing white root rot and collar rot disease in apple seedling under net house conditions.

4.11.1 Effectiveness of bacterial strains in preventing white root rot disease in apple seedlings/plants under net house conditions

White root rot disease, caused by *Dematophora necatrix* Hartig, is very serious soil-borne disease infecting temperate fruits such as apple, pear and avocado (Duan *et al.* 1990; Sharma *et al.* 2005). The root rot diseases are mainly incited by soil-borne pathogens, which are difficult to control through chemical means alone as the efficacy of chemicals is affected by several factors in complex system like soil. Besides, such management practice results in soil and water pollution as well as causes inhibition/ suppression of beneficial soil microflora. Concern about the adverse consequences of chemicals on environment has forced researchers to seek alternatives to chemicals. One of the viable options available is the use of biocontrol agents against soil-borne pathogens (Ashwani *et al.* 2004; Harman, 2006). Therefore, the present investigation was aimed to assess the impact of biocontrol agents on white root rot disease vis-à-vis health of apple seedlings under net house conditions.

In planta biocontrol experiment was initiated in 2013 with twenty apple seedlings (six month old) in total were taken per treatment for computing of data based on per cent disease incidence and per cent disease severity (based on foliar symptoms) and the per cent disease control was calculated accordingly.

The general observation was that, the first sign of wilt appeared on apple seedlings after fifteen days of fungal challenge over un-inoculated control and intensified gradually. The leaves, twigs or the whole plant, turned brown and later died.

The following treatments (applied in 2013) were assayed for their effect in preventing white root disease in apple seedlings under net house conditions.

1. Un-inoculated seedlings
2. Un-inoculated seedlings + Fungal challenge (FC) 180 days after plantation
3. *B. licheniformis* CKA1 seed treated raised seedlings + Soil drenching (SD) with CKA1 after planting + FC 180 days after plantation

4. *B. methylotropicus* CKAM seed treated raised seedlings + Soil drenching (SD) with CKA1 after planting + FC 180 days after plantation
5. *S. plymuthica* G4 (3) seed treated raised seedlings + Soil drenching (SD) with CKA1 after planting + FC 180 days after plantation
6. *B. methylotropicus* RG1 (3) seed treated raised seedlings + Soil drenching (SD) with CKA1 after planting + FC 180 days after plantation
7. Conjoint application of all four strains+ Soil drenching (SD) with after planting + FC 180 days after plantation

Four most potential PGPR strains were selected for *planta* experiment on apple so that the best antagonistic strain will be selected to use as biocontrol agent. Seedling root dip treatment in liquid-based culture of four efficient bacterial antagonists alone and in their consortia significantly reduced the disease severity compared with the positive control (untreated but pathogen inoculated) (Table 20, Plates 10, 11, 12 and 13). Disease ratings were observed twice during the experiment: first at two week after the pathogen challenge and again after four weeks of pathogen challenge. Liquid-based culture of strain CKA1 was found to be the most promising treatment for biocontrol with minimum 20.00 % disease incidence observed four weeks after the pathogen challenge and this was found to be highly significant as compared to other treatments. Disease severity index in the positive control was 84 % whereas it ranged from 20.00% to 84.00% in antagonists treated-plants. Strain CKA1 had the most significant effect in reducing the disease severity by 80%. The consortium of four strains also had no effect in achieving biocontrol efficacy against fungal pathogen.

From the biocontrol experimental results, 40.00 % disease incidence was observed in seedlings treated with PGPR strain G4(3) after four week of fungal challenge. Treatment with inoculation of strain G4(3) resulted in reducing the disease severity by 60.00%.

Seedlings raised by treatment with strain CKAM and strain RG1(3) root dip and with subsequent fungal challenge, resulted with 90.00% disease incidence after four week of fungal challenge. Treatment and inoculation of these strains resulted in reducing the disease severity by 10.00%.

Conjoint application of all the four strain was found not effective in controlling root rot disease of apple. In the present study, *B. licheniformis* strain CKA1, efficiently suppressed white root rot of apple caused by *Dematophora necatrix*. Biocontrol bacteria colonizing the

roots have been previously reported to compete and interact with the indigenous rhizobacterial population and with the phytopathogenic fungi. Some *Bacillus subtilis* strains when applied to seeds or mixed with soil, have been reported to provide crop protection mostly due to direct control of soil borne pathogens through efficient production of various fungitoxic chemicals (Phae *et al.* 1990; Shoda, 2000; Leclere *et al.* 2005). Visualization of root colonization at mycelia level is important for establishment of control of fungal infection in addition to the aerial symptoms on various parts of apple seedlings. Absence of white mycelia growth on healthy roots of bacterial treated seedlings, even after fungal challenge, is a clear proof of fungal disease control of white root rot of apple by *B. licheniformis* strain CKA1.

Ieki *et al.* (1969) have reported the inhibition of *R. necatrix* by different isolates of *Trichoderma* spp. Species of *Trichoderma* viz., *T. harzianum* and *T. hamatum* isolated from naturally roots rot infected root inhibited the growth of the fungus *R. necatrix* (Freeman *et al.*, 1986.) The use of antagonistic *Trichoderma* spp. against the disease has also been explored along with soil solarization (Sztejnberg *et al.* 1987). Reduction of root rot by using the antagonists like *T. harzianum*, *T. koningii* and *T. viride* has also been noticed under pot culture studies (Sharma, 1993). Sharma (2000) have also reported that fungal antagonists *T. harzianum* and *T. viride* and bacterial antagonists *Pseudomonas fluorescens* and *Bacillus* spp. were effective in controlling root rot under pot culture studies. Similarly, Mahajan (2013) had also reported the inhibition of *D. necatrix* by *Bacillus licheniformis* under *in vivo* and *in vitro* conditions. *Bacillus licheniformis* have resulted in 100% biocontrol efficacy of root rot of apple under net house studies.

Besides antagonistic fungi and bacteria the arbuscular mycorrhizal fungi (AMF) have also been used against soil borne diseases. Bharat and Bhardwaj (2001) while studying the interactions between AMF and root rot pathogens *D. necatrix* on apple seedlings under pot culture found that the apple seedlings previously inoculated with local AMF isolate of *Glomus* spp. showed less root rot severity as compared to the seedlings which were not inoculated with *Glomus* spp. The mycorrhizal seedlings also exhibited increased growth.

Mehta *et al.* (2014) carried out the similar study where they used the strain *B. methylotropicus* isolated from apple as a biocontrol agent against different fungal pathogens. *B. methylotropicus* exhibited highest protection against fungal pathogen *S. rolfsii*

(58 %) followed by *F.oxysporum* (54.5 %), *D. necatrix* (52.7 %) and *P. aphanidermatum* (36.3 %), respectively under net house condition.

Various mechanisms account for the ability of biocontrol PGPR to control plant pathogens, including competition for iron and other nutrients, niche exclusion, induced systemic resistance (ISR), and production of antimicrobial metabolites such as antibiotics, siderophores and IAA. For effective biocontrol, these antimicrobial metabolites must be delivered at a right time and site by an efficient root colonization process. *B. licheniformis* strain CKA1 used in the present study had shown its ability to act as an efficient root colonizer which could be seen by large population exhibiting similar colony morphotype both in rhizosphere soil and endorhizobacterial count.

Table: 20 Biocontrol efficacy of bacterial antagonists towards *Dematophora necatrix* under net house conditions

Isolates	Disease incidence (%)		Disease severity index (%)		Biocontrol efficacy (%)		Antagonist population after trial ($10^6 \times$ cfu g)
	2 week after pathogen challenge	4 week after pathogen challenge	2 week after pathogen challenge	4 week after pathogen challenge	2 week after pathogen challenge	4 week after pathogen challenge	
<i>Dematophora necatrix</i> (positive control)	70.00 (56.79)	96.00 (78.68)	50.00 (44.98)	84.00 (66.42)	-		-
<i>B.licheniformis</i> (CKA1)	0 (0.00)	20.00 (26.48)	0.00 (0.00)	20.00 (26.38)	100.00	80.00	73.00
<i>S.plymuthica</i> G4(3)	10.00 (18.37)	40.00 (39.21)	13.30 (21.35)	27.50 (31.60)	85.70	60.00	61.00
<i>B.methylotropicus</i> (CKAM)	40.00 (39.37)	90.00 (71.59)	26.66 (31.02)	64.00 (53.10)	42.85	10.00	46.00
<i>B.methylotropicus</i> (RG1(3)	70.00 (56.79)	90.00 (71.59)	36.66 (37.24)	62.00 (51.92)	0.00	10.00	33.00
Consortium	70.00 (56.77)	96.00 (79.09)	50.00 (44.98)	84.00 (66.42)	0.00	0.00	31.00
CD_{0.05}	3.21	4.96	3.10	3.91			5.14

$$\text{Per cent disease incidence} = \frac{\text{Number of diseased plants}}{\text{Total number of plants observed}}$$

$$\text{Disease severity index} = \frac{\sum(\text{Rating} \times \text{Number of plants rated})}{\text{Total number of plants} \times \text{highest rating}} \times 100$$

Similarly, Pasini *et al.* (2015) had documented the efficacy of *Trichoderma atroviride* SC1 as a biocontrol agent against *R. necatrix*. It reduced white root rot incidence on apple seedlings only if treatment was applied at least 1 week before planting. Application of potent biocontrol agents plays important role as it is required for root colonization.



***D. necatrix* fungal inoculum preparation**



Colonization of fungus *Denmatophora necatrix* in untreated apple plants

Plate 10. An overview of inoculum preparation and in planta colonization by *Denmatophora necatrix*



Plate 11. Initial symptoms of root rot of apple after fungal colonization in control



Plate 12. (A) An overview of biological control of root rot of apple under house conditions; Antagonistic activity of CKA1 against *D. nectrix* by (B, C) agar streak method (D) 10% cell free supernatant under lab conditions



Plate 13. Biocontrol efficacy of selected bacterial strains against *D. necatrix* under net house conditions

4.11.2 Effectiveness of *bacterial* strains in preventing collar root rot disease in apple seedlings/plants under net house conditions

Crown rot, also known as collar rot is prevalent in all apple growing regions of the world. It is caused by a oomycete *Phytophthora cactorum*. The disease causes extensive losses sometimes resulting in death of trees. The infection starts from the collar region and spread mostly to underground parts and the above ground stem. Bark at the soil level becomes slimy and rots resulting in cankered area (Bharat, 2011). The attacked trees show chlorotic foliage with red colouration of veins and margins. The causal agent is known to survive in the orchard soils as chlamydospores in plant debris or soil. The pathogen produces oospores which serve as the source of primary inoculums. Moderate temperature and high soil moisture favor the disease.

The following treatments (applied in 2013) were assayed for their effect in preventing collar root disease in apple seedlings under net house conditions.

1. Un-inoculated seedlings
2. Un-inoculated seedlings + Fungal challenge (FC) 180 days after plantation
3. *B. licheniformis* CKA1 seed treated raised seedlings + Soil drenching (SD) with CKA1 after planting + FC 180 days after plantation
4. *B. methylotropicus* CKAM seed treated raised seedlings + Soil drenching (SD) with CKAM after planting + FC 180 days after plantation
5. *S. plymuthica* G4 (3) seed treated raised seedlings + Soil drenching (SD) with G4(3) after planting + FC 180 days after plantation
6. *B. methylotropicus* RG1 (3) seed treated raised seedlings + Soil drenching (SD) with RG1(3) after planting + FC 180 days after plantation
7. Conjoint application of all four strains+ Soil drenching (SD) after planting + FC 180 days after plantation

All the four strains used for *in planta* experiments for the control of root rot disease of apple were also screened for biocontrol of collar rot disease of apple under net house conditions. Seedling root dip treatment with liquid-based culture of four efficient bacterial antagonists alone and in their consortia significantly reduced the disease severity compared with the positive control (untreated but pathogen inoculated) (Table 21). Disease ratings were observed twice during the experiment: first at two week after the pathogen challenge and again at four weeks after the pathogen challenge. Liquid-based culture of *B. licheniformis* strain CKA1 and *S. plymuthica* strain G4(3) were found to be the most promising treatments

for biocontrol with minimum 30.00 % disease incidence after four weeks after the pathogen challenge. Disease severity index in the positive control was 60.0 % whereas it ranged from 30.00% to 60.00% in antagonists treated-plants. Strain *B. licheniformis* CKA1 and *S. plymuthica* strain G4(3) had the most significant effect in reducing the disease severity by 50.00%. The consortium of four strains also had no effect in achieving biocontrol efficacy against fungal pathogen.

B. methylotropicus strain CKAM and *B. methylotropicus* strain RG1(3) resulted in biocontrol with 50.00 % disease incidence observed after four week of fungal challenge. Treatment and Inoculation of these two strains resulted in reducing the disease severity by 16.66%. Bacterial antagonists such as *Pseudomonas* spp., *Bacillus subtilis* were also found affective against *Phytophthora* crown rot (Janisiewicz and Covey, 1983; Utkhede, 1984).

For the management of this disease similar attempts have been made by using chemicals, cultural practices and through host resistance. But the use of biological agents for controlling *P. cactorum* has also been tested by various workers. Some bacterial antagonists have also been found effective in checking the disease. Utkhede and Smith (1997) showed that bacterium *Enterobacter aerogenes* was antagonistic to *P. cactorum*. When this antagonistic bacterial cells were applied as soil drench, reduced the infection of *P. cactorum* in McIntosh apple seedlings.

Strong antifungal potential of *E. aerogenes* and *Bacillus* sp. against *P. cactorum* had been also observed by Gupta and Utkhede (1986) and the treatments of apple plants with these antagonists even managed the crown rot infection under field conditions (Utkhede, 1987). Effect of soil drenching of apple trees with *E. aerogenes* was evaluated for three years by Utkhede and Smith (1991) in British Columbia with good control of the disease. Apple trees treated with strain B8 of *E. aerogenes* for two consecutive years not only reduced *P. cactorum* infection but also increased the plant height (Levesque *et al.* 1993), which are in agreement with the present study where the treatment of *B. licheniformis* CKA1 not only reduces the disease severity but also found to increase the plant height.

Sharma *et al.* (2014) also evaluated the use of different combinations of individually effective management inputs viz., biocontrol agents, bio-resources and fungicides against collar rot disease of apple caused by *Phytophthora cactorum*. The study indicates that a combined application of *Trichoderma harzianum*-1 (TH1), *Enterobacter aerogenes*- 2 (EA-2)

and metalaxyl MZ (0.25%) provided complete control when applied 7 days prior to inoculation under pot and 20 days before planting under nursery conditions, whereas under orchard conditions it increased the shoot length up to 32.2 cm as compared 6.1 cm in untreated plants. Next best treatments were an integrated application of *T. viride*-5 (TV5) + *Bacillus subtilis* 11 (BS11) + metalaxyl MZ (0.25%) and TH1+ EA-2+ mancozeb (0.4%) in order to control collar rot.

Apart from fungal antagonist, Roiger and Jeffer (1991) evaluated *Trichoderma* spp. against *Phytophthora* crown rot of apple and found that *T. virens*, *T. koningii* and *T. harzianum* were effective in controlling the disease. Other species of *Trichoderma*, *T. longibrachiatum*, *T. viride* also proved effective in controlling *P. cactorum* in apple (Kumar, 2002).

Table:21 Biocontrol efficacy of bacterial antagonists towards *Phytophthora cactorum* under net house conditions

Isolates	Disease incidence (%)		Disease severity index (%)		Biocontrol efficacy (%)		Antagonist population after trial (10 ⁶ × cfu g)
	2 week after pathogen challenge	4 week after pathogen challenge	2 week after pathogen challenge	4 week after pathogen challenge	2 week after pathogen challenge	4 week after pathogen challenge	
<i>Phytophthora cactorum</i> (positive control)	50.00 (44.92)	60.00 (50.75)	30.00 (33.18)	53.33 (46.89)	-		-
<i>B.licheniformis</i> (CKA1)	20.00 (26.50)	30.00 (33.19)	20.00 (26.53)	20.00 (26.52)	60.00	50.00	69.00
<i>S.plymuthica</i> G4(3)	20.00 (26.54)	30.00 (33.19)	15.00 (22.75)	23.30 (28.80)	60.00	50.00	60.00
<i>B.methylotropicus</i> (CKAM)	40.00 (39.21)	50.00 (44.98)	23.30 (28.63)	43.33 (41.14)	20.00	16.66	43.00
<i>B.methylotropicus</i> (RG1(3)	40.00 (39.19)	50.00 (44.98)	30.00 (33.93)	40.00 (39.21)	20.00	16.66	38.00
Consortium	50.00 (44.98)	60.00 (50.75)	30.00 (33.19)	53.33 (46.89)	0.00	0.00	30.00
CD _{0.05}	3.19	2.52	2.37	3.02			5.01

$$\text{Per cent disease incidence} = \frac{\text{Number of diseased plants}}{\text{Total number of plants observed}}$$

$$\text{Disease severity index} = \frac{\sum (\text{Rating} \times \text{Number of plants rated})}{\text{Total number of plants} \times \text{highest rating}} \times 100$$

Various mechanisms account for the ability of biocontrol PGPR to control plant pathogens, including competition for iron and other nutrients, niche exclusion, induced systemic resistance (ISR), and production of antimicrobial metabolites such as antibiotics, siderophores and IAA. For effective biocontrol, these antimicrobial metabolites must be

delivered at a right time and site by an efficient root colonization process. The strain *B. licheniformis* strain CKA1 used in the present study is an efficient root colonizer as shown by the large population both in rhizosphere soil and endorhizobacterial count.

Therefore results of present study demonstrate that *B. licheniformis* strain CKA1 has ability to prevent white root rot and collar root rot disease in apple under net house conditions and could be used as efficient plant growth promoting rhizobacteria with strong biocontrol efficacy.

4.12 OPTIMIZATION OF CULTURAL CONDITIONS FOR IAA PRODUCTION BY *Bacillus licheniformis* CKA1

Phytohormone-like acting compounds such as indole-3-acetic acid (IAA), cytokinins, and gibberellins previously have been suggested to be involved in the phytostimulatory action exerted by the plant-beneficial rhizobacterium (Bloemberg and Lugtenberg, 2001; Bottini *et al.* 2004). Auxin production has been proposed as a major means of attaining early growth promotion. Lambrecht *et al.* (2000); Fogaca and Fett-Neto (2005), have independently proposed that exogenous production of IAA by PGPR influences the rooting capacity and the promotion of root elongation and shoot growth of inoculated plant. Bacterial IAA stimulates the root development of host plant, which results in better absorption of water and nutrients from the soil (Fatima *et al.* 2009; Ahemad and Khan, 2011). The present findings suggest that optimization of cultural conditions for IAA production is a good tool to select effective PGPR for biofertilizer technology.

IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Mutluru and Konada, 2007). Moreover, isolates from the rhizosphere are more efficient auxin producers than isolates from the bulk soil (Sarwar and Kremer, 1992).

The property of synthesizing indole acetic acid is considered as an effective tool for screening beneficial organisms as there have been reports suggesting that IAA producing bacteria have profound effect on plant growth (Govindarajan *et al.* 2007; Kuklinsky *et al.* 2004).

IAA production in presence of tryptophan was measured spectrophotometrically at 535 nm for *B. licheniformis* strain CKA1. Development of pink colour after 30 minutes

indicated IAA production. The use of the technique for the detection of IAA using the Van Urk Salkowski reagent is an important option for qualitative and semi-qualitative determination that assure the presence of the hormone in the supernatant of bacterial cultures or liquid formulations of biological inoculants. The amount of IAA produced by the bacteria was within the detection limits of Salkowski reagent (Ehmann, 1977).

4.12.1 Effect of incubation period on IAA production by endophytic bacterial strain CKA1

The experiment was conducted in order to determine the optimum incubation period for bacterial isolate i.e.CKA1 on IAA production in LB medium. The results revealed that an increase in IAA production was observed up to 96 h of incubation i.e. 20.23 µg/ml with corresponding viable count (86×10^6 CFU/ml) as well as drop in final pH 7.00 to 5.20 and thereafter a significant decrease was observed up to 120 h (Fig. 31). Earlier reports had also shown that IAA production starts after 48 h of incubation and maximum reached at 10 days of incubation with a decline during late stationary phase of growth (Chaiharn and Lumyong, 2011). Erturk *et al.* (2010) had also reported the IAA production up to 48-168 h of incubation which was reported earlier (Chaiharn and Lumyong, 2011).

4.12.2 Effect of temperature on IAA production by endophytic bacterial strain CKA1 in LB medium at 96 h of incubation

The effect of different incubation temperatures (25, 30, 35, 40 and 45°C) on IAA production. The results revealed that maximum IAA production was observed at 35°C (23µg/ml) which corresponded to maximum viable count (112.00×10^6 CFU/ml) as well as maximum drop in final pH from 7.0 to 4.65. Increase in temperature beyond 35°C resulted in significant decrease in IAA production and viable count, with the minimum IAA production of 15.41µg/ml at 45°C and corresponding minimum viable count of 88×10^6 CFU/ml (Fig.32). Dasgupta *et al.* (2015) had studied the effect of temperature on IAA production and their findings were in concomitance with the findings presented in here where the minimum increase in IAA is only upto 37°C of temperature. The reason for decrease in IAA production at high temperature may be due to the influence of culture conditions, growth range and availability of substrates that varied greatly among different species (Vijila, 2000).

4.12.3 Effect of pH on IAA production by endophytic bacterial strain CKA1 in LB medium at 96 h of incubation

The experiment was conducted in order to determine the effect of pH on endophytic bacterial isolate CKA1 for IAA production in LB medium and at 96 h of incubation (Fig. 33). The results in the present study revealed that maximum IAA was produced at pH 7 (25.43 µg/ml) with maximum viable count of 108×10^6 CFU/ml and final pH of 4.50. However significant decrease in IAA production was observed by either increasing or decreasing the pH from 7.0. The decrease in the level of IAA might be due to the release of IAA degrading enzymes such as IAA oxidase and peroxidase, has been reported in some organisms such as *Rhizobium* species (Datta and Basu, 2000). In contrast at pH 9 to 10, *Streptomyces* sp. that had shown production of maximum IAA by Harikrishan *et al.* (2014).

Maximum IAA production at neutral pH (7.0) has been suggested in literature also. It was also observed by Dasgupta *et al.* (2015) that the acidic or high alkaline conditions are not suitable for IAA production, in same context strain CKA1 shows maximum IAA production at neutral pH (7.0)

4.12.4 Effect of inoculum size on IAA production by endophytic bacterial isolate CKA1 in LB medium at 96 h of incubation

The results (Fig. 34) revealed that among different concentration of inoculum size, maximum IAA production was found at 1.5% inoculum size i.e 27.10 µg/ml with corresponding viable count i.e. 131×10^6 CFU/ml at final pH of 4.25. Minimum IAA production was observed at 0.25% inoculum size (22.43 µg/ml) with viable count 89×10^6 CFU/ml and corresponding decrease in pH from 7.0 to 4.70.

4.12.5 Effect of different concentration of tryptophan for IAA production and by endophytic bacterial strain CKA1 in LB medium at 96 h of incubation

Data (Fig. 35) revealed that there was a gradual decrease in the final pH with increase in the concentration of tryptophan. Maximum IAA production was observed at 1.00% of tryptophan (29.20 µg/ml) along with maximum viable count 123×10^6 CFU/ml and final pH 4.38. Minimum IAA production was observed at 0.1% of tryptophan (19.53 µg/ml) with corresponding viable count 112×10^6 CFU/ml and final pH 5.0. Production of IAA varies greatly among different species and is influenced by culture conditions, growth stages and availability of substrate (Vijila, 2000). Bacterial strain isolated from the rhizosphere of various plants have been shown to produce IAA in pure culture (Barea *et al.* 1976). Results in

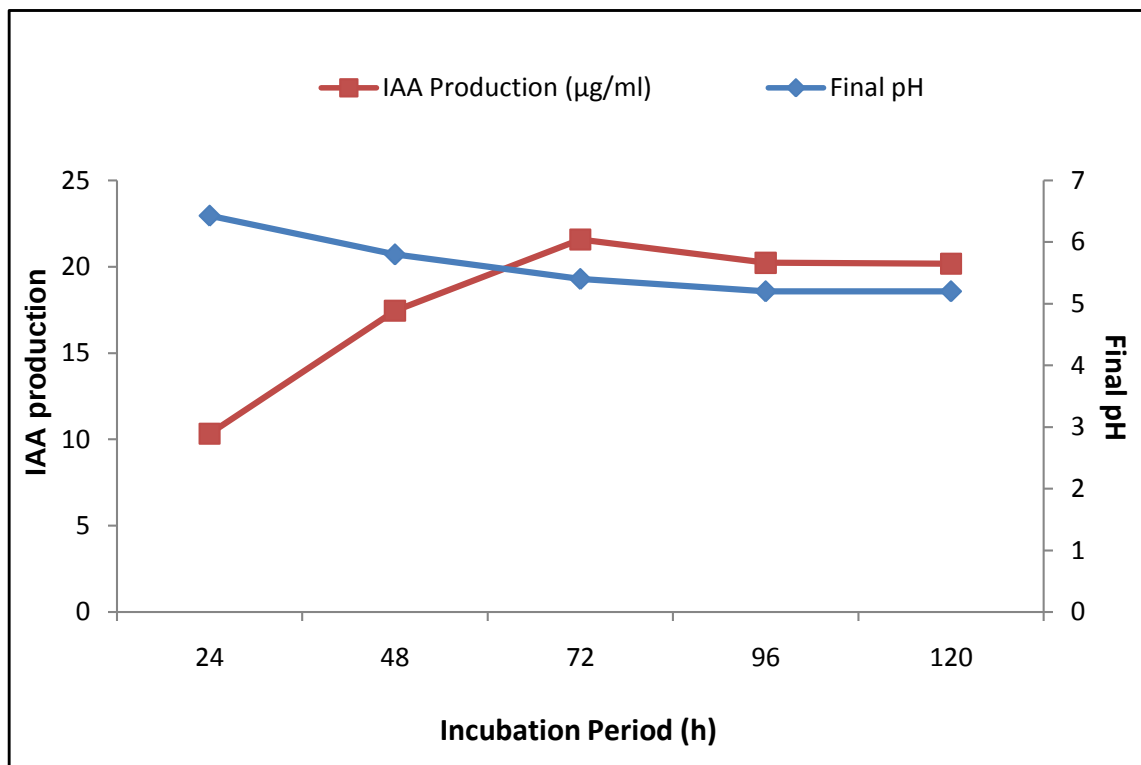


Figure: 31 Effect of incubation period on IAA production in Luria Bertani by endophytic bacterial strain CKA1

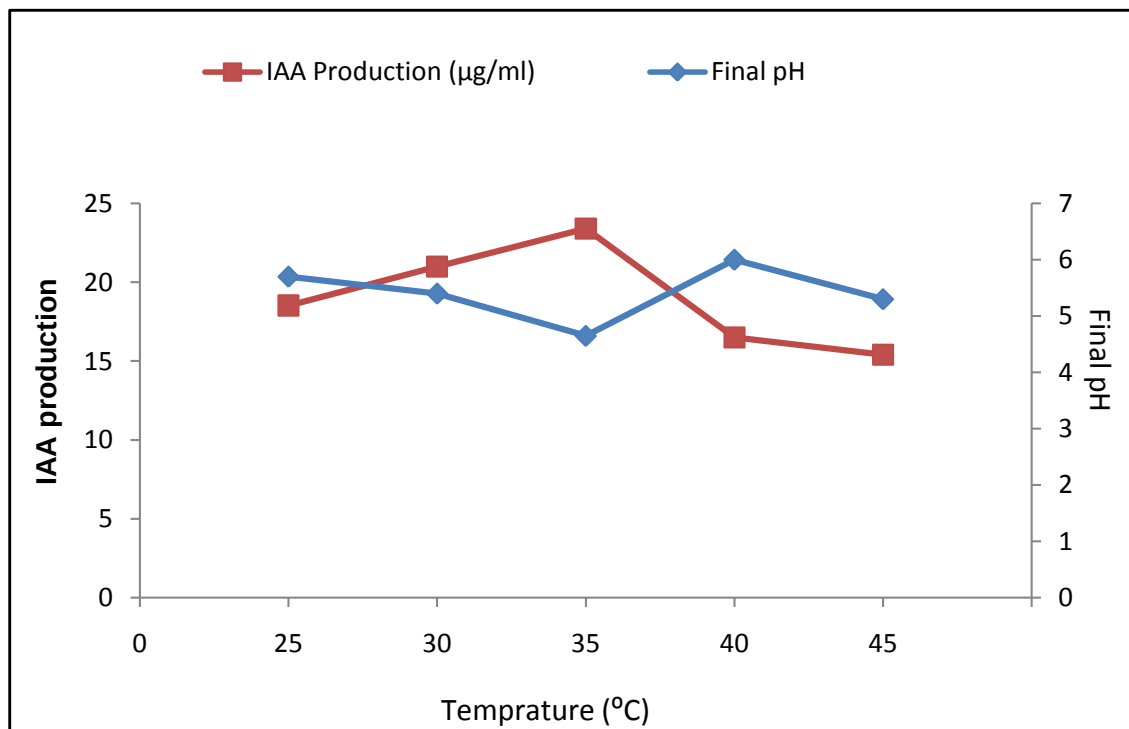


Figure:32 Effect of temperature on IAA production by endophytic bacterial strain CKA1 in Luria Bertani medium at 96 h of incubation

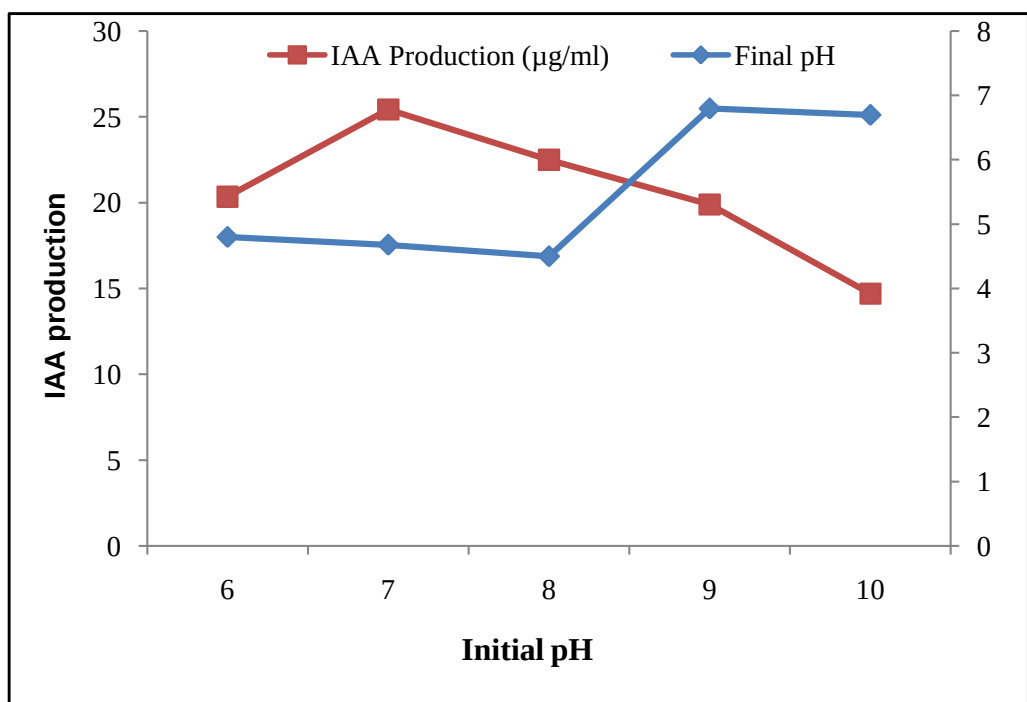


Figure 33 Effect of pH on IAA production in Luria Bertani medium by endophytic strain CKA1 after 72 hours of incubation at 35°C

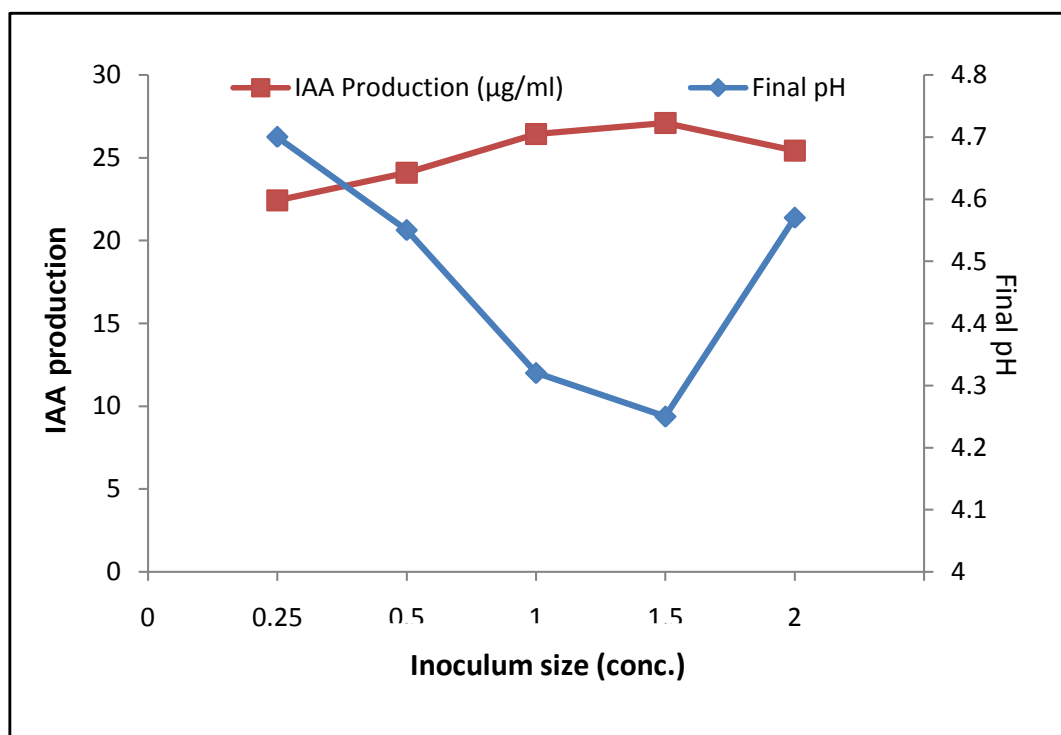


Figure 34 Effect of inoculum size on IAA production in Luria Bertani medium by endophytic strain CKA1 after 72 hours of incubation at 35°C

the present study are in concomitance with literature that had shown higher IAA production in the presence of precursor, L-tryptophan (Chaiharn and Lumyong, 2011).

4.12.6 Effect of different components of LB medium on IAA production by selected bacterial isolate CKA1 at 96 h of incubation

The experiment was conducted in order to determine the effect of different components of LB media on endophytic bacterial strain i.e. CKA1 for IAA production at 96h of incubation. The results revealed that maximum IAA was produced in the absence of NaCl (25.80µg/ml) with maximum viable count of 111×10^6 CFU/ml and final pH of 4.85. Minimum IAA production was observed in the absence of tryptophan (10.54µg/ml) with a viable count of 121×10^6 CFU/ml and final pH of 5.80 (Fig. 36).

The property of synthesizing IAA is considered as effective tool for screening beneficial microorganisms suggesting that IAA producing bacteria have profound effect on plant growth (Wahyudi *et al.* 2011). Inoculation with IAA producing bacteria induces the proliferation of lateral roots and root hairs. Fatima *et al.* (2009) had registered similar effect on germination rate, roots, and shoot growth of plant by the use of PGPR. The result of our finding suggested that simultaneous screening of PGPR isolates for *in vitro* IAA production and growth promotion is a good tool to select efficient PGPR for biofertilizer technology. Therefore, the isolate was studied for its effect on plant growth under controlled conditions

4.12.7 Evaluation and assessment of extracted IAA by thin layer chromatography and high performance liquid chromatography

Production of IAA by most efficient endophytic bacterial strain CKA1 was further confirmed by extraction of crude IAA and subsequent thin layer chromatography (TLC) analysis. TLC of ethyl acetate extracts from strain CKA1 culture that showed a clear pink spot at the R_f value 0.71 corresponding to standard reference of R_f value 0.79 (Fig. 37). A specific spot from the extracted IAA production was found correspond with a standard spot of IAA with similar R_f value. Similar results were documented by Chaiharn and Lumyong, (2011) in which TLC of ethyl acetate extracts from *Klebsiella* SN 1.1 cultures had shown a clear pink spot at the R_f value (0.7) corresponding to standard IAA. Pant and Agarwal (2014) also confirmed the IAA production by thin layer chromatography in which ethyl acetate extract of *Bacillus* showed a clear pink spot at the R_f value (0.9) corresponding to standard run IAA on TLC.

HPLC analysis was conducted to identify and quantify the IAA production precisely. As shown in Fig. 38(B), ethyl acetate extract from the culture filtrate of the strain CKA1 and corresponding reference used as standard (Fig. 38(A)) showed peak at almost the similar retention time (9.0 min).

Similar study on optimization of cultural conditions for maximum production of IAA was carried out by Harikrishanan *et al.* (2014). The strain *Streptomyces* sp. VSMGT1014 ability to produce IAA was confirmed by TLC and further confirmed by the results obtained from HPLC analysis.

The property of synthesizing indole acetic acid is considered as an effective tool for screening beneficial microorganism as there have been reports suggesting that IAA producing bacteria having profound effect on plant growth (Kuklinsky *et al.* 1999; Ali and Hashin , 2007) The improved plant growth by PGPR is due to its ability to produce phytohormones such as indole acetic acid (IAA) (Patten and Glick, 2002), gibberellic acid (Mahmoud *et al.* 1984) and cytokinin (Tien *et al.* 1979).

The work done by Verma *et al.* (2010) suggested that high proportion of rhizo microorganisms are able to produce plant growth hormone, *i.e.*, indole acetic acid, which acts to stimulate plant growth and provides it with more branching and larger surface area. Thus, the IAA producing strain CKA1 was further studied for their effect on plant growth under controlled conditions.

4.13 CLONING AND SEQUENCING OF GLUCOSE DEHYDROGENASE (GDH) - ENCODING GENE (*gdh*) IN *Bacillus licheniformis* CKA1

The present study was conducted in order to test the presence of the gene (*gdh*) encoding the enzyme responsible for gluconic acid production in *Bacillus licheniformis* CKA1. PCR amplification experiments were conducted using specific primers GDHf1, GDHf2, GDHr for *gdh* gene mentioned in material method section 3.10.2.

The optimized temperature profile obtained after standardization using PCR was: an initial denaturation step of 95⁰C for 2 min. followed by 35 cycles of denaturation at 95⁰C for 45 sec., annealing at 55⁰C for 45 sec. and extension at 72⁰C for 1 minute, final extension step of 72⁰C for 10min followed by holding the reaction at 4⁰C for 5 min.

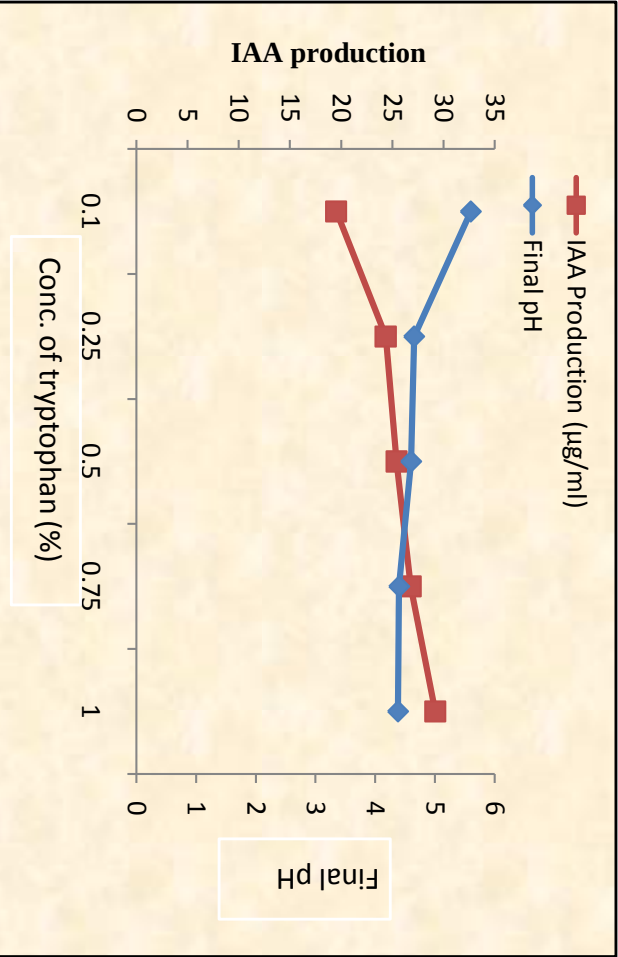


Figure 35. Effect of different concentration of tryptophan on IAA production in Luria Bertani medium by endophytic strain CKA1 after 72 hours of incubation at 35°C

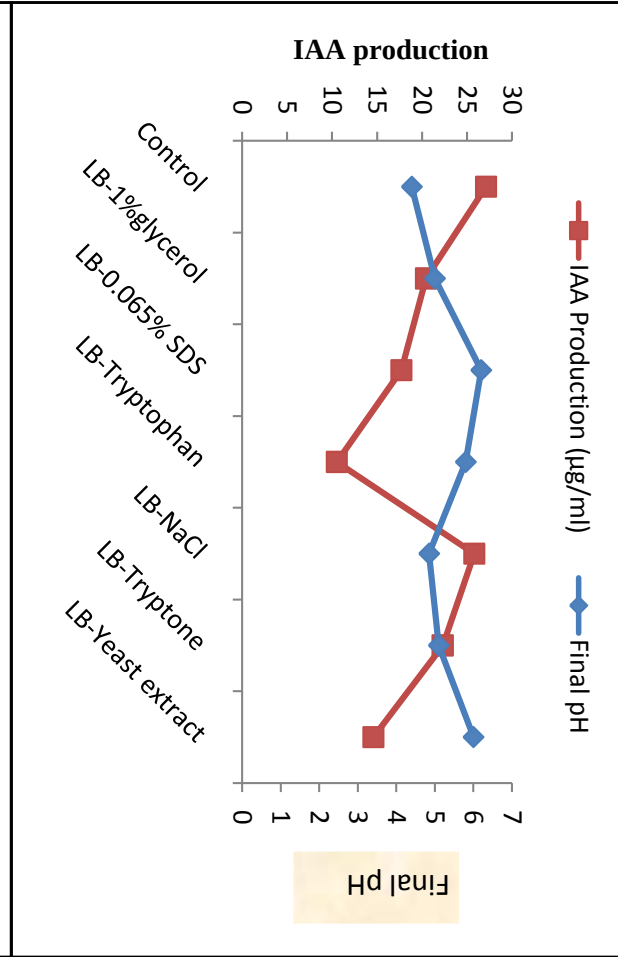


Figure 36. Effect of different components of Luria Bertani medium on IAA production by endophytic strain CKA1 after 72 hours of incubation at 35°C

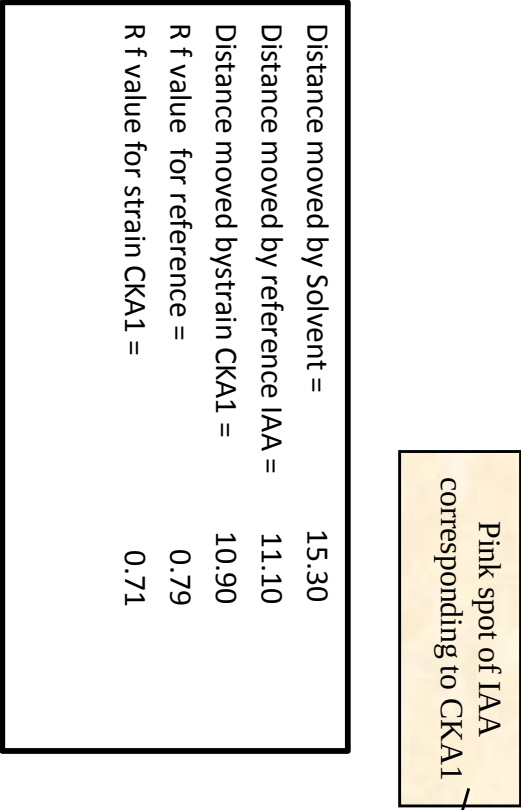


Figure 37. Detection of IAA production in TLC by *B. licheniformis* strain CKA1 compared with standard IAA

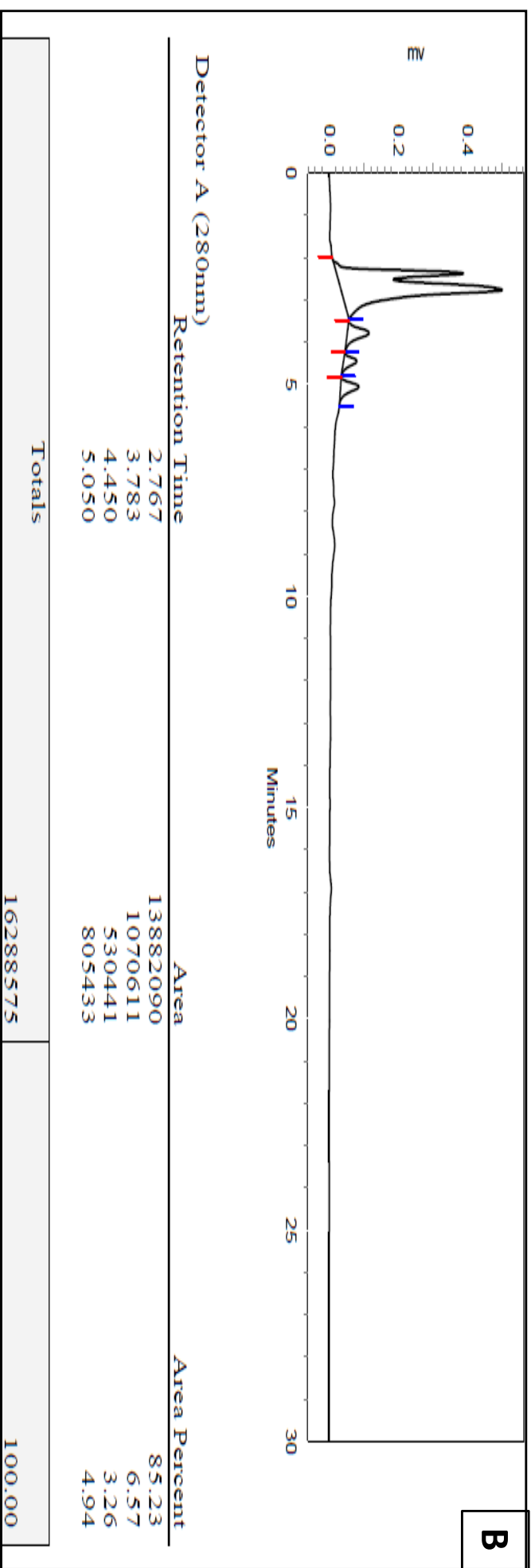
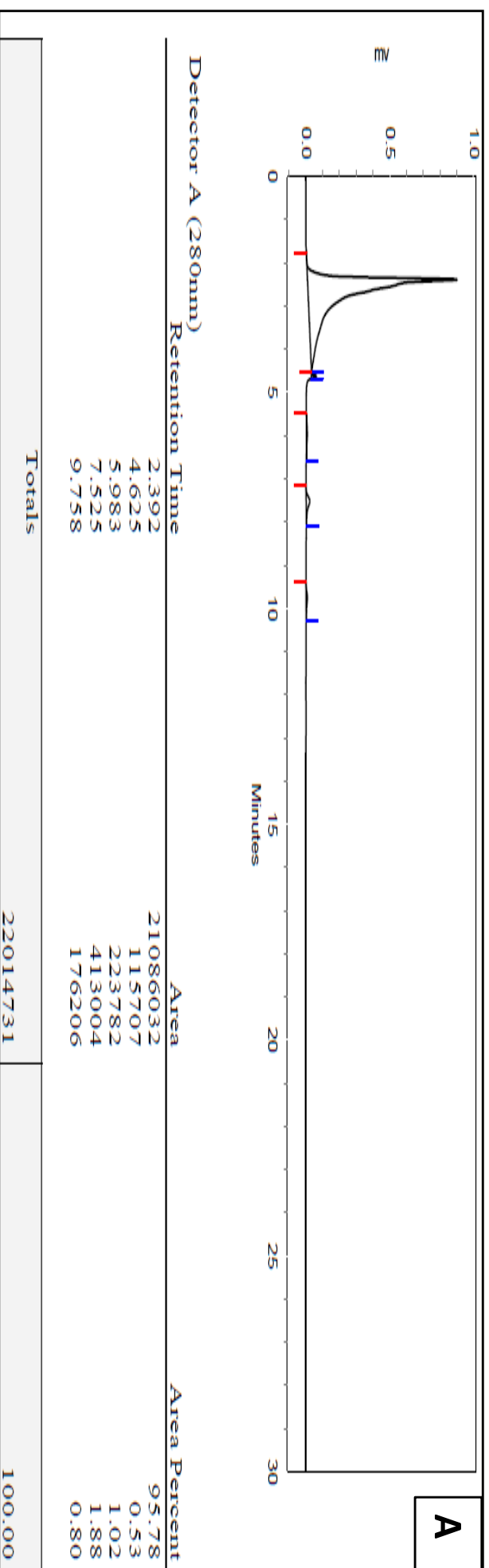


Figure 38. Comparison of HPLC profile of IAA production by CKA1 with authentic standard : (A) Standard IAA (B) IAA production by *B.licheniformis* strain CKA1

An amplicon of expected size i.e. ~ 650-700 bp was observed on 1.0% agarose gel indicating the presence of *gdh* gene in *B. licheniformis* strain CKA1 (Plate 14). Excised amplicon of ~ 650-700 bp from gel was eluted as per protocol described in chapter 3. PCR product was then cloned in pGEM-T easy vector system (Plate 15) and transformed into competent cells of *E. coli* (DH5 α). Positive clone containing insert was then identified using colony PCR (Plate 16). Plasmid was then isolated from selected positive colony and was sequenced. Sequence of 650 bp corresponding to *gdh* gene of *B. licheniformis* strain CKA1 was obtained.

Sequence of glucose dehydrogenase (*gdh*) gene of *Bacillus licheniformis* CKA1 was analysed with top ten hits as obtained in BLASTn analysis. Sequence analysis revealed that *gdh* gene of *Bacillus licheniformis* CKA1 showed maximum homology (97.61%) with *gdh* gene of *Bacillus* sp. JS (Accession number. CP003492) followed by 93.68 per cent homology with *Bacillus subtilis* subsp. *subtilis* RO-NN-1 (Accession number CP002906).

Table. 22 Relatedness of *gdh* gene of *B.licheniformis* CKA1 to closely affiliated known *gdh* nucleotide sequence from database

S. No	Nearest Homologue	Accession number	Identity (%)
1	<i>Bacillus</i> sp. JS	CP003492	97.61
2	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> RO-NN-1	CP002906	93.68
3	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> strain BSD-2	CP013654	93.49
4	<i>Bacillus subtilis</i> strain UD1022	CP011534	93.49
5	<i>Bacillus subtilis</i> HJ5	CP007173	93.49
6	<i>Bacillus subtilis</i> subsp. <i>subtilis</i> str. BAB-1	CP004405	93.49
7	<i>Bacillus subtilis</i> XF-1	CP004019	93.49
8	<i>Bacillus subtilis</i> subsp. <i>inaquosorum</i> strain DE111	CP013984	91.65
9	<i>Bacillus subtilis</i> subsp. <i>natto</i> strain CGMCC 2108	CP014471	91.12
10	<i>Bacillus subtilis</i> subsp. <i>natto</i> BEST195 DNA	AP011541	91.12

Phylogenetic tree based on *gdh* sequences (Fig. 39) validated that the *gdh* gene of *B.licheniformis* CKA1 clustered closely with *gdh* gene of other reported isolates of *Bacillus subtilis* subsp. *subtilis* strain BSD-2 with high boot strap value (100%).

The mineral phosphate-solubilization exhibited by phosphate solubilizing bacteria has been traditionally associated with the production of low molecular-weight organic acids, mainly gluconic and 2-keto-gluconic acids (Rodriguez and Fraga, 1999; Deubel *et al.* 2000; Farhat *et al.* 2009). Since the production of organic acids is considered to be the principal mechanism for mineral phosphate solubilization, it is assumed that any gene involved in organic acid synthesis might have an effect on their character. Gluconic acid production is

catalysed by periplasmic oxidation of glucose by membrane bound glucose dehydrogenase (GDH).

The findings in the present study indicate that *B.licheniformis* CKA1 solubilizes phosphorus which is mediated by metabolic production of organic acids. The cloning and sequencing of *gdh* gene in *B.licheniformis* CKA1 as reported in the present study, therefore hints towards the acid production theory involving the gluconic acid biosynthesis by the glucose dehydrogenase (GDH) enzyme and the co-factor, pyrroloquinoline quinone (PQQ). Mobilization of insoluble phosphates has been reported by researchers to be as a consequence of the production of gluconic acid, which results from the extracellular oxidation of glucose via the quinoprotein glucose dehydrogenase (Rodriguez *et al.* 2000; Goldstein *et al.* 2003; Patel *et al.* 2008; Choi *et al.* 2008; Park *et al.* 2009; Sashidhar and Podile, 2010).

4.14 EFFECT OF *Bacillus licheniformis* CKA1 INOCULATION ON VEGETATIVE GROWTH OF APPLE CULTIVARS IN ORCHARDS AT DIFFERENT LOCATION

4.14.1 Cultivar: Red Chief planted at replant sites

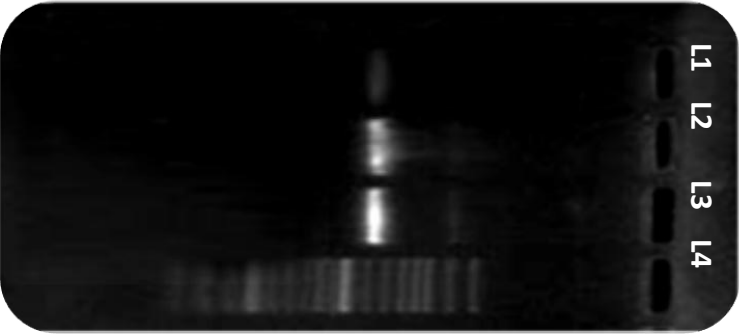
The experiment was conducted in order to evaluate the effect of CKA1 inoculation on apple plants (cv. Red Chief) at replant sites of apple orchard at different location. The result in table 23 revealed that growth parameters varied significantly with variation in location. Maximum mean average plant height (105.77cm) irrespective of location was observed with plants raised with CKA1 inoculation which was significantly higher than un-inoculated control.

Interaction studies revealed that CKA1 inoculation resulted in maximum plant height (122.56cm) at location Nanni, Matiana which was significantly higher than uninoculated control and at par with plant height at location Sablog, Matiana (120.00cm).

Maximum mean average shoot diameter (15.15mm) irrespective of location was observed with plants raised with CKA1 inoculation which was significantly higher than un-inoculated control.

Interaction studies revealed that CKA1 inoculation resulted in maximum shoot diameter (18.68cm) at location Sablog, Matiana which was significantly higher than

Molecular characterization of strain CKA1 for glucose dehydrogenase enzyme (GDH) - encoding gene (*gdh*)



L1: ~ 650 bp amplicon of *gdh* gene amplified with primers GDHf(1) and GDHr

L2: ~ 650 bp amplicon of *gdh* gene amplified with primers GDHf(2) and GDHr

L3: ~ 650 bp amplicon of *gdh* gene amplified as result of nested PCR

L4: 100bp ladder

Plate 14. Amplification of ~ 650 bp amplicon of glucose dehydrogenase enzyme (GDH)-encoding gene (*gdh*) in *B. licheniformis* strain CKA1 using nested PCR as observed on 1.0% agarose gel

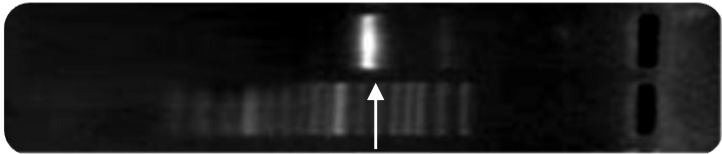
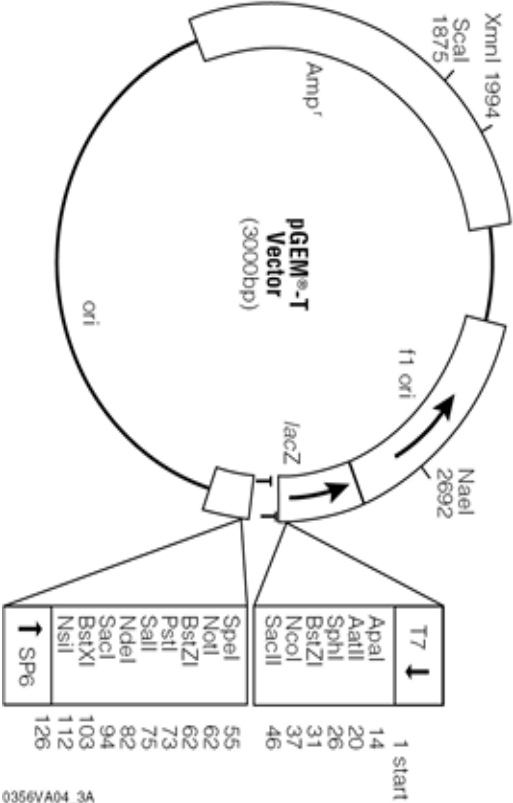


Plate 15. Cloning of ~ 650 bp PCR product in pGEM-T vector

uninoculated control and shoot diameter at location Nanni, Matiana (15.81mm), Kyari, Kotkhai (15.96mm) and RHRS, Mashobra.

Table 23. Effect of *B.licheniformis* strain CKA1 inoculation on vegetative growth of apple plants (cv. Red Chief) at replant sites of apple orchard at different locations

Location	Average Plant Height(cm)*			Average shoot diameter(mm)*			Average leaf surface area(cm ²)*		
	CKA1 inoculated*	Un-inoculated control	Mean	CKA1 inoculated	Un-inoculated control	Mean	CKA1 inoculated	Un-inoculated control	Mean
RHRS, Mashobra	67.59	52.71	60.15	10.15	9.66	9.90	24.06	23.39	23.72
Nanni, Matiana	122.56	85.93	104.24	15.81	13.37	14.59	32.27	30.19	31.23
Sablog, Matiana	120.00	73.93	96.96	18.68	14.66	16.67	31.46	28.53	29.99
Kyari, Kotkhai	112.93	75.72	94.32	15.96	13.08	14.52	28.81	25.94	27.37
Mean	105.77	72.07		15.15	12.69		29.15	27.01	

*Data average of three year trials (2013 -2015)

CD _{0.05}	Location (L)	:	1.64	Location (L)	:	0.42	Location (L)	:	0.65
	Height (H)	:	2.32	Shoot diameter (SD)	:	0.60	Leaf surface area (LSA)	:	0.92
	LxH	:	3.27	LxSD	:	0.85	LxLSA	:	1.10

Maximum leaf surface area (29.15cm²) irrespective of location was observed with plants raised with CKA1 inoculation which was significantly higher than un-inoculated control.

Interaction studies revealed that CKA1 inoculation resulted in maximum leaf surface area (cm) at location Nanni, Matiana, which was significantly higher than uninoculated control and at location Sablog, Matiana (31.46cm²), Kyari, Kotkhai (28.81 cm²) and RHRS, Mashobra (24.06 cm²).

CKA1 inoculation resulted in significant increase in growth parameters at all the locations, accounting for 66.21, 49.14, 42.62 and 28.22 per cent increase over un-inoculated control in case of plant height at Sablog, Matiana, Kyari, Kotkhai, Nanni, Matiana and RHRS, Mashobra, respectively; 27.42, 22.00, 18.24 and 5.07 per cent increase over un-inoculated control in case shoot diameter at Sablog, Matiana, Kyari, Kotkhai, Nanni, Matiana and RHRS, Mashobra, respectively (Fig. 40).

Fatima *et al.* (2009) had documented higher increase in root and shoot parameters by the use plant growth promoting rhizobacteria.

4.14.2 Cultivar: Red Chief planted at new plantation sites

The experiment was conducted in order to evaluate the effect of CKA1 inoculation on apple plants (cv. Red Chief) at new plantation sites of apple orchard at different locations. The result in table 24 revealed that growth parameters varied significantly with variation in location. Maximum mean average plant height (209.91cm) irrespective of location was observed with plants raised with CKA1 inoculation which was significantly higher than un-inoculated control (140.95cm).

Interaction studies revealed that CKA1 inoculation resulted in maximum plant height (238.63cm) at location Sablog, Matiana which was significantly higher than plant height at location Kufarbagh, Kotkhai (158.24cm).

Maximum mean average shoot diameter (28.47mm) irrespective of location was observed with plants raised with CKA1 inoculation which was significantly higher than un-inoculated control (24.12mm).

Interaction studies revealed that CKA1 inoculation resulted in maximum shoot diameter (28.81cm) at location Sablog, Matiana which was statistically at par with shoot diameter at location Kufarbagh, Kotkhai (28.15 mm) (Fig.41).

Table 24. Effect of most efficient *B.licheniformis* strain CKA1 inoculation on vegetative growth of apple plants (cv.Red Chief) at new plantation sites at different locations

Location	Cultivar	Average Plant Height (cm)*			Average shoot diameter (mm)*			Average leaf surface area (cm ²)*		
		CKA1 inoculated	Un-inoculated control	Mean	CKA1 inoculated	Un-inoculated control	Mean	CKA1 inoculated	Un-inoculated control	Mean
Sablog, Matiana	Red Chief	238.63	181.19	198.43	28.81	23.49	27.15	23.82	21.83	22.82
Kufarbagh, Kotkhai		158.24	123.66	152.42	28.15	22.76	25.45	23.30	20.91	22.10
		209.91	140.95		28.47	24.12		23.56	21.36	

*Data average of three year trials (2013 -2015)

CD _{0.05}	Location (L)	:	6.71	Location (L)	:	0.55	Location (L)	:	1.08
	Height (H)	:	6.71	Shoot diameter (SD)	:	0.55	Leaf surface area (LSA)	:	0.43
	LxH	:	9.49	LxSD	:	0.78	LxLSA	:	1.14

Maximum mean leaf surface area (23.56cm²) irrespective of location was observed with plants raised with CKA1 inoculation which was significantly higher than un-inoculated control.

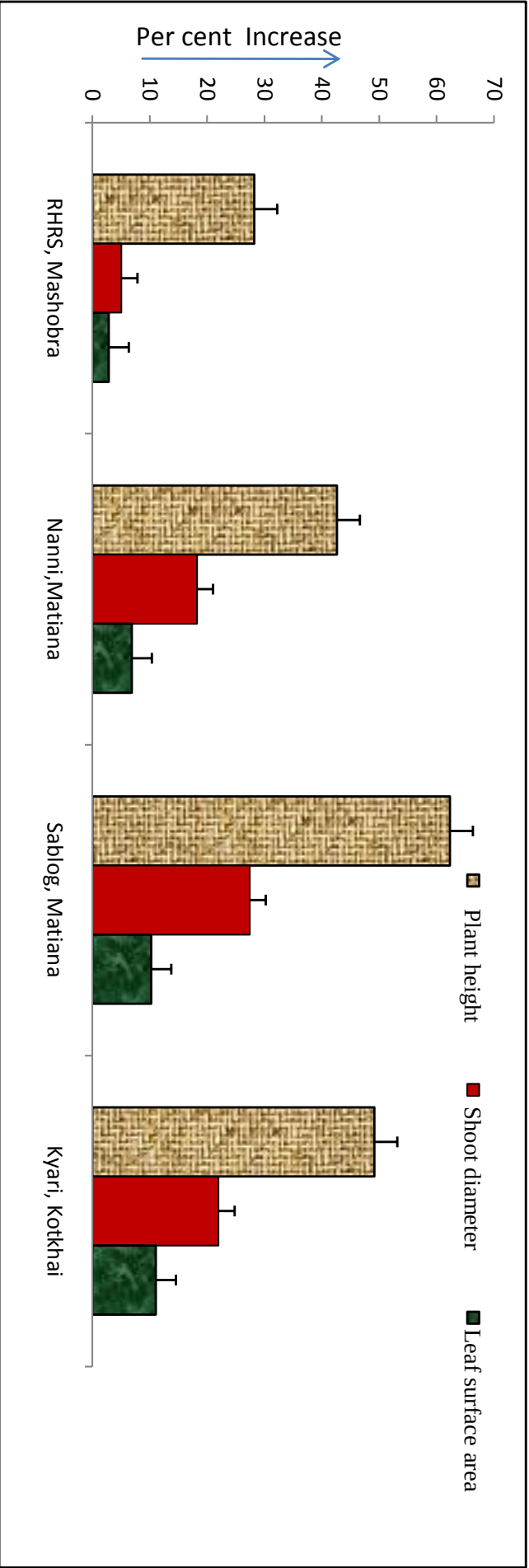


Figure 40. Per cent increase in growth parameters of apple plants (cv. Red Chief) over un-inoculated control at different replant locations

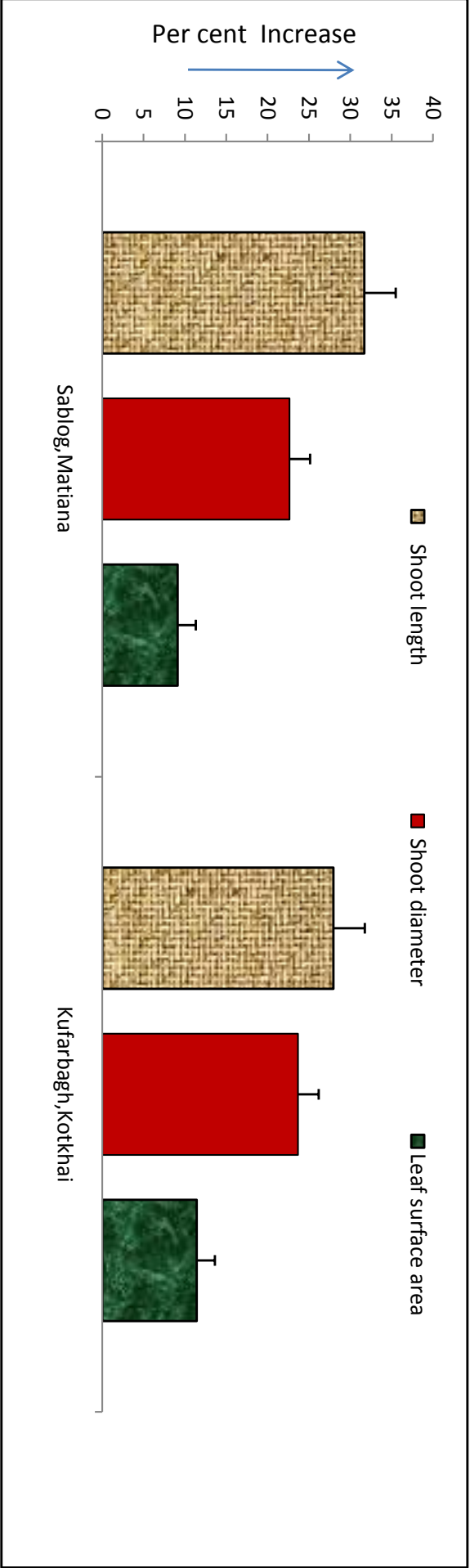


Figure 41. Per cent increase in growth parameters of apple plants (cv. Red Chief) over un-inoculated control at new plantation sites of different locations

Interaction studies revealed that CKA1 inoculation resulted in maximum leaf surface area (23.82cm²) at location Sablog, Matiana which was significantly higher than uninoculated control and statistically at par with leaf surface of apple plants at location Kufarbagh, Kotkhai (23.30cm²).

CKA1 inoculation resulted in significant increase in growth parameters at all the locations, accounting for 31.70 and 27.96 per cent increase over un-inoculated control in case of plant height at Sablog, Matiana, and Kufarbagh, Kotkhai respectively; 22.64 and 23.68 per cent increase over un-inoculated control in case shoot diameter at Sablog, Matiana, and Kufarbagh, Kotkhai respectively and 9.11 and 11.42 per cent increase over un-inoculated control in case of leaf surface area at Sablog, Matiana, and Kufarbagh, Kotkhai respectively (Fig. 41).

Similarly promotion in growth parameters and yield of various other crops in response to inoculation with PGPR have been reported by other workers (Gravel *et al.* 2007; Gholami *et al.* 2009).

4.14.3 Cultivar: Top Red planted at replant sites

The experiment was conducted in order to evaluate the effect of CKA1 inoculation on apple plants (cv. Top red) at replant sites of apple orchard different location. The result in table 25 revealed that growth parameters varied significantly with variation in location. Maximum mean average plant height (91.51cm) irrespective of location was observed with plants raised with CKA1 inoculation which was significantly higher than un-inoculated control.

Table 25. Effect of *B. licheniformis* strain CKA1 inoculation on vegetative growth of apple seedlings (cv. Top Red) at replant sites of apple orchard at different locations

Location	Average Plant Height(cm)*			Average shoot diameter(mm)*			Average leaf surface area(cm ²)*		
	CKA1 inoculated	Un-inoculated control	Mean	CKA1 inoculated	Un-inoculated control	Mean	CKA1 inoculated	Un-inoculated control	Mean
Sablog, Matiana	97.37	53.70	75.53	14.97	11.79	13.38	35.16	30.52	32.82
Nanni, Matiana	113.95	69.75	91.85	19.62	14.80	17.21	33.16	29.35	31.26
Kyari, Kotkhai	63.23	54.72	58.96	19.78	15.47	17.63	29.86	27.06	28.51
Mean	91.51	59.39		18.12	14.02		32.76	28.97	

*Data average of three year trials (2013 -2015)

CD _{0.05}	Location (L)	:	0.85	Location (L)	:	1.66	Location (L)	:	0.53
	Height (H)	:	1.04	Shoot diameter (SD)	:	2.03	Leaf surface area (LSA)	:	0.65
	LxH	:	1.47	LxSD	:	2.34	LxLSA	:	0.91

Interaction studies revealed that CKA1 inoculation resulted in maximum plant height (113.35cm) at location Nanni, Matiana which was significantly higher than plant height at other location.

Maximum mean average shoot diameter (18.12mm) irrespective of location was observed with plants raised with CKA1 inoculation which was significantly higher than uninoculated control.

Interaction studies revealed that CKA1 inoculation resulted in maximum shoot diameter (19.62cm) at location Nanni, Matiana which was significantly higher than uninoculated control and statistically at par with shoot diameter at location Kyari, Kotkhai (19.78 mm).

Maximum mean leaf surface area (32.76cm²) irrespective of location was observed with plants raised with CKA1 inoculation which was significantly higher than un-inoculated control.

Interaction studies revealed that CKA1 inoculation resulted in maximum leaf surface area (35.16cm) at location Sablog, Matiana which was significantly higher than uninoculated control and at location Nanni, Matiana (33.16cm²) and Kyari, Kotkhai (29.86 cm²).

CKA1 inoculation resulted in significant increase in growth parameters at all the locations, accounting for 81.32, 66.36 and 15.15 per cent increase over un-inoculated control in case of plant height at Sablog, Matiana, Nanni, Matiana and Kyari, Kotkhai respectively; 26.97, 32.56 and 27.86 per cent increase over un-inoculated control in case shoot diameter at Sablog, Matiana, Nanni, Matiana and Kyari, Kotkhai respectively and 15.20, 12.98 and 10.34 per cent increase over un-inoculated control in case of leaf surface area at Sablog, Nanni and Kyari respectively (Fig.42).

A number of workers have also reported beneficial effects of plant growth promoting rhizobacteria for improving plant growth under normal as well as stressful environment (Belimov *et al.* 2009; Heidari and Golpayegani, 2012; Nadeem *et al.* 2010a,b).

4.14.4 Cultivar: Super Chief planted at replant sites

The experiment was conducted in order to evaluate the effect of CKA1 inoculation on apple plants (cv. Super Chief) at replant sites of apple orchard different location. The result in

table 26 revealed that growth parameters varied significantly with variation in location. Maximum mean average plant height (88.91cm) irrespective of location was observed with plants raised with CKA1 inoculation which was significantly higher than un-inoculated control.

Table 26. Effect of *B. licheniformis* strain CKA1 inoculation on vegetative growth of apple seedlings (cv. Super Chief) at replant sites of apple orchard at different locations

Location	Average Plant Height(cm)*			Average shoot diameter(mm)*			Average leaf surface area(cm ²)*		
	CKA1 inoculated	Un-inoculated control	Mean	CKA1 inoculated	Un-inoculated control	Mean	CKA1 inoculated	Un-inoculated control	Mean
Sablog, Matiana	91.47	70.98	81.22	13.08	11.35	12.21	22.92	21.20	22.06
Nanni, Matiana	87.64	71.20	79.43	13.13	10.89	11.97	22.88	20.89	21.88
Kyari, Kotkhai	87.60	68.02	77.81	12.42	10.86	11.64	22.48	21.09	21.78
Mean	88.91	70.07		12.87	11.00		22.76	21.06	

*Data average of three year trials (2013 -2015)

CD _{0.05}	Location (L)	:	0.46	Location (L)	:	1.26	Location (L)	:	0.69
	Height (H)	:	0.57	Shoot diameter (SD)	:	1.37	Leaf surface area (LSA)	:	0.31
	LxH	:	0.80	LxSD	:	1.62	LxLSA	:	0.84

Interaction studies revealed that CKA1 inoculation resulted in maximum plant height (91.47cm) at location Sablog, Matiana which was significantly higher than uninoculated control and plant height at other locations.

Maximum mean average shoot diameter (12.87mm) irrespective of location was observed with plants raised with CKA1 inoculation which was significantly higher than un-inoculated control.

Interaction studies revealed that CKA1 inoculation resulted in maximum shoot diameter (13.13cm) at location Nanni, Matiana which was significantly higher than uninoculated control and at par with shoot diameter at location Sablog, Matiana (13.13mm) and Kyari, Kotkhai (12.42mm).

Maximum leaf surface area (22.76cm²) irrespective of location was observed with plants raised with CKA1 inoculation which was significantly higher than un-inoculated control.

Interaction studies revealed that CKA1 inoculation resulted in maximum leaf surface area (22.92cm²) at location Sablog, Matiana which was significantly higher than uninoculated control and at par with location Nanni, Matiana (22.88cm²), Kyari, Kotkhai (22.48cm²).

CKA1 inoculation resulted in significant increase in growth parameters at all the locations, accounting for 28.86, 23.08 and 28.78 per cent increase over un-inoculated control in case of plant height at Sablog, Matiana, Nanni, Matiana and Kyari, Kotkhai respectively; 15.24, 20.56 and 11.36 per cent increase over un-inoculated control in case shoot diameter at Sablog, Matiana, Nanni, Matiana, and Kyari, Kotkhai, respectively and 8.11, 9.52 and 6.59 per cent increase over un-inoculated control in case of leaf surface area at Sablog, Matiana, Nanni, Matiana and Kyari, Matiana, respectively (Fig. 43).

PGPR inoculation strongly influenced plant height, shoot diameter and leaf surface area during the early stages of growth. Indicating the tested PGPR strains are capable of promoting plant growth in the early years of planting.

Three years of trial under orchard conditions showed that bacterial inoculation and cultivar, significantly affected the shoot number and leaf area. Similar results were reported with bacterial applications in rabbit eye blueberry (De Silva *et al.* 2000) in mulberry (Sudhakar *et al.* 2000), and in apple (Aslantas *et al.* 2007).

It can be concluded, that CKA1 application increased vegetative growth by promoting the production of, for example, auxins (Jeon *et al.* 2003; Egamberdiyeva, 2005), cytokinins (García de Salamone *et al.* 2001) and gibberellins (Gutiérrez-Mañero *et al.* 2001). Many studies have been performed to determine the effects of bacterial applications on vegetative growth in apricot (Esitken *et al.* 2003); in wheat (Khalid *et al.* 2004); in sweet cherry (Esitken *et al.* 2006); in sugar beet (Cakmakci *et al.* 2006); and in apple (Aslantas *et al.* 2007). In these studies it was documented that bacteria applications increased annual shoot length and plant height. The plant growth enhancement effects of bacteria used in this study on apple, could be explained with N₂-fixing, P-solubilizing, indole-3- acetic acid (IAA) producing capacity and also antimicrobial effects (Sahin *et al.* 2000; Abbasi *et al.* 2001; Cakmakci *et al.* 2001; Esitken *et al.* 2003).

Overall, the response to inoculation with PGPR isolate varied with cultivars and locations. However, the treated plants were better as compared to untreated. The plant growth promoting effect of bacterial applications appeared to be related to phytohormone production and phosphate solubilization activities of the PGPR strain tested (Burd *et al.* 2000). Further studies are necessary to determine the residual effect of PGPR treatments on the growth, fruit yield and quality of apple trees as well as soil microbial communities in the future over longer time periods.

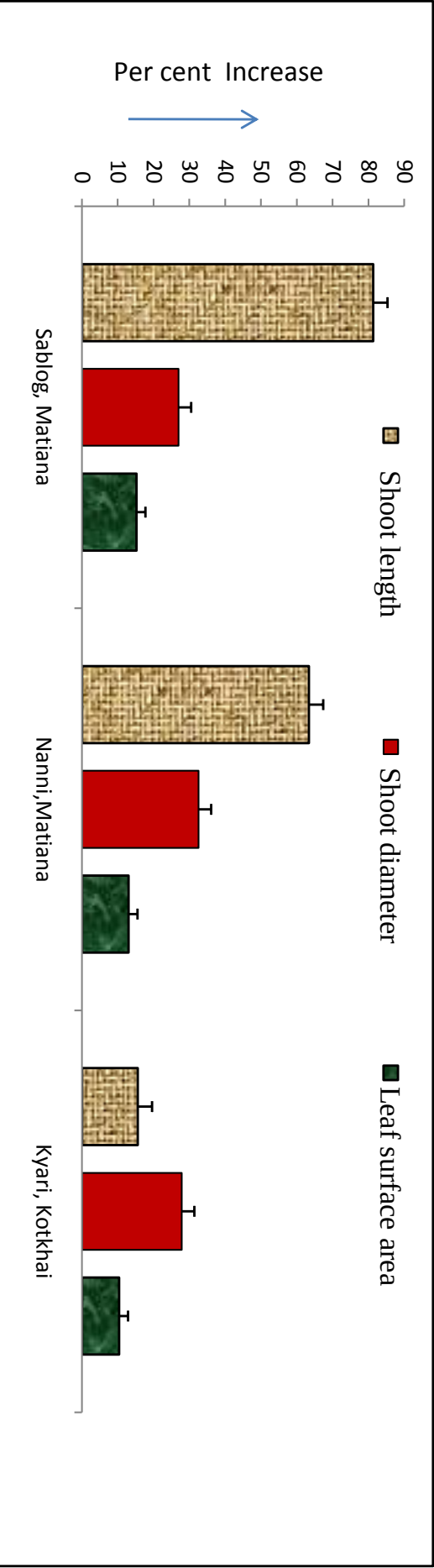


Figure 42. Per cent increase in growth parameters of apple plants (cv. Top Red) over un-inoculated control at different replant locations

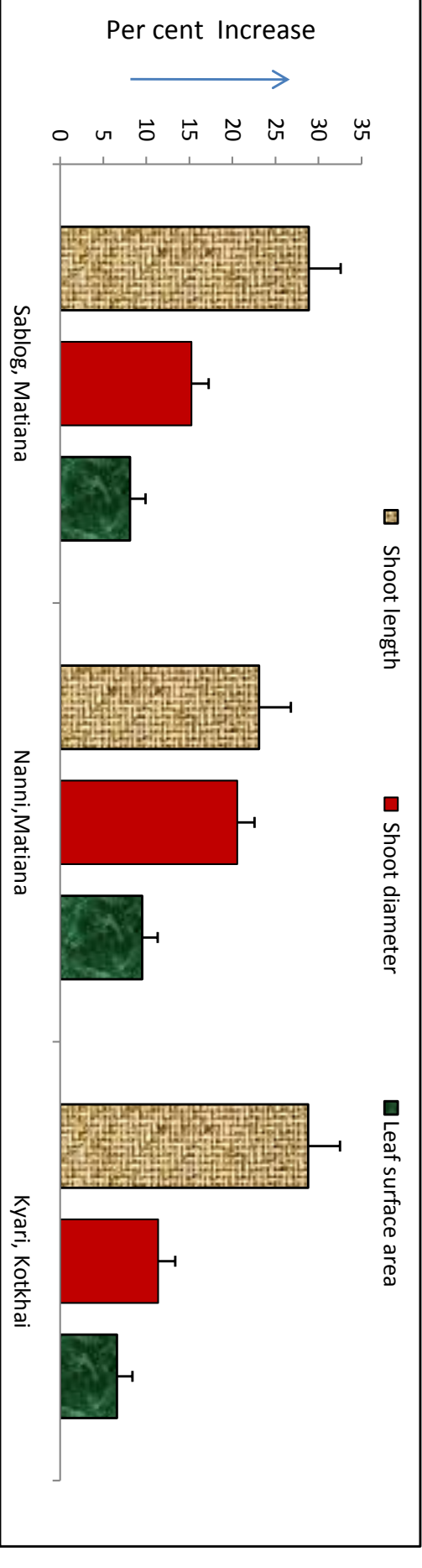


Figure 43. Per cent increase in growth parameters of apple plants (cv. Super Chief) over un-inoculated control at replant locations

Obtaining maximum benefits on farms from plant growth promoting biofertilizers will require a systematic strategy designed to fully utilize all these beneficial factors, allowing crop yields to be maintained or even increased while fertilizer applications are reduced (Kennedy *et al.* 2004). The results indicated that selected PGPR strain CKA1 are able to promote tree growth and reduce the need for chemical fertilizers for young apple trees.

4.15 PLANT GROWTH RESPONSE (VEGETATIVE AND NUTRIENT PARAMETERS) OF APPLE PLANTS TO *Bacillus licheniformis* CKA1 INOCULATION IN ORCHARDS AT DIFFERENT LOCATION AFTER THREE YEAR OF PLANTATION

Effect of *B. licheniformis* strain CKA1 inoculation on measured parameters of vegetative growth and per cent nutrient (NPK) in leaves was evaluated in apple orchard at different locations on apple trees after 3 years of plantation. Liquid culture of CKA1 was applied through soil drenching in the basin of apple trees for two consecutive times every year i.e. once in March, and then again in the month of July. Data was recorded in the month of October every year.

4.15.1 Cultivar: Red Chief at replant site

Data observed on measured parameters of vegetative growth components appended in table 27 revealed a significant increase in plant height, average shoot diameter and average leaf surface area over un-inoculated apple trees over a period of three years. A significant increase in average plant height, shoot diameter and leaf surface area in case of CKA1 inoculated apple trees was observed in month of October for all the three years for all the four locations. Based on the values of three year data on average increase in 20.10, 51.10, 67.10 and 50.10 per cent increase in case of plant height, 17.40, 22.48, 30.67 and 22.79 per cent increase in case of shoot diameter and 13.58, 7.93, 13.99 and 13.43 per cent increase in leaf surface area was observed in CKA1 inoculated trees at RHRS, Mashobra, Nanni, Matiana Sablog, Matiana, and Kyari, Kotkhai, respectively over uninoculated trees, over a period of three years.

4.15.2 Cultivar: Red Chief at new plantation sites

Based on the data obtained from trial carried out in 2013, where repeated inoculations of CKA1 were made, significant increase in plant height, shoot diameter and leaf surface was observed over un-inoculated control (Table 28).

Significant increase in average plant height in case of CKA1 inoculated apple trees at new plantation sites was observed every year at all location Kufarbagh, Kotkhai), accounting for an overall 31.19 and 14.03 per cent increase at Sablog, Matiana and Kufarbagh, Kotkhai respectively over un-inoculated trees.

In CKA1 inoculated apple trees, significant increase in average shoot diameter was observed every year in both locations accounting for an overall 24.72 and 25.12 per cent increase at Sablog, Matiana and Kufarbagh, Kotkhai respectively over un-inoculated trees.

In CKA1 inoculated apple trees, significant increase in average increase in leaf surface area was observed resulting in 14.40 and 16.21 per cent increase was observed in CKA1 inoculated trees at Sablog, Matiana and Kufarbagh, Kotkhai respectively over un-inoculated trees.

The more beneficial effect on plant growth in new plantation soils by CKA1 most likely resulted from plant growth promotion by the bacteria and contributed to increased soil fertility. In the present study, the growth promoting effect on apple seedlings/plants by *B. licheniformis* strain CKA1 appears to be direct, with possible involvement of plant growth regulators IAA, phosphate solubilization and nitrogen fixation. This is supported by the fact that disease symptoms were not reported throughout the experiment and plants remained healthy both in bacterial treated plants at all the locations. Thus, indirect plant growth promoting effect as a result of biological control of deleterious microorganisms as reported for most of the studies on plant growth promoting rhizobacteria as biocontrol agent (Luna *et al.* 2002; Whipps, 2000; Compant *et al.* 2005) is clearly ruled out as far as the present study is concerned. Plant growth promoting rhizobacteria enhance plant growth by direct and indirect mechanisms, but the specific mechanisms involved have not all been well characterized (Nelson, 2004). The identification of genes and partial characterization of molecular mechanisms in *B. licheniformis* strain CKA1 highlights its ability to perform as single strain multifunctional plant growth promoting rhizobacterium not only in laboratory but also under field conditions under varied environmental conditions.

Apple cultivars showed different reactions to treatment with bacteria and the regional differences may also affect effectiveness of the bacteria. The inoculation with PGPR contributed to the increase in vegetative growth and nutrient content of apple when compared to the control, but it strongly depended on the cultivars and bacterial strain tested. Plant

Table:27 Effect of *B.licheniformis* strain CKA1 inoculation on vegetative growth of apple seedlings (cv. Red Chief) at replant sites of different locations

Location	Treatment	Average plant height(cm)					Average shoot diameter(mm)					Average leaf surface area(cm ²)				
		2013	2014	2015	Mean	2013	2014	2015	Mean	2013	2014	2015	Mean	2013	2014	2015
RHRS, Mashobra	Un-inoculated control	44.00	52.80	51.34	49.38	7.2	10.12	12.95	10.09	23.38	23.12	24.41	23.63			
	CKA1 inoculated	54.36	59.68	75.74	63.26	8.75	11.81	14.95	11.83	26.13	26.50	27.89	26.84			
	t-value	7.08	6.33	21.47		2.09	2.13	2.09		2.37	2.61	2.83				
Nanni, Matiana	Un-inoculated control	68.78	77.41	89.60	78.59	9.02	14.08	16.94	13.34	29.76	31.53	32.11	31.13			
	CKA1 inoculated	98.57	119.50	138.21	118.75	10.67	17.89	20.47	16.34	31.75	33.43	35.64	33.60			
	t-value	26.95	43.08	38.8		2.18	4.21	3.48		2.09	2.21	2.96				
Sablog, Matiana	Un-inoculated control	56.25	75.10	92.46	74.60	9.41	16.87	18.90	15.06	24.34	28.79	30.48	27.87			
	CKA1 inoculated	98.00	129.00	147.00	124.66	12.73	19.56	26.76	19.68	28.70	30.95	35.74	31.79			
	t-value	40.53	58.13	64.00		4.63	3.10	7.41		4.89	2.75	6.29				
Kyari, Kotkhai	Un-inoculated control	69.40	77.16	85.30	77.28	8.98	12.43	20.83	14.08	23.59	25.35	27.89	25.61			
	CKA1 inoculated	90.25	119.06	138.50	115.93	10.71	15.84	25.33	17.29	26.31	29.54	31.32	29.05			
	t-value	18.4	40.29	52.99		4.34	5.89	5.12		3.58	5.49	4.71				

Table:28 Effect of *B.licheniformis* strain CKA1 inoculation on vegetative growth of apple seedlings (cv. Red Chief) at new plantation sites of different locations

Location	Cultivar	Treatment	Average plant height(cm)				Average shoot diameter(mm)				Average leaf surface area(cm ²)			
			2013	2014	2015	Mean	2013	2014	2015	Mean	2013	2014	2015	Mean
Sablog, Matiana	Red Chief	Un-inoculated control	156.50	179.50	214.57	183.52	15.43	23.47	32.80	23.90	18.83	21.15	22.50	20.82
		CKA1 inoculated	189.00	234.50	298.41	240.63	20.56	29.68	39.21	29.81	21.67	23.31	26.48	23.82
		t-value	33.73	60.77	35.33		5.45	6.76	7.35		3.28	3.14	4.95	
Kufarbagh Kotkhai	Red Chief	Un-inoculated control	74.40	114.23	187.50	139.64	15.69	22.90	32.10	23.56	19.90	21.34	21.50	20.91
		CKA1 inoculated	108.00	139.42	230.31	159.24	19.43	29.52	39.50	29.48	22.56	24.55	25.79	24.30
		t-value	37.85	28.00	47.06		4.89	8.52	7.99		3.36	4.05	5.30	

growth responses were variable and depended on the inoculant strain, apple cultivar and growth parameters evaluated. The effect of PGPR, as a complex process, depends on bacterial strains and population, plant bacterial strain combination, plant genotype, growth parameters evaluated, and environmental conditions as reported earlier by many researchers (Cakmakci *et al.* 2006,2007; Sahin *et al.* 2000).

4.15.3 Cultivar: Early Red One at replant site

Data observed on measured parameters of vegetative growth components appended in table 29 revealed a significant increase in plant height, average shoot diameter and average leaf surface area over un-inoculated apple trees over a period of three years. A significant increase in average plant height, shoot diameter and leaf surface area in case of CKA1 inoculated apple trees was observed in month of October for all the three years for all the three locations. Based on the values of three year data on average increase in 35.46, 30.63 and 34.96 per cent increase in case of plant height; 37.79, 18.41 and 19.39 cent increase in case of shoot diameter and 3.74, 6.29 and 8.99 per cent increase in leaf surface area was observed in CKA1 inoculated trees at Sablog, Matiana; Jhalroo, Matiana and Nanni, Matiana, respectively over uninoculated trees, over a period of three years.

4.15.4 Cultivar: Top Red at replant site

Data observed on measured parameters of vegetative growth components appended in table 30 revealed a significant increase in plant height, average shoot diameter and average leaf surface area over un-inoculated apple trees over a period of three years. Similar trend was observed in case of cultivar Top Red as observed in case of cultivar Red Chief and Early Red planted at replant sites of apple orchard at different locations with a significant increase in average plant height in case of CKA1 inoculated apple trees was observed in month of October for all the three years Based on mean vales of three year data on average increase in 86.41, 63.36 and 82.56 per cent increase in case of plant height; 27.95, 32.11 and 26.16 per cent increase in case of shoot diameter and 17.98, 15.71 and 15.10 per cent increase in leaf surface area was observed in CKA1 inoculated trees at Sablog,Matiana, Nanni,Matiana and Kyari, Kotkhai respectively over uninoculated trees.

4.15.5 Cultivar: Super Chief at replant site

Data observed on measured parameters of vegetative growth components appended in table 31 revealed a significant increase in plant height, average shoot diameter and average

leaf surface area over un-inoculated apple trees over a period of three years. Similar trend was observed in case of cultivar Super Chief as observed in case of cultivar Red Chief, Early Red and Top Red planted at replant sites of apple orchard at different locations with a significant increase in average plant height in case of CKA1 inoculated apple trees was observed every year at all the three years Based on mean values of three year data on average increase in 30.72, 24.85 and 29.00 per cent increase in case of plant height; 17.57, 23.26 and 18.30 per cent increase in case of shoot diameter and 9.89, 11.77 and 13.00 per cent increase in leaf surface area was observed in CKA1 inoculated trees at Sablog, Matiana, Nanni, Matiana and Kyari, Kotkhai respectively over un-inoculated trees.

Although plant growth promoting rhizobacteria have been reported to influence plant growth, yield and nutrient uptake by an array of mechanism, the specific traits by which PGPR promote plant health are limited to the expression of one or more traits expressed at a given environment of plant microbe interaction. In the present studies, novel strain of *B. licheniformis* strain CKA1 that posses the capability for production of growth stimulating hormone; solubilization of phosphate; siderophore production; IAA production and nitrogen fixation was evaluated for growth promotion of apple seedlings under net house, nursery conditions and in apple orchard. *B. licheniformis* strain CKA1 is the first known example of an endophytic phosphate solubilizing bacteria isolated from apple seedlings to exhibit multiple plant growth promoting activities.

The growth-promoting strain CKA1 persisted reasonably well in the apple root environment, and its population level was affected by the inoculum concentration applied, environmental conditions at particular location and by the stage of plant development. During the long-term experiment consisting of repeated inoculation at 10^{10} CFU ml⁻¹, strains CKA1 had a population level of 10^6 CFU/ g dry weight of soil at basin of the young plants at the end of three years.

In the present study, re-isolations of a single morphotype (results presented latter) from roots of seedlings indicate the better root colonizing efficiency of *B. licheniformis* strain CKA1. Green house and field studies have demonstrated that treatment of seeds and seedlings of crops with single strain has lead to significant increase in growth and yield (Çakmakç *et al.* 2007; Larcher *et al.* 2003). The seed treatment of *B. licheniformis* strain CKA1 consistently increased the various plant attributes such as shoot length, root length and total dry weight of plants over uninoculated control. The results in the present study show

that *B. licheniformis* strain CKA1 could become endophytically established in roots of apple seedlings both under controlled conditions as well as fluctuating field conditions at different locations.

Although endophytes have been confirmed and localized in various parts and are postulated to play an important role in sustainable agricultural development (Tilak *et al.* 2005; Pandey *et al.* 2005), the mechanisms for their endophytic establishment are not well known. It has been suggested that the bacteria could enter through fissures created by the emergence of lateral roots or actively dissolving the cell wall component to gain entry (Reinhold Hurek and Hurek, 1998). It is possible that strain CKA1 might be using either of the two mechanisms to colonize the internal root tissues. The highly significant correlation between the endophytic bacterial population and shoot length, shoot fresh weight and shoot dry weight suggest the major role of *B. licheniformis* strain CKA1 in enhancing the early growth of apple seedlings. Whether the bacteria elaborate products that directly promote plant growth and/or effect the rhizosphere micro flora by antagonism has also been discerned. The contention that plant growth promotion by *B. licheniformis* strain

CKA1 is not merely related to the reduction of population in rhizosphere rather than the production of plant growth promoting substances is supported by the finding that roots of the seedlings used for plant growth studies were not associated with fungal pathogen. Contrary to this observation, enhanced growth of bacterized onion seedlings with *Bacillus subtilis* was not correlated with the relative population of root surface or root zone soil (Reddy and Rahe, 1989).

The increased growth and biomass in case of apple seedlings/plants treated with *B. licheniformis* strain CKA1 may be attributed to the cumulative effect of phosphate solubilization, nitrogen fixation and production of plant growth regulators, the molecular mechanisms of which have been partially characterized in the present study. The increase in growth and biomass yield by the use of *Bacillus* spp. have been reported for various crops (Kundu *et al.* 2002; Brown and Hamilton 1993; Kloepper *et al.* 1989) including apple (Shirkot and Sharma, 2005). The solubilization of insoluble phosphate in the rhizosphere is the most common mode of action involved in PGPRs that enhance nutrient availability to plants (Richardson, 2001). In a study it has been found that phosphate solubilization is an effective mechanism of maize growth promotion and root colonization by the phosphate solubilizing strain of *Rhizobium leguminosarum* pv. *phaseoli* (Chabot *et al.* 1998).

Besides having P-solubilization activity, *Bacillus* sp. And *Pseudomonas* sp. secretes plant growth regulators (PGRs) such as auxins, cytokinins and gibberellins (Anith *et al.* 1999). Auxins are quantitatively the most abundant phytohormones secreted by the PGPRs and it is generally agreed that auxin production rather than phosphate solubilization and siderophore production is major factor responsible for the stimulation of root growth. It is assumed that increase in the growth of shoot and root as compared to un-inoculated control may be as a result of production of plant growth regulators by *B. licheniformis* strain CKA1. Many of the growth responses associated with the use of strain CKA1 may be merely the result of increase in root growth. A more extensive root system, in treated seedlings would provide more contact surface for nutrient uptake. Further, the increased growth rate may allow the roots to escape certain root diseases. *Phyllobacterium* spp. significantly promoted *Brassica napus* total root length up to 50 per cent by increasing the lateral root density through out the primary root and growth rate of mature lateral roots (Larcher *et al.* 2003). There is strong evidence to support the hypothesis that alterations in plant growth and development in response to inoculum may be due to microbial production of IAA (indole-3-acetic acid) (Selvadurai *et al.* 1991).

4.15.6 Cultivar: Red Velox grafted on different rootstocks at replant sites

Data observed on measured parameters of vegetative growth components of two year old Red Velox grafted on different rootstock at location Thanedhar is appended in table 32 and plate 17 and 18, revealed a significant increase in plant height, average shoot diameter and average leaf surface area over un-inoculated apple trees over a period of three years. Similar trend was observed in case of grafted rootstock as observed in case of cultivar Red Chief, Early Red, Super Chief and Top Red planted at replant sites of apple orchard at different locations with a significant increase in average plant height in case of CKA1 inoculated apple trees was observed every year at all the three years Based on mean vales of three year data on average increase in 52.40 and 49.22 per cent increase in case of plant height; 44.87 and 33.99 per cent increase in case of shoot diameter and 25.87 and 19.33 per cent increase in leaf surface area was observed in CKA1 inoculated rootstocks MM111 and MM106 respectively location at location Thanedhar over un-inoculated rootstocks.

Several researchers have reported that fertilization of apple and sour cherry rootstock with organic fertilizers and amendments of different origin i.e bacterial cultures and composition is effective in producing of high quality plants (Zygmunt *et al.* 2012).

Table:29 Effect of *B.licheniformis* strain CKA1 inoculation on vegetative growth of apple seedlings (cv. Early Red) at replant sites of different locations

Location	Treatment	Average plant height(cm)					Average shoot diameter(mm)					Average leaf surface area(cm ²)				
		2013	2014	2015	Mean		2013	2014	2015	Mean		2013	2014	2015	Mean	
Sablog, Matiana	Un-inoculated control	48.62	71.51	96.23	72.12		8.90	12.42	18.37	13.23		24.45	25.50	26.12	25.35	
	CKA1 inoculated	72.10	103.33	117.68	97.70		11.30	16.51	26.90	18.23		29.90	28.60	29.40	29.30	
	t-value	30.24	40.52	28.89			3.78	5.60	11.55			5.49	3.85	3.09		
Jhalroo, Matiana	Un-inoculated control	35.46	69.07	88.41	64.31		8.70	11.63	19.90	13.41		24.06	25.21	27.90	25.72	
	CKA1 inoculated	46.00	94.34	111.69	84.01		11.50	14.95	21.21	15.88		25.94	26.59	29.50	27.34	
	t-value	10.64	26.83	24.89			6.12	10.02	2.33			2.97	2.09	2.34		
Nanni, Matiana	Un-inoculated control	58.50	72.55	93.49	74.84		9.28	13.79	19.84	14.30		23.21	24.13	28.38	25.24	
	CKA1 inoculated	79.50	104.75	118.80	101.01		10.94	18.63	21.61	17.06		25.67	26.50	30.36	27.51	
	t-value	25.28	33.33	28.84			2.11	6.20	2.37			2.78	2.54	2.15		

Table:30 Effect of *B.licheniformis* strain CKA1 inoculation on vegetative growth of apple seedlings (cv. Top Red) at replant sites of different locations

Location	Treatment	Average plant height(cm)				Average shoot diameter(mm)				Average leaf surface area(cm ²)			
		2013	2014	2015	Mean	2013	2014	2015	Mean	2013	2014	2015	Mean
Sablog, Matiana	Un-inoculated control	43.00	50.75	65.67	53.14	9.79	11.90	15.89	12.52	26.56	28.58	33.42	29.52
	CKA1 inoculated	75.50	87.20	134.50	99.06	11.46	15.70	20.90	16.02	34.88	31.90	37.71	34.83
	t-value	48.47	48.22	67.93		2.17	5.18	5.98		10.3	3.46	4.64	
Nanni, Matiana	Un-inoculated control	54.50	74.21	80.55	69.75	12.79	15.84	17.79	15.35	26.53	30.86	34.67	30.68
	CKA1 inoculated	101.50	111.67	128.69	113.95	17.61	20.83	22.42	20.28	30.80	35.89	39.81	35.50
	t-value	37.79	30.44	56.36		6.66	6.65	5.44		5.41	6.90	6.29	
Kyari, Kotkhai	Un-inoculated control	39.47	47.80	76.89	54.72	11.23	15.40	22.79	16.47	25.72	27.00	28.70	27.14
	CKA1 inoculated	76.10	97.01	126.60	99.90	15.60	19.35	27.41	20.78	29.70	31.80	32.23	31.24
	t-value	48.71	49.91	61.8		5.7	5.03	6.6		5.45	5.75	4.79	

Table:31 Effect of *B.licheniformis* strain CKA1 inoculation on vegetative growth of apple seedlings (cv. Super Chief) at replant sites of different locations

Location	Treatment	Average plant height(cm)				Average shoot diameter(mm)				Average leaf surface area(cm ²)			
		2013	2014	2015	Mean	2013	2014	2015	Mean	2013	2014	2015	Mean
Sablog, Matana	Un-inoculated control	43.40	78.44	91.15	70.99	8.50	11.65	14.51	11.55	21.30	22.50	23.82	22.54
	CKA1 inoculated	50.71	99.00	128.71	92.80	9.56	13.50	17.70	13.58	23.21	24.53	26.57	24.77
	t-value	9.24	24.56	33.56		3.01	2.65	5.04		2.69	2.74	3.07	
Nanni, Matana	Un-inoculated control	42.68	77.70	94.23	71.53	7.70	12.40	14.59	11.56	19.36	21.11	22.20	20.89
	CKA1 inoculated	51.89	98.16	117.89	89.31	9.80	15.77	17.18	14.25	21.80	23.75	24.50	23.35
	t-value	12.18	19.83	29.36		3.51	5.25	3.62		3.22	3.72	3.63	
Kyari, Kotkhai	Un-inoculated control	41.60	75.46	89.00	68.68	7.90	10.50	13.88	10.76	19.50	21.75	21.70	21.00
	CKA1 inoculated	53.47	93.68	118.66	88.60	9.32	12.73	16.15	12.73	22.85	23.56	24.78	23.73
	t-value	13.86	29.96	31.02		2.4	3.03	3.62		2.64	3.65	4.16	

Table:32 Effect of *B.licheniformis* strain CKA1 inoculation on vegetative growth of apple rootstocks at replant sites of apple orchard at location Thanedhar

Location	Cultivar	Treatment	Average shoot length(cm)				Average shoot diameter(mm)				Average leaf surface area(cm ²)			
			2013	2014	2015	Mean	2013	2014	2015	Mean	2013	2014	2015	Mean
	Red Velox on MM111	Un-inoculated control	54.87	108.83	179.35	114.35	18.63	24.60	29.31	24.18	26.60	26.67	28.95	27.40
		CKA1 inoculated	93.80	177.56	251.50	174.28	22.21	34.38	48.50	35.03	32.83	34.24	36.41	34.49
		t-value	43.06	74.75	83.28		5.05	10.46	21.85		7.8	9.22	8.24	
Thanedhar	Red Velox on MM106	Un-inoculated control	82.33	118.20	159.57	120.03	18.32	25.68	32.10	25.36	26.47	27.51	29.83	27.93
		CKA1 inoculated	115.75	190.12	231.50	179.12	21.79	35.31	44.84	33.98	33.18	33.41	33.42	33.33
		t-value	39.67	73.27	95.32		4.21	12.75	16.34		8.59	7.09	4.84	

The inoculation modes with PGPR and rootstock plays a very important role in the effects observed. Thus, plant growth promoting bacteria may interact synergistically with stock and cultivar selected. Our results also suggest that a synergistic, metabolic interaction involving existing rootstock cultivar and PGPR strain CKA1. Both apple cultivar and rootstock tested responded differently to inoculation with rhizobacterial strains at same location. Growth promotion effects were seen early in young apple trees development, and these subsequently translated into higher yield, which was earlier reported also by Dobbelaere *et al.* (2002).

4.16 EFFECT OF *Bacillus licheniformis* STRAIN CKA1 INOCULATION ON SOIL NUTRIENT CONTENT IN APPLE ORCHARDS AT DIFFERENT LOCATION

In CKA1 inoculated apple, based on mean values of three year data on N content in soil was 284.8, 289.81, 289.7, 262.00 at location Sablog, Matiana, Thanedhar, Nanni, Matiana and Kyari, Kotkhai before CKA1 inoculation. CKA1 inoculation resulted in the significant higher N of the soil (338.50, 327.00, 341.00, 338.40 at location Sablog, Matiana, Thanedhar, Nanni, Matiana and Kyari, Kotkhai after CKA1 inoculation), accounting for 18.85 per cent, 12.83 per cent, 15.04 per cent and 29.16 per cent increase in N over un-inoculated control at location Sablog, Matiana, Thanedhar, Nanni, Matiana and Kyari, Kotkhai respectively (Fig.44).

Based on mean values of three year data, P content of soil was 41.00 47.00, 46.50, 49.00 at location Sablog, Matiana, Thanedhar, Nanni, Matiana and Kyari, Kotkhai before CKA1 inoculation. CKA1 inoculation resulted in the significant higher P of the soil (65.85, 47.87, 52.68 and 34.69 at location Sablog, Matiana, Thanedhar, Nanni, Matiana and Kyari, Kotkhai after CKA1 inoculation), accounting for 18.85 per cent, 12.83 per cent, 15.04 per cent and 29.16 per cent increase in P over un-inoculated control at location Sablog, Matiana, Thanedhar, Nanni, Matiana and Kyari, Kotkhai respectively (Fig. 44)).

Similar trend was observed in case of K content of soil in CKA1 inoculated orchard at location Sablog, Matiana, Thanedhar, Nanni, Matiana and Kyari, Kotkhai accounting for 11.70 per cent, 22.19 per cent, 13.56 per cent and 16.68 per cent increase respectively over uninoculated control.

In this study, inoculation with *B. licheniformis* strain CKA1 not only improved the growth of seedlings but also increased the per cent nitrogen, phosphorus and potassium



Plate 17. Effect of CKA1 inoculation on growth of apple plants at farmer's field Thanedhar (A) first year after inoculation (B) Second year after inoculation

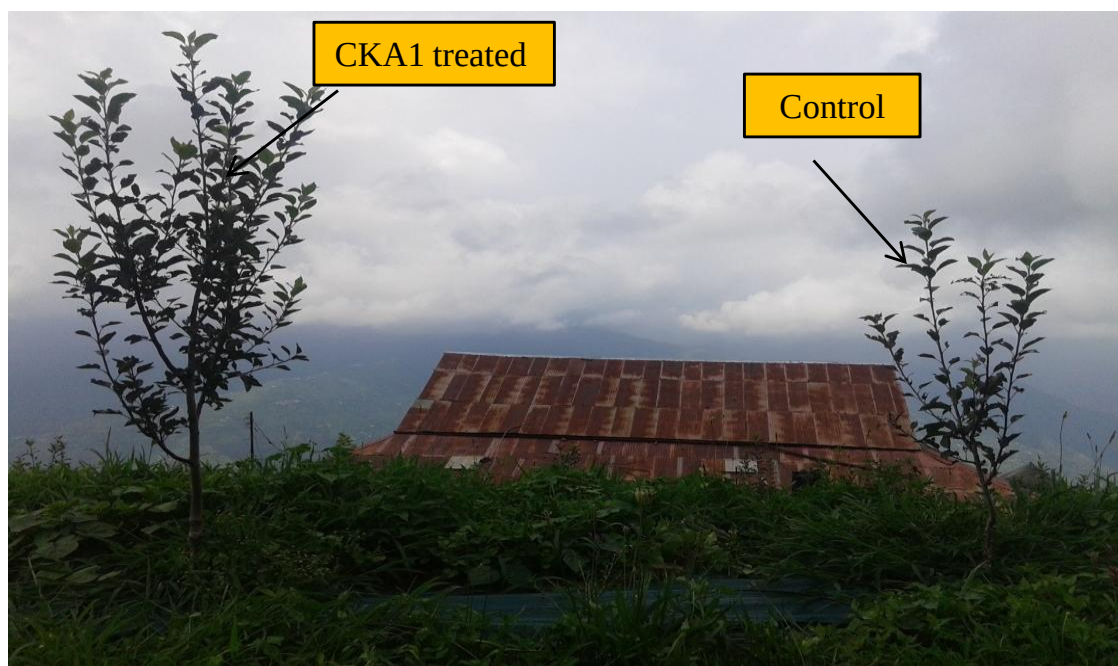


Plate 18. Effect of CKA1 inoculation on growth of apple plants at farmer's field at farmer's field Thanedhar

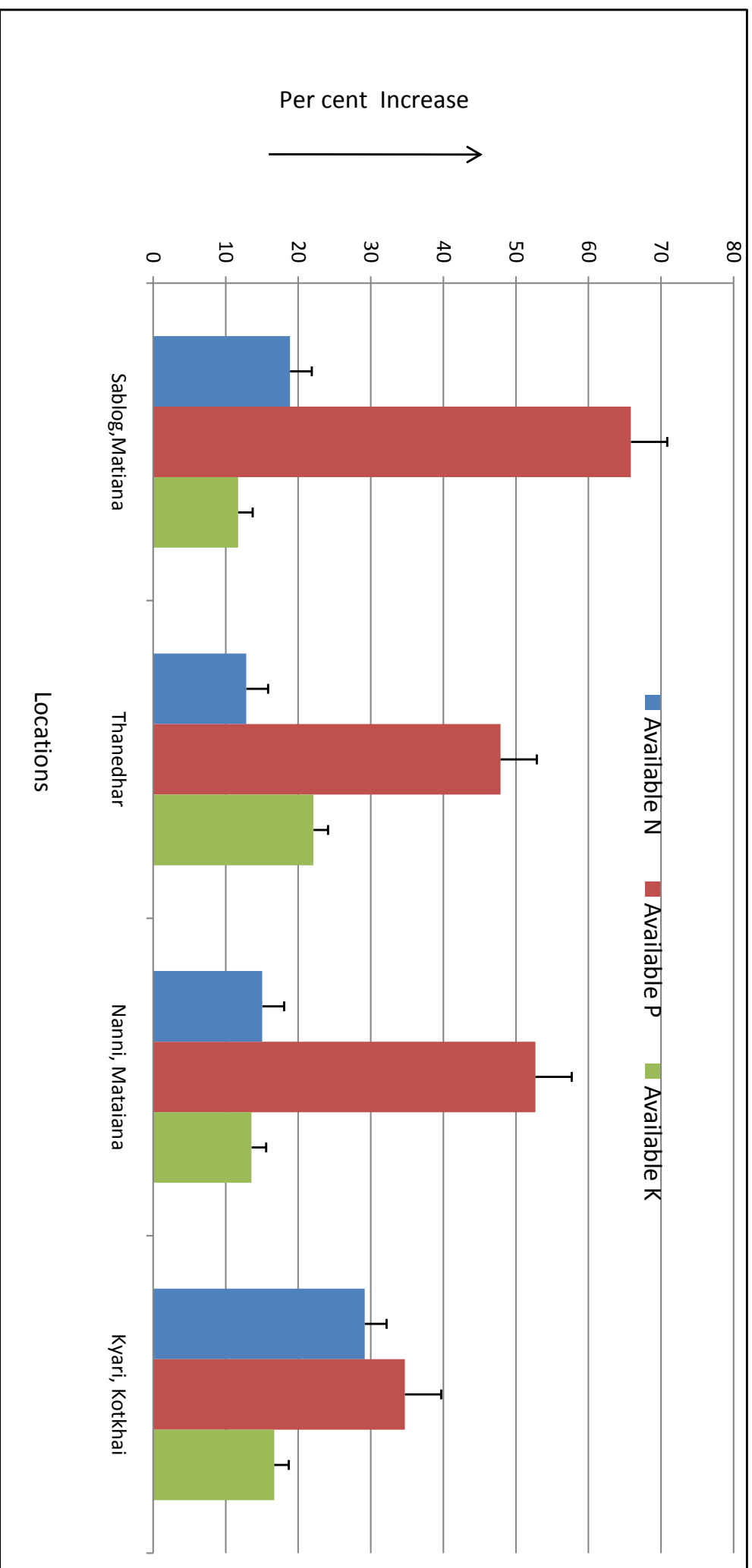


Figure 44. Per cent increase in available NPK of soil by CKA1 treatment at different location

(NPK) contents which were significantly more in inoculated seedlings than in uninoculated seedlings. This increase may have been caused by nitrogen fixation and phosphate solubilization ability of PGPR used in this study. It is also noted that larger the total rhizosphere population more is the accumulation of nitrogen, phosphorus and potassium (NPK). Increased nutrient uptake associated with seed treated plants may be the results of more root-shoot ratio. Certain plant growth promoting rhizobacteria, affect the development and function of roots by improving uptake of minerals such as nitrate, phosphate, potassium, and water uptake (Tilak *et al.* 2005). The acquisition of nutrients from soils is governed by root growth and its interaction with the abiotic and biotic components of soil. This interaction is manifest largely by the physical, chemical and biological properties of the rhizosphere (Hinsinger *et al.* 2009). Through better understanding of rhizosphere interactions and how roots associate with soil microorganisms there is opportunity for enhancing the efficiency of nutrient uptake by plants (Rengel and Marschner, 2005).

Direct enhancement of mineral uptake due to increase in specific ion fluxes at the root surface in the presence of plant growth promoting rhizobacteria has also been reported (Bertrand, 2000; Bashan and Levanony, 1991). PGPR have been referred to have great impact on nitrogen nutrition by increasing NO_3^- uptake capacity, indirectly as a consequence of stimulated lateral root development and possibly directly by stimulating NO_3^- transport systems (Mantelin and Touraine, 2003).

Plant growth promotion provided by PGPR strains was related to improved root development and enhanced nodulation, resulting in better nutrient uptake capability and increased N supply in the plant (Bai *et al.* 2003). An increase in root length and the volume of soil foraged by the roots, which leads to an enhanced nutrient uptake, is the most commonly proposed explanation for the beneficial effects of PGPR on plant growth. The improvement in mineral nutrition would explain the promotion of plant growth. This rationale is consistent with the observation that plants inoculated with *Azospirillum* take up NPK and micronutrients more efficiently from the soil (Okon and Vanderleyden, 1997). The increased uptake of phosphorus by inoculated apple plants in the present study suggests that *B. licheniformis* strain CKA1 supply phosphorus in a soluble form. The results are in concomitance with previously reported results where phosphorus uptake increased significantly (13-23%) in rice when inoculated with plant growth promoting rhizobial strain (Biswas *et al.* 2000). Kundu and Gaur (1984) tested the effect of phosphorus solubilizing bacteria on yield and phosphorus

absorption by rice and reported 25 per cent increase in the yield. The mechanism of action suggested was enhancement of phosphorus solubilization, with a subsequent increase in uptake of phosphorus by plants. This might hold true for apple seedlings treated with *B. licheniformis* strain CKA1.

4.17 PLANT GROWTH RESPONSE (YIELD, POMOLOGICAL TRAITS, FRUIT CONTENT AND PLANT NUTRIENT CONTENT) OF APPLE TREES TO *Bacillus licheniformis* CKA1 INOCULATION IN APPLE ORCHARDS

Apple trees of 20-25 years old inoculated with liquid based culture of *B. licheniformis* strain CKA1 were selected for analysis of yield, pomological parameters, fruit content and plant nutrient content in apple orchard at different locations (RHRS, Mashobra, Sablog, Matiana Nanni, Matiana and Kyari Kotkhai).

Liquid culture of CKA1 was applied through soil drenching in basin of apple trees alone, foliar spray at pink bud stage and soil drenching in combination with foliar spray for three consecutive years i.e. once in February and again in month of July every year. Pirlak *et al.* (2007) had reported significant increase in growth and yield of apple by foliar and floral spray of PGPR on apple trees.

4.17.1 Yield

Data observed on measured parameters of yield, appended in Table 33 revealed a significant increase in average yield over uninoculated apple trees (control) over a period of three years.

Significant increase in average yield in case of CKA1 inoculated apple trees was observed over un-inoculated apple trees (Table 33). Foliar spray at full bloom in combination with root inoculation resulted in significantly highest yield (28 kg/tree at RHRS, Mashobra (Plate 19); 103.75 kg/tree at Sablog, Matiana (Plate 20); 20.23 kg/tree at Nanni, Matiana (Plate 22) and 110 kg/tree at Kyari, Kotkhai) over uninoculated control (18.00 kg/tree at RHRS, Mashobra; 80.00 kg/tree at Sablog, Matiana; 11.63kg/tree at Nanni, Matiana and 51.00 kg/tree at Kyari, Kotkhai) and other treatments(i.e root inoculation alone (22.00kg/tree at RHRS, Mashobra; 100.25kg/tree at Sablog, Matiana; 16.66kg/tree at Nanni, Matiana and 66.30 kg/tree at Kyari, Kotkhai) and Foliar spray (24.00kg/tree at RHRS,Mashobra; 100.88kg/tree at Sablog, Matiana; 19.33kg/tree at Nanni, Matiana and 81.60 kg/tree at Kyari, Kotkhai)) at four different locations (Plate 21).



Control



CKA1 treated

Plate 19 An overview of field trial on the effect of *B. licheniformis* strain CKA1 on apple trees cv. Staking delicious at RHRS Mashobra apple orchard



Plate 20. An overview of field trial on the effect of *B. licheniformis* strain CKA1 on yield of apple cv. Starking delicious at farmer's field, Sablog, Matiana



Plate 21. Effect of CKA1 inoculation strategies (viz. soil drenching, Foliar spray, soil drenching + Foliar spray) on apple in terms of yield different locations

CKA1 application strategies accounting for an overall 5.26, 25.31, 43.25 and 30.00 per cent increase in case of root inoculation at location RHRS, Mashobra (Fig. 45), Sablog, Matiana, Nanni, Matiana and Kyari, Kotkhai respectively; 26.31, 26.10, 66.20, 60.00 per cent increase in case of foliar spray at full bloom stage at location RHRS, Mashobra, Sablog, Matiana, Nanni, Matiana and Kyari, Kotkhai respectively; 47.36, 29.68, 73.94 and 116.66 per cent increase in case of foliar spray at full bloom stage along with root inoculation at location RHRS, Mashobra, Sablog, Matiana, Nanni, Matiana and Kyari, Kotkhai respectively (Plate 44).

Many PGPR strains have been identified as having the ability to produce the plant growth regulators indole-3-acetic acid, cytokinin and/or other plant hormones in the rhizosphere, which play an important role in plant growth promotion and yield (Bent *et al.* 2001; Cook *et al.* 2001; Gadagi *et al.* 2004; Fogaca and Fett-Neto, 2005; Khalid *et al.* 2004; Lambrecht *et al.* 2000; Ngugi *et al.* 2005; Probanza *et al.* 1996). It is well known that PGPR strains that produce plant hormones can stimulate plant cell elongation or cell division, and/or change bacterial 1- aminocyclopropane-1-carboxylate (ACC) deaminase activity (Patten and Glick, 2002), which prevents the production of plant growth-inhibiting hormone, ethylene (Penrose *et al.* 2001).

The yield and plant growth enhancement effects of bacteria used in this study on apple could be explained due to its inherent N₂-fixing ability, P-solubilization and IAA producing ability. The positive effects of CKA1 on the yield of crops such as apricot, tomatoes, sugar beet, and barley were explained by N₂-fixation ability, P solubilization and antimicrobial substance production (Sahin *et al.* 2000; Abbasi *et al.* 2001; Cakmakci *et al.* 2001; Esitken *et al.* 2002, 2003) and by IAA production (Esitken *et al.* 2002, 2003). In the present study, we have also found that the different strategies of CKA1 inoculation increased yield of apple. Floral and foliar bacterial applications at time of full bloom and after flowering (T stage of the fruit, cell-division phase) stimulated fruit set and fruit development by IAA.

B.licheniformis strain CKA1 was found to be positively correlated with plant growth, similar to the reports documented by Franke *et al.* (2015), where GP16 was found positively correlated with plant growth.

Table 33. Effect of CKA1 inoculation strategies on apple in terms of yield and pomological properties at different locations as an average of 2013 to 2015

Treatments	Yield/tree (kg)	Weight (g)	Breadth (mm)	Length (mm)	Volume (cm ³)	TSS (%)	Titration Acidity (%)
A) Location: RHRS, Mashobra							
Root inoculation	20.00	169.00	69.75	54.10	141.00	7.70	0.65
Foliar spray	24.00	170.00	72.70	58.40	167.00	7.90	0.66
Root inoculation +Foliar Spray	28.00	172.00	78.50	62.31	168.00	7.90	0.67
Control	19.00	154.00	64.77	54.00	114.00	6.50	0.65
CD_{0.05}	1.76	1.55	0.96	1.31	1.10	0.32	0.02
B) Location: Sablog, Matiana							
Root inoculation	100.25	173.00	72.50	64.50	140.00	8.00	0.63
Foliar spray	100.88	186.50	79.00	68.00	180.00	8.30	0.65
Root inoculation+Foliar Spray	103.75	192.00	80.20	69.50	210.00	8.30	0.64
Control	80.00	170.00	65.11	60.20	124.00	6.70	0.65
CD_{0.05}	1.63	1.36	1.97	1.05	1.36	0.26	0.08
(C) Location Nanni, Matiana							
Root inoculation	16.66	165.00	70.50	63.50	150.00	7.50	0.64
Foliar spray	19.33	178.00	73.00	68.00	180.00	8.00	0.68
Root inoculation+Foliar Spray	20.23	180.30	76.00	69.50	184.00	8.00	0.68
Control	11.63	161.50	63.87	58.00	119.00	6.60	0.64
CD_{0.05}	1.74	1.12	0.84	1.62	4.51	1.42	0.04
(D) Kyari, Kotkhai							
Root inoculation	66.30	171.00	73.00	61.00	143.00	7.70	0.67
Foliar spray	81.60	185.00	75.00	67.00	165.00	7.90	0.68
Root inoculation +Foliar Spray	110.50	189.00	81.00	68.50	175.00	7.90	0.69
Control	51.00	167.00	66.70	58.00	117.00	6.50	0.65
CD_{0.05}	2.75	5.09	5.28	1.24	1.85	0.29	0.02



Plate 22. An overview of field trial on the effect of *B. licheniformis* strain CKA1 on yield of apple cv. Starking delicious at farmer's field, Nanni, Matiana

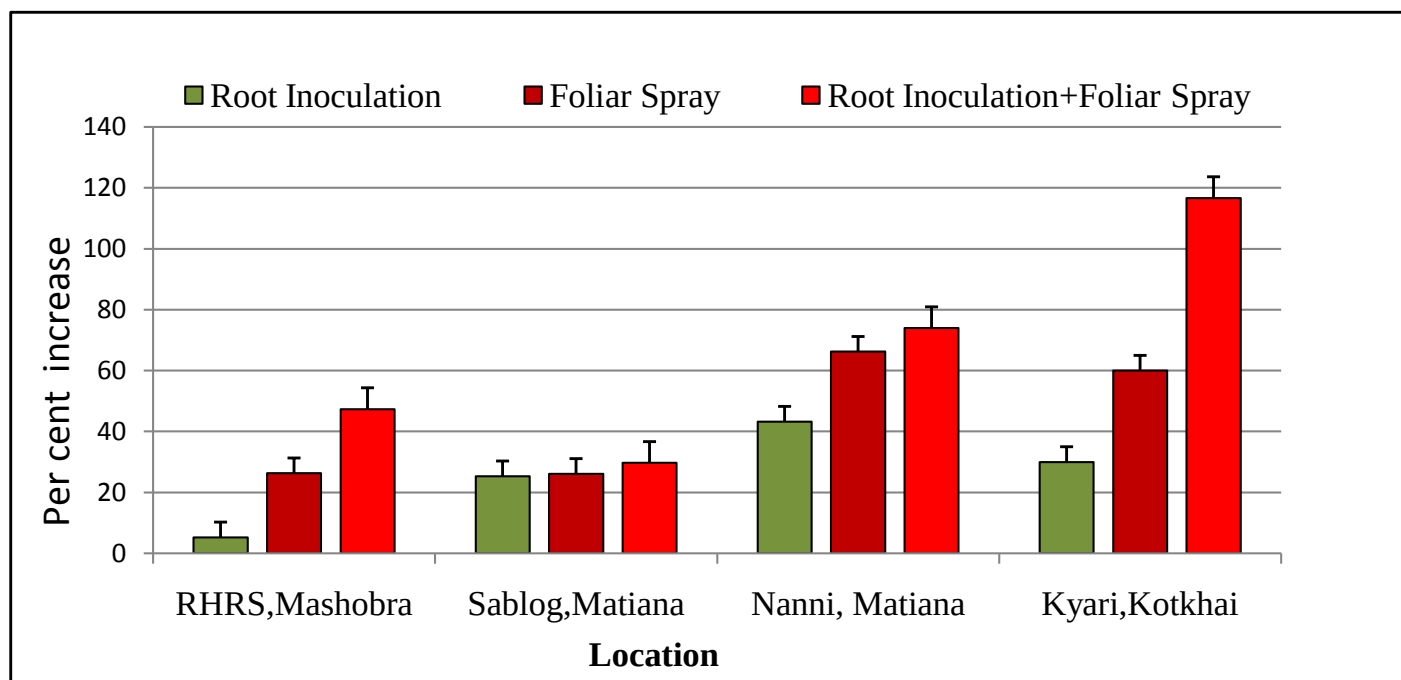


Figure 45. Effect of CKA1 inoculation on per cent increase in yield over uninoculated at different location

Although IAA was analyzed before and after bacteria application, it is well known that some auxins exert positive effects on fruit set and development in various plant species, as strawberry, fig, citrus, and olive. Cytokinins also promote cell division in the presence of auxins (Davies, 2004). Thus, producing IAA by CKA1 may have positive effects on fruit development in apple, which is supported by various studies on IAA production with positive effect on apple plants (Jakobsen, 1986; Smith and Read, 1997; Sundra *et al.* 2002; Shen *et al.* 2004).

Growth promotion effects were seen early in young apple trees development, and these subsequently translated into higher yield, which was also reported by Dobbelaere *et al.* (2002). PGPR inoculation strongly influenced shoot length and diameter and yield during the early stages of growth. Thus indicates that the tested PGPR strain *B. licheniformis* strain CKA1 is capable of promoting apple plant growth in the early years of plantation. Results presented in the present study are in concomitance with Aslantas *et al.* (2007) who reported that newly planted apple trees inoculated with OSU-142, OSU-7, BA-8 and M-3 PGPR resulted in fruit yield by 116.4, 88.2, 137.5 and 73.7 per cent, respectively. Pirlak *et al.* (2007) reported floral and foliar application of plant growth promoting rhizobacteria (PGPR) to apples increased yield, growth, and nutrient element contents of leaves. The results of this study suggested that *Pseudomonas* BA-8 and *Bacillus* OSU-142 alone or in combination had the potential to increase the yield, growth, and nutrition of apple trees.

In the present study, *B. licheniformis* strain CKA1 inoculation not only increased growth but also yield of apples trees. Neilsen *et al.* (2001) observed that the optimum amount of nitrogen application for young dwarf apple trees was estimated to be less than 10 g/year/tree. Furthermore, apple trees grown with sufficient soil nitrogen can grow vigorously and produce fruits for long-term without supplementing nitrogen fertilization. Excessive nitrogen application increases growth and leaf nitrogen content, but negatively affects fruit quality (Komamura *et al.* 2000). In addition to, based on tree vigour, budgets for N, P, and K and soil N status, N concentration of medium would be adequate for maiden apple trees, without over-fertilizing and excessive growth (Ro and Park, 2000). The amount of shoot growth is often used as an indicator of plant N requirement in. The growth of 25–30 cm indicates a good N fertilization program in young apple trees locally (Guleryuz *et al.* 1998).

In the present study, the shoot growth of inoculated trees with *B. licheniformis* strain CKA1 was in the range, which indicates the PGPR inoculation match tree N requirement.

These observations indicate that mechanisms of growth promotion via N₂ fixation and other mechanisms such as phytohormone production, improved nutrient uptake balance, go hand in hand, thus highlighting the multifunctional plant growth promoting activities of *B. licheniformis* strain CKA1.

4.17.2 Fruit pomological traits

Data appended in Table 33 revealed that for all the pomological properties, effect of the CKA1 treatment was statistically significant. The CKA1 treatment of root inoculation and foliar spray resulted in highest fruit weight at all the four locations (172.00g at RHRS, Mashobra, 192.00g at Sablog, Matiana, 20.23g at Nanni, Matiana and 110.00g at Kyari, Kotkhai) which was significantly higher than uninoculated control (154g at RHRS, Mashobra, 170g at Sablog, Matiana, 161g at Nanni, Matiana and 167g at Kyari, Kotkhai). Effect of foliar spray of PGPR on pomological properties of apple is also suggested by Pirlak *et al.* (2007).

The root inoculation along with foliar spray of CKA1 treatment had highest fruit breadth (78.50mm at RHRS, Mashobra, 80.20mm at Sablog, Matiana, 76.00mm at Nanni, Matiana and 81.00mm at Kyari, Kotkhai), fruit length (62.31mm at RHRS, Mashobra, 69.50mm at Sablog, Matiana, 69.50mm at Nanni, Matiana and 68.50mm at Kyari, Kotkhai), fruit volume (168.00cm³ at RHRS, Mashobra, 210cm³ at Sablog, Matiana, 184.00cm³ at Nanni, Matiana and 175.00 cm³ at Kyari, Kotkhai) which are significantly higher than fruit breadth (64.77 mm at RHRS, Mashobra, 65.11mm at Sablog, Matiana, 63.87mm at Nanni, Matiana and 66.70 mm at Kyari, Kotkhai), fruit length (54.00mm at RHRS, Mashobra, 60.20 mm at Sablog, Matiana, 58.00 mm at Nanni, Matiana and 58.00mm at Kyari, Kotkhai), fruit volume (114.00cm³ at RHRS, Mashobra, 124.00cm³ at Sablog, Matiana, 119.00cm³ at Nanni, Matiana and 117.00cm³ at Kyari, Kotkhai) of control at all the locations.

PGPR treatment as foliar spray and in its form of soil drenching are known to show effect on different pomological parameters as suggest by many researchers earlier (Orhan *et al.* 2006; Pirlak *et al.* 2007; Pirlak and Kose, 2009)

4.17.3 Fruit chemical analysis

The results obtained from the bacterial treatments on fruit chemical composition is displayed in Table 33. Chemical analysis was performed on the same fruit on which

pomological analysis was carried out. CKA1 root inoculation along with foliar spray and foliar spray alone had highest total soluble solid (7.90% at RHRS, Mashobra, 8.30% at Sablog, Matiana, 8.00% at Nanni, Matiana and 7.90 % at Kyari, Kotkhai) which was significantly higher than control (6.50% at RHRS, Mashobra, 6.70% at Sablog, Matiana, 6.60% at Nanni, Matiana and 6.50% at Kyari, Kotkhai).

Similar trend was observed in case of titrable acidity which was highest in case of CKA1 root inoculation along with foliar spray at all the location, although neither of these is significantly different than control.

Similar increase in TSS and titrable acidity by bacterial treatment had been reported in wheat (Ebrahim and Ally, 2004) and barley and sugar (Cakmakci *et al.* 2001).

4.17.4 Per cent nutrient (NPK)

Data observed on measured parameters of per cent nutrient (NPK) appended in Table 34 revealed a significant increase in per cent NPK over un-inoculated apple trees over a period of three years.

In CKA1 inoculated apple trees (root inoculation + foliar spray at full bloom), based on mean values of three year data on per cent N content in leaves was 2.08 per cent at location RHRS, Mashobra, 2.11 per cent at location Sablog, Matiana, 2.07 per cent at location Nanni, Matiana and 2.28 per cent at location Kyari, Kotkhai which differed significantly from that observed in un-inoculated apple trees (1.88 per cent at RHRS, Mashobra, 1.83 per cent at Sablog, 1.91 per cent at Nanni, Matiana and 1.95 per cent at Kyari, Kotkhai).

Table 34 Measured parameters of per cent nutrient (NPK) in leaves 20-25 year old apple trees (cv. Starking delicious) at different location as an average of 2013-2015

Treatments	RHRS, Mashobra			Sablog, Matiana			Nanni, Matiana			Kyari, Kotkhai		
	N (%)	P (%)	K (%)	N (%)	P (%)	K (%)	N (%)	P (%)	K (%)	N (%)	P (%)	K (%)
Root inoculation+Foliar Spray	2.08	0.22	1.18	2.11	0.25	1.58	2.07	0.20	1.62	2.28	0.27	1.60
Control	1.88	0.13	0.91	1.83	0.15	1.16	1.91	0.17	1.01	1.95	0.19	1.25
t- value	3.44	2.45	5.11	3.45	2.11	3.45	1.98	3.25	2.56	3.40	2.54	4.23

Based on mean values of three year data, per cent P content in leaves of CKA1 inoculated apple trees was 0.22 per cent at location RHRS, Mashobra, 0.25 per cent at location Sablog, Matiana, 0.20 per cent at location Nanni, Matiana and 0.27 per cent at location Kyari, Kotkhai which differed significantly from that observed in un-inoculated apple trees (0.13 per cent at location RHRS, Mashobra, 0.15 per cent at location Sablog, Matiana, 0.17 per cent at location Nanni and 0.19 per cent at location Kyari, Kotkhai).

Similar trend was observed in case of K content in leaves of CKA1 inoculated trees with 1.18 per cent at location RHRS, Mashobra, 1.58 per cent at location Sablog, Matiana, 1.62 per cent at location Nanni, Matiana and 1.60 per cent at location Kyari, Kotkhai. These values of per cent K differed significantly from those observed in uninoculated apple trees (0.91 per cent at location RHRS, Mashobra, 1.16 per cent at location Sablog, Matiana, 1.01 per cent at location Nanni, Matiana and 1.25 per cent at location Kyari, Kotkhai).

The higher N and P content in the *Bacillus licheniformis* CKA1 treated trees might results from the N₂-fixation ability and P-solubilizing ability of the bacteria, respectively, as reported earlier (Sahin *et al.* 2000; Cakmakci *et al.* 2001; Esitken *et al.* 2003; Orhan *et al.* 2006; Aslantas *et al.* 2007; Karakurt *et al.* 2009 and Karakurt and Aslantas, 2010). It is well known that increasing N content in the plant results in greater uptake of nutrient elements such as P, K, Ca, and Mg from soil (Marschner, 1995). Earlier studies have reported that the percentage of N, P and K was significantly and/or relatively higher in the bacteria-treated plants, which may be explained by N₂-fixing and P-solubilization activity of the bacterial population.

4.18 POPULATION DYNAMICS OF INOCULATED *B. licheniformis* STRAIN CKA1

4.18.1 Rhizospheric and endospheric population dynamics of inoculated *B. licheniformis* strain CKA1 and its comparative study with morphologically identical reisolated bacterial strains using ARDRA

Population densities of bacteria colonizing rhizosphere soil and root endosphere of CKA1 inoculated apple seedlings in net house, apple nursery, apple orchard at RHRS, Mashobra and farmers apple orchard at Sablog, Matiana, Jhalroo, Matiana Nanni, Matiana, Thanedhar, Kufarbagh, Kotkhai Kyari, Kotkhai are presented in Table 35 and 36 (Plate 23, 24). Based on colony morphology, count of bacterial isolates matching *B. licheniformis* strain CKA1 were made and based on which Simpson's index of Dominance (D) was computed.

Representative isolates were selected and compared with *B. licheniformis* strain CKA1 based on ARDRA profile developed earlier in the study, results appended in plate 25, 26 and 27.

Similar to the present study, Mahajan (2013) and Tahir *et al.* (2008) have also used ARDRA profile to study the establishment of inoculated PGPR in the rhizosphere of apple trees.

Net House

In net house, total rhizosphere and endosphere bacterial populations in CKA1 inoculated apple seedlings were 94.53×10^6 CFU/g soil and 32.64×10^4 CFU/g root respectively. Rhizosphere populations identical to *B. licheniformis* strain CKA1 was 52.54×10^6 CFU/g soil with a Simpson's index of Dominance (D) of 1.51 (Plate 23). This may imply that rhizobacteria had more competitive ability to survive and affect the growth of inoculated plants in the presence of indigenous microflora (Khalid *et al.* 2004). Thus, suggesting the establishment and persistence of *B. licheniformis* strain CKA1 in rhizosphere of apple trees.

Bacterial population identical to *B. licheniformis* strain CKA1 in root endosphere (Plate 24) was 28.56×10^4 CFU/g root accounting for 3.80 Simpson's index of Dominance (D).

RI-R-NH and RI-E-NH were selected as representative isolates.

Apple nursery field

Total rhizosphere bacterial population in CKA1 inoculated apple seedlings was 98.68×10^6 CFU/g soil of which the population matching *B. licheniformis* strain CKA1 was 51.57×10^6 CFU/g soil accounting for 1.35 Simpson's index of Dominance (D).

In the root endosphere of CKA1 inoculated apple seedlings, total bacterial population was 26.00×10^4 CFU/g root. Population matching *B. licheniformis* strain CKA1 was 20.15×10^4 CFU/g root accounting for 3.05 Simpson's index of Dominance (D).

RI-R-ANF and RI-E- ANF were selected as representative isolates

In the present study, tracing back of inoculated strains, from apple rhizosphere after the trial showed successful colonization by the inoculated bacteria. Colonization of apple

rhizosphere by bacterial isolates at the right place and time is essential for enhanced PGP activity. Determining the dynamics of root colonization by the introduced bacteria is essential for their effective use as it is critical in plant growth promotion and biological control (Kloepper & Beauchamp, 1992). Moreover, successful interaction between the plant and inoculated PGPR depends on sufficient population density, rhizosphere competence, root colonizing ability and PGP ability of the bacteria (Lugtenberg & Dekkers, 1999).

Apple orchard RHRS, Mashobra

Total culturable rhizospheric bacterial population in CKA1 inoculated apple trees was 110.54×10^6 CFU/g soil of which the population matching *B. licheniformis* strain CKA1 was 78.67×10^6 CFU/g soil accounting for 2.51 Simpson's index of Dominance (D).

In the root endosphere of CKA1 inoculated apple seedlings, total bacterial population was 33.57×10^4 CFU/g root. Population matching *B. licheniformis* strain CKA1 was 24.61×10^4 CFU/g root accounting for 2.60 Simpson's index of Dominance (D).

RI-R-M and RI-E-M were selected as representative isolates.

Farmers apple orchard, Sablog Matiana

Total culturable rhizospheric bacterial population in CKA1 inoculated apple trees was 117.53×10^6 CFU/g soil of which the population matching *B. licheniformis* strain CKA1 was 88.56×10^6 CFU/g soil accounting for 2.81 Simpson's index of Dominance (D).

Total endophytic bacterial population reisolated from the CKA1 inoculated apple seedlings was 23.45×10^4 CFU/g root. Population matching *B. licheniformis* strain CKA1 was 18.00×10^4 CFU/g root accounting for 2.88 Simpson's index of Dominance (D).

RI-R-S and RI-E- S were selected as representative isolates.

Similat to the present study, Hameeda *et al.* (2008) has also traced back the introduced phosphate-solubilizing bacteria in field trials from maize rhizosphere.

Farmers apple orchard, Jhalroo, Matiana

Total culturable rhizospheric bacterial population in CKA1 inoculated apple trees was 114.21×10^6 CFU/g soil of which the population matching *B. licheniformis* strain CKA1 was 84.00×10^6 CFU/g soil accounting for 2.60 Simpson's index of Dominance (D).

In the root endosphere of CKA1 inoculated apple seedlings, total bacterial population was 37.54×10^4 CFU/g root. Population matching *B. licheniformis* strain CKA1 was 28.50×10^4 CFU/g root accounting for 2.80 Simpson's index of Dominance (D).

RI-R-J and RI-E- J were selected as representative isolates.

Farmers apple orchard, Nanni, Matiana

Total culturable rhizospheric bacterial population in CKA1 inoculated apple trees was 104.21×10^6 CFU/g soil of which the population matching *B. licheniformis* strain CKA1 was 86.41×10^6 CFU/g soil accounting for 2.80 Simpson's index of Dominance (D).

Total culturable endophytic bacterial population colonizing CKA1 inoculated apple seedlings was 27.50×10^4 CFU/g root. Population matching *B. licheniformis* strain CKA1 was 22.50×10^4 CFU/g root accounting for 3.32 Simpson's index of Dominance (D).

RI-R-N and RI-E- N were selected as representative isolates.

Sharma *et al.* (2015) also traced back of inoculated strains, from tomato rhizosphere after the trial using the isolation method and resemblance of the morphotype obtained from the soil samples with the inoculated parent stain and thus showed successful colonization by the inoculated bacteria.

Farmers apple orchard, Thanedhar

Total culturable rhizospheric bacterial population in CKA1 inoculated apple trees was 121.69×10^6 CFU/g soil of which the population matching *B. licheniformis* strain CKA1 was 89.12×10^6 CFU/g soil, accounting for 3.40 Simpson's index of Dominance (D).

In the root endosphere of CKA1 inoculated apple seedlings, total bacterial population was 31.00×10^4 CFU/g root. Population matching *B. licheniformis* strain CKA1 was 26.80×10^4 CFU/g root, accounting for 3.69 Simpson's index of Dominance (D).

RI-R-KU and RI-E- KU were selected as representative isolates

Farmers apple orchard, Kyari, Kotkhai

Total culturable rhizospheric and endophytic bacterial population in CKA1 inoculated apple trees at Kyari, Kotkhai followed a similar trend as found in reisolation of culturable bacterial population from CKA1 inoculated apple trees from apple orchard at RHRS,

Table 35. Rhizosphere population dynamics of inoculated *B. licheniformis* strain CKA1 and its comparison with morphologically identical re-isolates based on Simpson's index of Dominance

Re-isolation source Rhizosphere Soil from CKA1 treated plants obtained from experiments conducted in (5 Samples from each experiment)	Total viable bacterial population on Nutrient agar (x10 ⁶ CFU /g soil)	Number of bacterial isolates matching <i>B. licheniformis</i> strain CKA1 based on colony morphology (x 0 ⁶ CFU /g soil)	Simpson's index of Dominance (D)*	Representative** isolate selected for molecular based identification
Net house	94.53	52.54	1.51	RI-R-NH
Apple nursery field, Nauri	98.68	51.57	1.35	RI-R-ANF
RHRS, Mashobra	110.54	78.67	2.51	RI-R-M
Sablog, Matiana	117.53	88.56	2.81	RI-R-S
Jhalroo, Matiana	114.53	84.00	2.60	RI-R-J
Nauri, Matiana	104.21	86.41	2.80	RI-R-N
Thanedhar	121.69	89.12	3.40	RI-R-T
Kyari, Kotkhai	113.76	81.68	2.60	RI-R-K
Kufarbagh, Kotkhai	98.53	79.54	3.20	RI-R-KU

** RI: Re-isolate; R: Rhizosphere; NH: Net house; ANF: Apple nursery field; M: Mashobra; S: Sablog; J: Jhalroo; N: Nauri; T: Thanedhar; K: Kyari; KU: Kufarbagh
 * Simpson's index of Dominance (D) $D = \frac{1}{\sum P_i^2}$ where P_i is the relative abundance of isolates calculated according to the following equation $P_i = \frac{n_i}{N}$
 Where, n_i , is the number of isolates matching *B. licheniformis* strain CKA1 and N, is the total rhizosphere soil bacterial population.

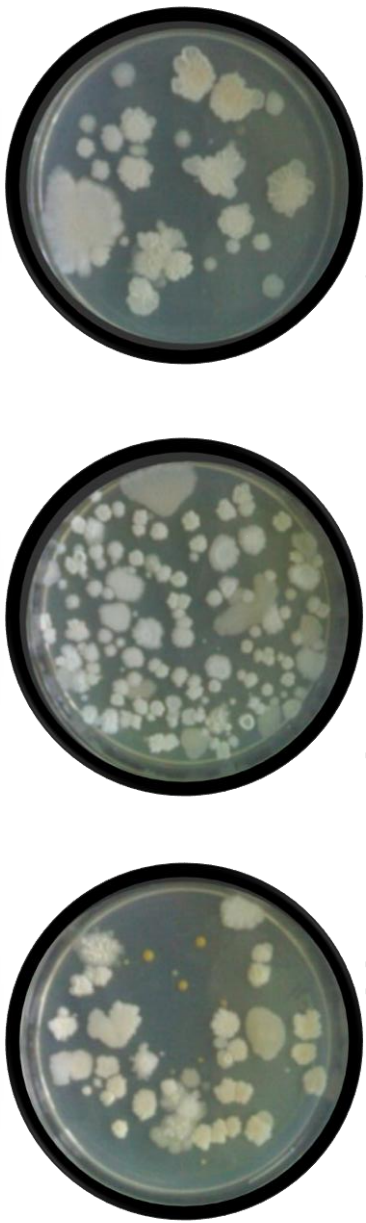


Plate 23. Total culturable rhizosphere soil bacterial population on nutrient agar dominated by bacterial colonies matching *B. licheniformis* strain CKA1

Table 36. Endosphere population dynamics of inoculated *B. licheniformis* strain CKA1 and its comparison with morphologically identical re-isolates based on Simpson's index of Dominance

Re-isolation source Rhizosphere Soil from CKA1 treated plants obtained from experiments conducted in (5 Samples from each experiment)	Total viable bacterial population on Nutrient agar (x10 ⁶ CFU /g soil)	Number of bacterial isolates matching <i>B. licheniformis</i> strain CKA1 based on colony morphology (x 0 ⁶ CFU /g soil)	Simpson's index of Dominance (D)*	Representative** isolate selected for molecular based identification
Net house	32.64	28.56	3.80	RI-E-NH
Apple nursery field, Naini	26.00	20.15	3.05	RI-E-ANF
RHRS, Mashobra	33.57	24.61	2.60	RI-E-M
Sablog, Matiana	23.45	18.00	2.88	RI-E-S
Jhalroo, Matiana	37.54	28.50	2.80	RI-E-J
Naini, Matiana	27.50	22.50	3.32	RI-E-N
Thanedhar	31.00	26.80	3.69	RI-E-T
Kyari, Kotkhai	26.53	21.57	3.28	RI-E-K
Kufarbagh, Kotkhai	31.00	28.00	4.05	RI-E-KU

** RI: Re-isolate; E: Endophytic; NH: Net house; ANF: Apple nursery field; M: Mashobra; S: Sablog; J: Jhalroo; N: Naini; T: Thanedhar; K: Kyari; KU: Kufarbagh

* Simpson's index of Dominance (D) $D = \frac{1}{\sum P_i^2}$ where P_i is the relative abundance of isolates calculated according to the following equation $P_i = \frac{n_i}{N}$

Where, n_i , is the number of isolates matching *B. licheniformis* strain CKA1 and N, is the total rhizosphere soil bacterial population.

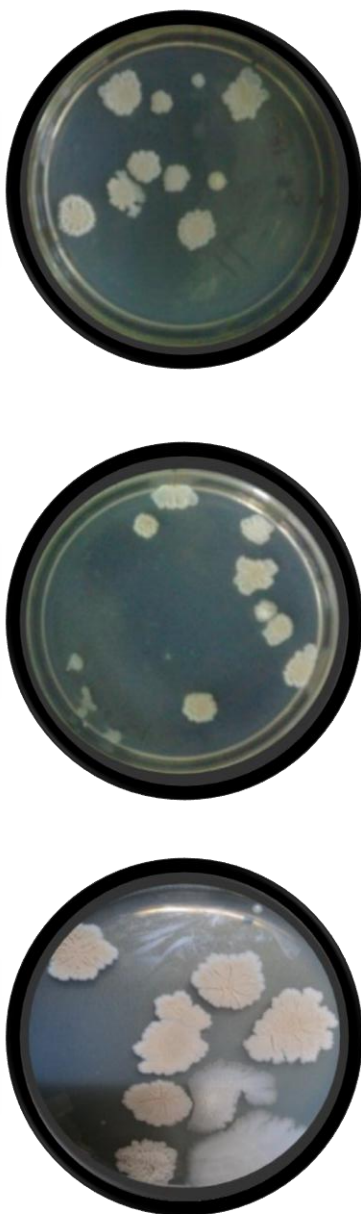


Plate 24. Total culturable endophytic bacterial population on nutrient agar dominated by bacterial colonies matching *B. licheniformis* strain CKA1

In the root endosphere of CKA1 inoculated apple seedlings, total bacterial population was 26.53×10^4 CFU/g root. Population matching *B. licheniformis* strain CKA1 (Plate 24) was 21.57×10^4 CFU/g root accounting for 3.28 Simpson's index of Dominance (D).

RI-R- K and RI-E-K were selected as representative isolates.

Farmers apple orchard, Kufarbagh, Kotkhai

Total culturable rhizosphere bacterial population in CKA1 inoculated apple trees was 98.53×10^6 CFU/g soil of which the population matching *B. licheniformis* strain CKA1 was 79.54×10^6 CFU/g soil accounting for 3.20 Simpson's index of Dominance (D).

In the root endosphere of CKA1 inoculated apple seedlings, total bacterial population was 31.00×10^4 CFU/g root. Population matching *B. licheniformis* strain CKA1 was 21.57×10^4 CFU/g root accounting for 4.05 Simpson's index of Dominance (D).

RI-R-KU and RI-E- KU were selected as representative isolates.

4.18.2 Comparative study of morphologically identical re-isolated bacterial strains with *B. licheniformis* strain CKA1 using ARDRA

Re-isolated rhizospheric bacterial strains viz. RI-R-NH, RI-R-ANF, RI-R-M, RI-R-S, RI-R-J, RI-R-N, RI-R-T, RI-R-K RI-R-KU and endophytic RI-E-NH, RI-E-ANF, RI-E-M, RI-E-S, RI-E-J, RI-E-N, RI-E-T, RI-E-K, RI-E-KU were compared with ARDRA profile of *B. licheniformis* strain CKA1.

Identical ARDRA finger prints of re-isolate strains with strain CKA1 presented in plate further confirmed the similar molecular identity of re-isolated stains with *B. licheniformis* strain CKA1 (Plate 25, 26 and 27).

The biotic parameters, especially the indigenous microflora and microfauna lead to a significant affect on the establishment and population densities of an introduced bacterial inoculant. The significantly higher bacterial population of culturable microbial communities in rhizosphere soil and endophytic rhizobacteria of treated plants over the untreated control irrespective of other treatment is an indication of the aggressive nature of PGPR in colonizing all parts of root system. Similarly, the numbers of phosphate solubilizing bacteria (PSB) in the rhizosphere of different crops were found higher with inoculation of PSB as compared to the un-inoculated control (Kundu *et al.* 2002). The presence of endophytic bacteria within the

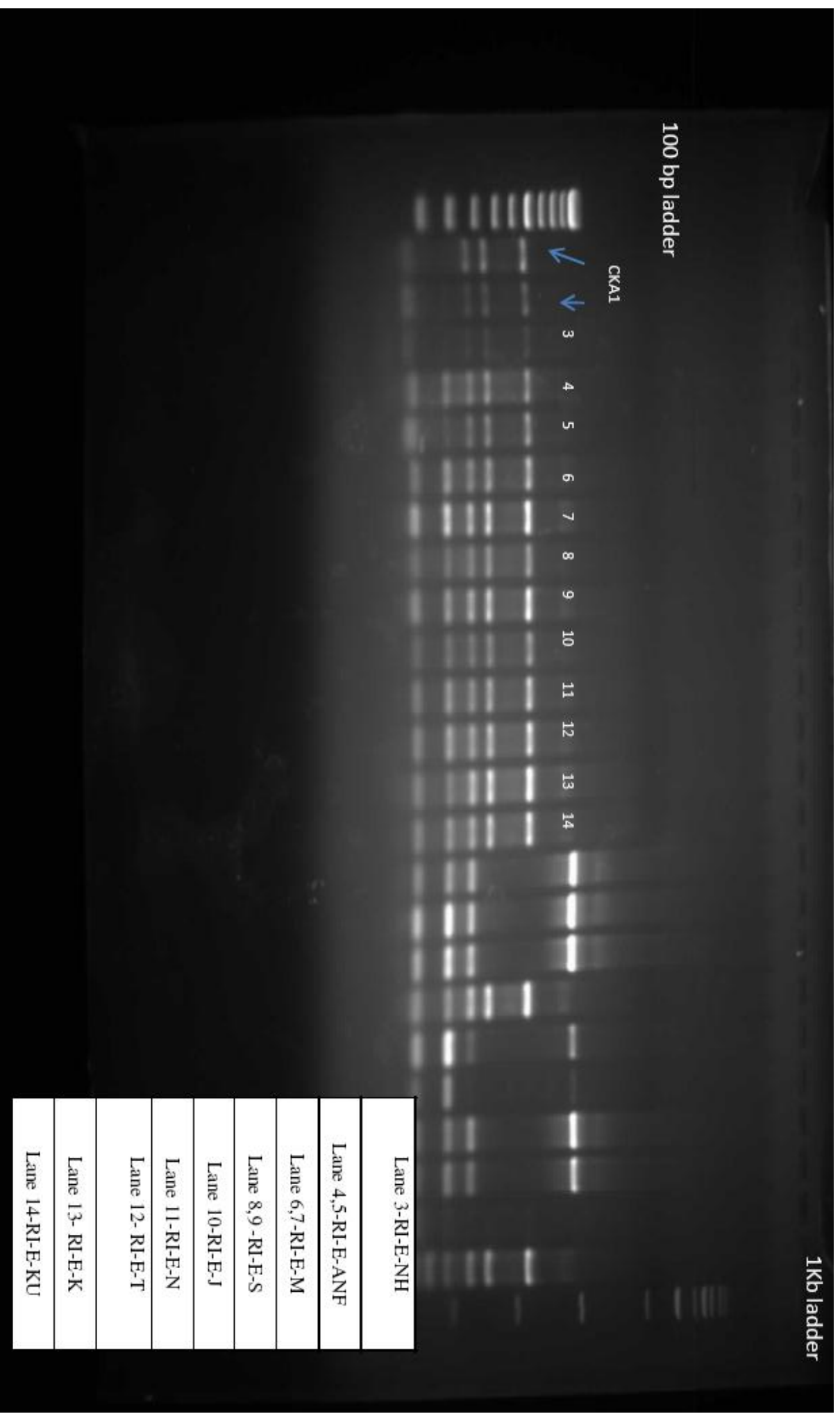


Plate 25. ARDRA based fingerprints of strain CKA1 and its comparison with re-isolates with *Hinf* I restriction enzymes

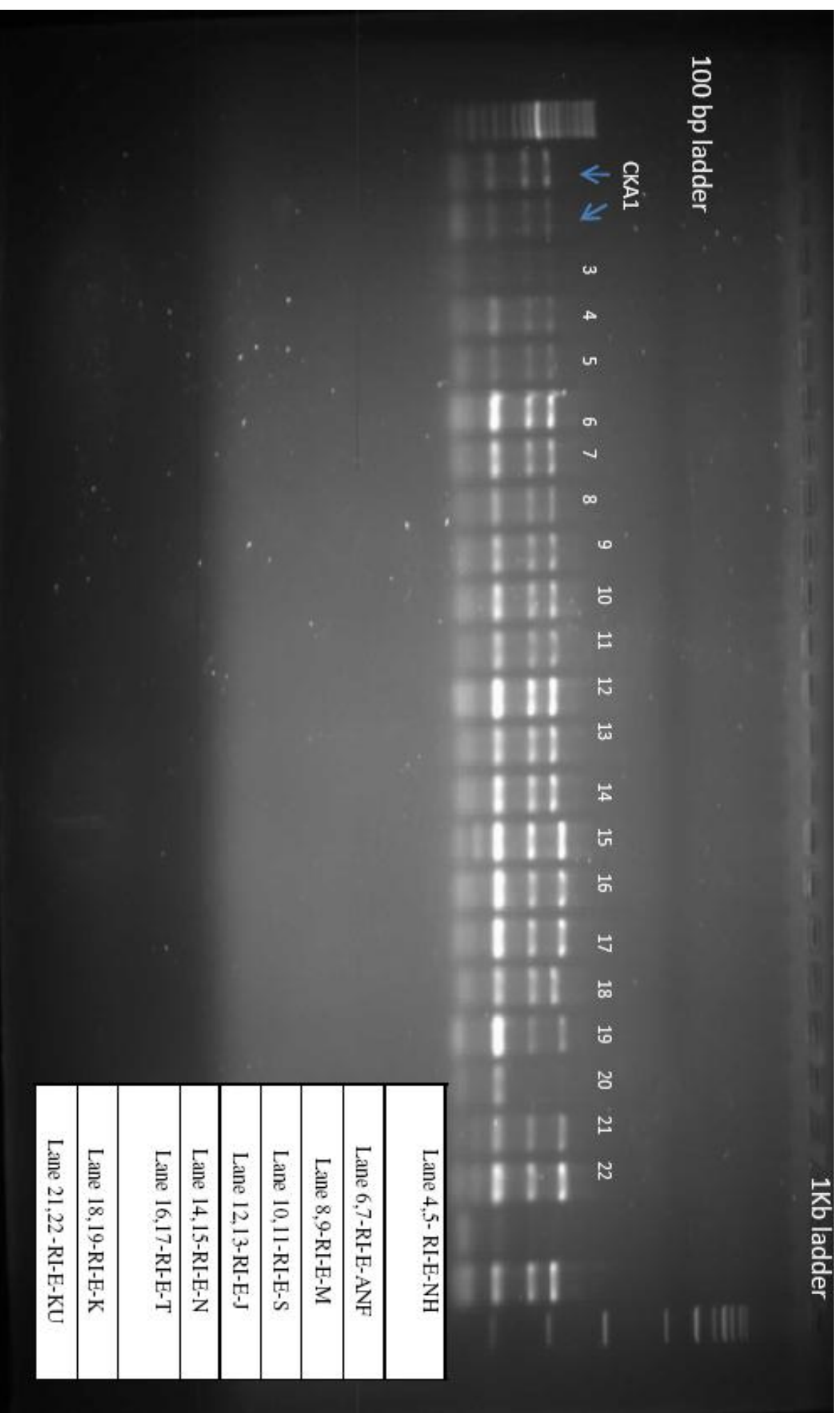


Plate 26. ARDRA based fingerprints of strain CKA1 and its comparison with re-isolates with *Hpa* II restriction enzymes

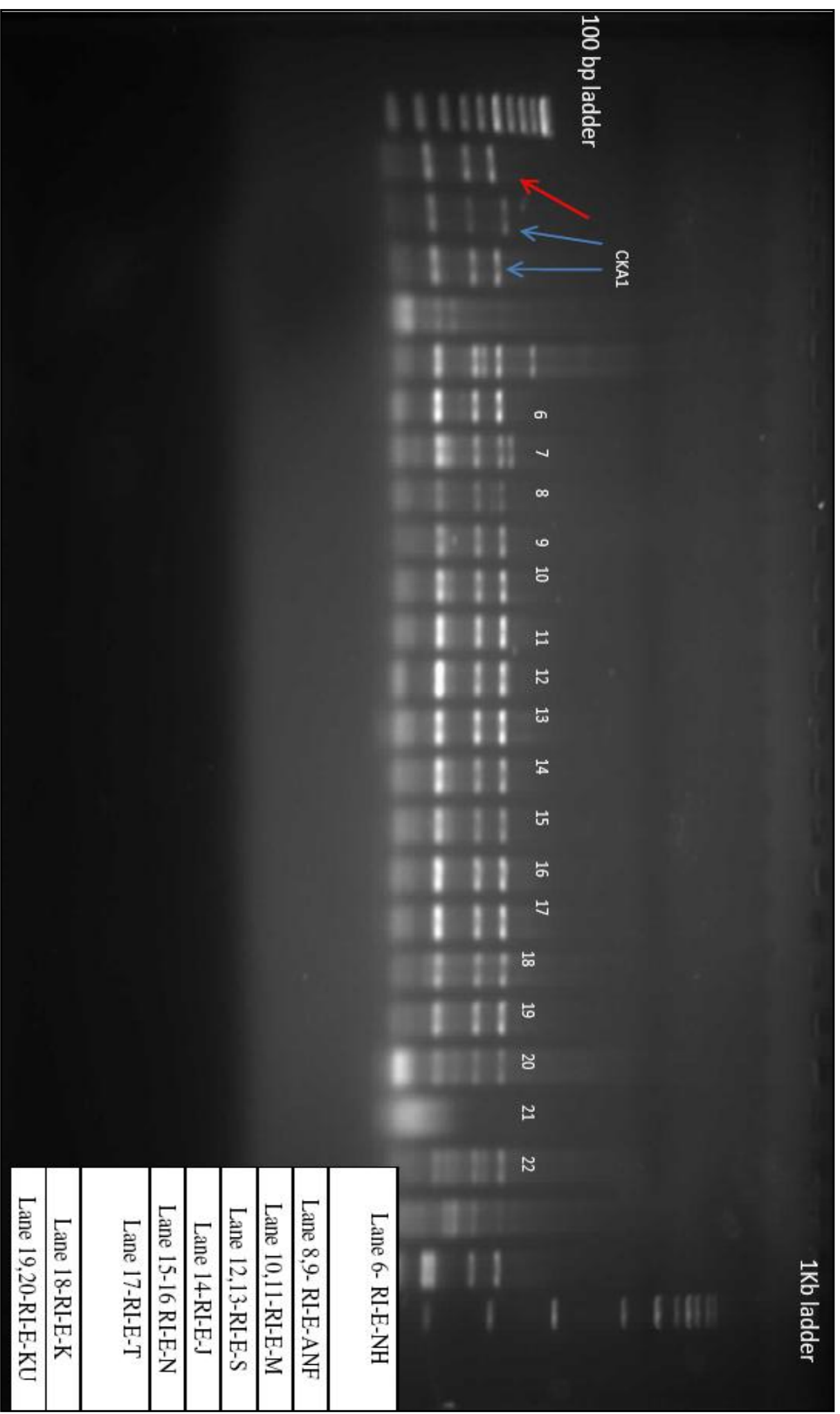


Plate 27. ARDRA based fingerprints of strain CKA1 and its comparison with re-isolates with *Taq I* restriction enzymes

roots of apple seedlings was investigated in the present study. To ensure that the endophytic colonization values of wet root determined in this study reflected only the numbers of cells in the interior of the plant tissues, a specific surface sterilization-trituration method was developed (discussed in materials and methods). This sterilization method should have killed and/or washed away the surface bacteria while the internal bacteria survived. Several bacteria have the ability to attach to root surfaces (rhizosphere) allowing them to derive maximum benefit from root exudates. *B. licheniformis* strain CKA1 investigated in the present study seems to be more specialized, as it possesses the ability to penetrate and reside the root tissues (endophyte) and have direct access to organic compounds in the apoplast. By occupying this preferential endophytic location, this PGPR strain does not face competition from its counterparts as encountered in the rhizosphere or in the soil.

Earlier workers have demonstrated the use of antibiotic resistant marker (Peer *et al.* 1990), construction of *gfp* and *gusA*-marked strains (Compant *et al.* 2004) for determination of the similarity of the strains. However, there have always been issues with the incorporation of genetically modified microorganisms in soil. Keeping in view the above limitation, application of ARDRA technique was employed in the present study to develop an environmentally friendly easy to use approach for monitoring *B. licheniformis* strain CKA1 in soil. Phenotypic characterization using RAPD and ARDRA of plant growth promoting rhizobacteria has been successfully employed by various researchers (Cello *et al.* 1997; Fischer *et al.* 2007; Lawongsa *et al.* 2008).

Chapter-5

SUMMARY AND CONCLUSION

The major findings of the present investigation entitled “**Study of rhizobacterial community structure and evaluation of plant growth promoting rhizobacterial application strategies to promote growth and yield in apple**” can be summarized under the following sub-headings:

5.1 Examination of the diversity of culturable bacterial communities colonizing the rhizosphere soil apple trees at ((a) healthy and root rot infected diseased apple orchards; (b) replant sites and new plantation sites) at different locations of Himachal Pradesh.

(a) Healthy apple and root rot infected diseased apple orchards:

1. In healthy apple orchards examination of functional rhizobacterial diversity indicated higher percentage of P-solubilizers followed by putative N- fixers and siderophore producers in apple rhizosphere. Whereas, in root rot infected diseased apple orchards, presence of lesser population of P-solubilizers, siderophore producers and putative nitrogen fixers was observed.
2. Culturable rhizobacteria, in healthy apple orchards an overall average of 76.41 per cent were P-solubilizers, whereas only 43.73 per cent were observed at root rot infected diseased apple orchards.
3. Siderophore producers in healthy apple orchards accounted for average of 47.08 per cent, whereas in root rot infected diseased apple orchards their contribution was 19.20 per cent.
4. An overall average contribution of culturable rhizobacterial population growing on nitrogen free medium in healthy orchards was 52.37 per cent, whereas 18.35 per cent were observed in root rot infected diseased apple orchards.
5. The occurrence of functionally better performing rhizobacteria in terms of their innate abilities of plant growth promotion from healthy sites hints towards the role of root exudates from healthy apple trees, that might exert a selective pressure on

the proliferation of specific microbes. The plausible reason, for the lower population densities of functional rhizobacteria in root rot infected diseased apple orchards might be due to the interactions of pathogenic microbes with host, especially in the root ecosphere.

6. A total of 66 isolates from eleven healthy apple orchards, 31 from seven root rot infected diseased apple orchard were selected and screened for multifarious plant growth promoting traits.
7. Significantly high variation was observed in antagonism against phytopathogens among isolates with 77.27%, 30.00%, 86.36%, 63.63% and 77.27% antagonistic activity against *Dematophora necatrix*, *Phytophthora* sp., *Fusarium oxysporum*, *Sclerotium rolfsii* and *Pythium aphanidermatum* respectively, in isolates of apple from healthy apple orchards. However, isolates from root rot infected diseased apple orchard exhibited 35.48%, 6.45%, 45.00%, 35.25% and 41.93% antagonistic activity against *Dematophora necatrix*, *Phytophthora* sp., *Fusarium oxysporum*, *Sclerotium rolfsii* and *Pythium aphanidermatum*, respectively.
8. Principal Component Analysis (PCA) was carried to evaluate correlations between apple associated rhizobacterial populations and different plant growth promoting traits in healthy and root rot infected diseased apple orchards.
9. The results, suggest that higher variation in population of phosphate solublizers is present at the location with root rot infected diseased apple trees, thus indicating the rhizobacterial mediated P-mobilization phenomenon is not a limiting factor in root rot infected diseased apple orchards.
10. Lower population variance of rhizobacterial antagonists to *Dematophora necatrix* are present at the location with root rot infected diseased apple trees, thus indicating the fact that, the fungal pathogen *Dematophora necatrix* significantly contributes to the disease in root rot infected diseased apple orchards.
11. Higher population variance of siderophore producers at healthy locations, suggest that the iron chelating properties of siderophore producers did not allow the pathogens to survive in healthy tree rhizosphere. Thus, the findings indicate population of siderophore producers is critical when comparing relationship

between apple rhizobacterial isolates (isolated from healthy apple and root rot infected diseased apple orchards) and plant growth promoting traits.

12. ARDRA based molecular diversity analysis among all the 66 isolates from healthy apple were grouped into 15 different phylotype at 75 % similarity level whereas, all 31 isolates from root rot infected diseased apple were grouped into 8 different phylotype.
13. The results indicated that the bacteria belonging to family Bacillaceae were amongst the most abundant in the rhizosphere of the apple trees at healthy as well as at root rot infected diseased apple orchards followed by the bacteria belonging to family Enterobacteriaceae.
14. Phylogenetic analysis confirmed the affiliation of the selected PGPR from healthy apple into *Bacillus*, *Cosenzaea*, *Plesiomonas*, *Providencia* and *Tatumella* as revealed by clustering of each genus to its corresponding group. Whereas, representative bacterial isolates from root rot infected diseased apple orchards were grouped into *Bacillus*, *Pantoea*, *Leminorella* and *Morganella* as revealed by clustering of each genus to its corresponding group.

(b) Replant sites and new plantation sites

1. At replant sites of apple orchards, examination of functional rhizobacterial diversity indicated higher percentage of functionally diverse rhizobacteria whereas, in new plantation sites, a functionally less diverse rhizobacterial population was observed.
2. Culturable bacterial population, in new plantation and replant sites of apple orchards accounted for an overall average of 72.43 per cent and 32.65 per cent P-solubilizers, respectively.
3. Siderophore producers in new plantation and replant sites of apple orchards accounted for average of 37.54 per cent and 9.39 per cent contribution, respectively.
4. An overall average contribution of rhizobacterial population growing on nitrogen free medium in new plantation sites of orchards was 38.48 per cent, whereas 26.38 per cent were observed in replant sites of apple orchards.

5. Lower population of P-solubilizers, putative nitrogen fixers and siderophore producers could be associated to the fact that in the soil of apple orchard with replant sites, there is low availability of nutrients and organic matter thus, resulting in a less favorable environment for the growth plants and for many species of microorganisms. Compared with new planting sites, where the soil is rich in nutrients the possibility of obtaining higher nutrient mobilizes in rhizosphere is high, as is also evident from the findings in the present study.
6. Significantly high variation was observed in antagonism against phytopathogens among isolates with 33.65%, 8.65%, 27.88%, 25.96% and 26.62% antagonistic activity against *Dematophora necatrix*, *Phytophthora* sp., *Fusarium oxysporum*, *Sclerotium rolfsii* and *Pythium aphanidermatum* respectively, in isolates from replant sites of apple orchards. However, isolates from new plantation sites of apple orchard exhibited 56.25%, 12.50%, 62.50%, 50.00% and 62.50% antagonistic activity against *Dematophora necatrix*, *Phytophthora* sp., *Fusarium oxysporum*, *Sclerotium rolfsii* and *Pythium aphanidermatum*, respectively.
7. Principal Component Analysis (PCA) was carried to evaluate correlations between apple associated rhizobacterial populations and different plant growth promoting traits in apple replant sites and new plantation sites of apple orchards.
8. Higher variance (34.03%) in siderophore producers was observed at new plantation sites, while a lower variance of 33.06% was observed in replant apple, which correlated lower populations of siderophore producers in replant apple. Lower populations variance of siderophore producers at replant apple locations; suggest that the lower iron chelating properties of rhizobacteria, allowed the pathogens to survive at replant apple sites, which can be attributed as the plausibly reason for poor establishment of apple seedlings at replant sites. Thus, population of siderophore producers is critical when comparing relationship between apple rhizobacterial isolates (isolated from new plantation sites and replant sites apple) and plant growth promoting traits.
9. At new plantation sites, maximum variance was exhibited by rhizobacterial isolates antagonistic to *Dematophora necatrix* (41.40%), which was followed by *Phytophthora* sp. (24.73%). Similarly, at replant sites maximum variance was

exhibited by rhizobacterial isolates antagonistic to *Dematophora necatrix* (28.68%), which was followed by *Phytophthora* sp. (21.04%).

10. Presence of lower population variance of rhizobacterial antagonists to *Dematophora necatrix* at the replant sites, indicate the fact that, the fungal pathogen *Dematophora necatrix* significantly contributes to the poor establishment of new apple seedlings at replant sites.
11. ARDRA based molecular diversity analysis among all the 104 isolates from fifteen healthy apple orchards, were grouped into 23 different phylotype at 75 % similarity level whereas, all 16 isolates from four root rot infected diseased apple orchards were grouped into 8 different phylotypes.
12. Phylogenetic analysis confirmed the dominance of genus *Bacillus* at replant sites of apple whereas, at new plantation sites bacteria belonging to genus *Bacillus*, *Pantoea* and *Pluralibacter* were observed as was revealed by clustering of each genus to its corresponding group.

5.2 Selection and comparison of different PGPR strains, for growth promotion and bio-control against phytopathogens based on *in planta* evaluations

1. Bacterial rhizospheric (strain G4(3) and strain RG1(3)) and endophytic (strain CKA1 and strain CKAM) strains isolated from rhizosphere/roots of apple (*Malus domestica* Borkh.) were selected in the present study based on their unique ability of exhibiting multifarious plant growth promoting (PGP) traits. viz. **nutrient mobilization** (P-solubilization, ability to fix atmospheric nitrogen, iron chelating ability); **phyto-hormone production** (indole-3-acetic acid production); **act as bio-protecting agent** (siderophore production, chitinase and protease enzyme production, and antifungal activity against soil borne fungal pathogens such as *Phytophthora* spp., *Fusarium oxysporum*, *Pythium aphanidermatum*, *Sclerotium rolfsii*, *corticium salmonicolour* and *Dematophora necatrix*).
2. Results based on morphological, physiological and biochemical tests suggested that the strain CKA1, RG1(3) and CKAM belonged to genus *Bacillus* and identified as strain CKA1 *Bacillus licheniformis*, *B. methylotropicus* strain RG1(3) and *B. methylotropicus* strain CKAM.

3. Based on genotypic and phylogenetic identification (16S ribosomal RNA (*rrn*) gene), bacterial endophyte (strain CKA1 and strain CKAM) isolated from apple roots were identified as *Bacillus licheniformis* strain CKA1 and *B. methylotropicus* strain CKAM; rhizospheric isolates (strain G4(3) and strain RG1(3)) were identified as *S. plymuthica* strain G4(3) and *B. methylotropicus* strain RG1(3), respectively .
4. Seeds bacterized with liquid formulation of all bacterial strains resulted in significantly higher per cent germination as compared to un-inoculated seeds.
5. Seed treatment with *B. licheniformis* strain CKA1, *S. plymuthica* strain G4 (3), *B. methylotropicus* strain CKAM, *B. methylotropicus* strain RG1 (3) and consortium accounted for 52.53, 18.09, 21.75, 38.22 and 36.40 per cent increase, respectively, in case of seed germination over un-inoculated control.
6. Seed treatment with *B. licheniformis* strain CKA1, *S. plymuthica* strain G4 (3), *B. methylotropicus* strain CKAM, *B. methylotropicus* strain RG1 (3) and consortium accounted for 72.80, 14.42, 29.90, 57.60 and 44.83 per cent increase over un-inoculated control, respectively in case of shoot length; 29.07, 15.00, 16.66, 24.52 and 21.66 per cent increase over un-inoculated control, respectively, in case shoot diameter; 56.76, 26.25, 33.42, 42.44 and 38.42 per cent increase, respectively, over un-inoculated control in case of root length; 23.47, 10.54, 18.06, 20.44, 18.55 per cent increase, respectively, over un-inoculated in case of leaf surface area.
7. Under net house condition, in case of one year treated apple seedlings, among all the PGPR treatments, *B. licheniformis* strain CKA1 accounted for maximum per cent increase in shoot length (25.81%), shoot diameter (33.99%), root length (21.46%) and leaf surface area (43.90%).
8. Evaluation of different PGPR strains and their consortium on vegetative growth of two month old transplanted apple seedling under nursery field conditions after six months of plantation, revealed that *B. licheniformis* strain CKA1 treated seedlings resulted in maximum 44.21, 86.04, 34.71 per cent increase in shoot length, shoot diameter and leaf surface area, respectively.

9. Evaluation of different PGPR strains and their consortium revealed that maximum shoot length, shoot diameter and leaf surface area, after nine months of grafting was observed in *B. licheniformis* CKA1 treated plants (cv. Red Chief, Top Red and cv. Oregon spur) followed by *B. methylotropicus* RG1(3) treatment.
10. Studies based upon comparison of different PGPR strains and their consortia conclusively indicate that *B. licheniformis* strain CKA1 (with multiple PGPR traits) proved to be statistically most effective in improving the plant growth parameters of apple plants and therefore led to its selection for subsequent studies on growth yield of apple under field condition.
11. Selected bacterial strains were evaluated for its effect in managing white root rot and collar rot disease in apple seedling/plants under net house conditions.
12. Among the different PGPR strains and their consortia, *B. licheniformis* strain CKA1 had the most significant effect in reducing the disease severity with biocontrol efficacy of 80% in case of white root rot disease in apple seedlings.
13. Liquid-based culture of *B. licheniformis* strain CKA1 and *S. plymuthica* strain G4(3) were found to be the most promising treatments for biocontrol of collar rot disease in apple seedlings caused by *P. cactorum* with minimum 30.00 % disease incidence after four weeks after the pathogen challenge.
14. Ability of bacterial strains to act as nutrient mobilizer was evaluated by characterizing its ability to solubilise tri-calcium phosphate. Ability of *B. licheniformis* strain CKA1 to act as nutrient mobilizer was evaluated by characterizing its ability to solubilise tri-calcium phosphate. Partial characterization of molecular mechanism involved in tri-calcium phosphate solubilise was studied by molecularly characterizing *B. licheniformis* strain CKA1 for enzyme glucose dehydrogenase (GDH)-encoding gene (*gdh*)
15. Ability of bacterial to act as phytohormone producer was evaluated by characterizing its ability to produce IAA. An attempt was made to identify the crude IAA produced by most efficient *B. licheniformis* strain CKA1 using TLC and HPLC analysis.

5.3 Multi-location field trial based evaluation of selected, single strain rhizobacterium based bio-formulation, with multifarious plant growth promoting traits on growth and yield of apple, employing different application strategies

1. Plant growth response to seed treatment with liquid based formulation of *B. licheniformis* strain CKA1 resulted in significant increase in shoot, root, leaf surface and per cent nutrient (NPK) of apple seedlings under field condition in both fresh and replant sites of apple orchards at different locations.
2. Apple cultivars varied response to different PGPR treatments under field conditions with bacteria might be due to the difference in agro climatic conditions where the trials were executed. The inoculation with PGPR contributed to the increase in vegetative growth and nutrient content of apple when compared to the control, but it strongly depended on the cultivars and different bacterial strains tested.
3. Effect of liquid formulation of CKA1 on apple yield, pomological traits, and fruit content and nutrient parameters was also evaluated at different locations. Comparison of different application strategies of liquid formulation viz. soil drenching in basin of apple trees alone, foliar spray at pink bud stage and soil drenching in combination with foliar spray for three consecutive years revealed that maximum apple yield/tree (28.00kg at RHRS, Mashobra; 103.75kg at Sablog, Matiana; 20.23kg at Nanni, Matiana; 110.50kg at Kyari, Kotkhai) was observed when apple trees were treated with CKA1 using a combination of root inoculation and foliar spray.
4. The CKA1 treatment of root inoculation and foliar spray resulted in highest fruit weight at all the four locations (172.00g at RHRS, Mashobra, 192.00g at Sablog, Matiana, 20.23g at Nanni, Matiana and 110.00g at Kyari, Kotkhai) which was significantly higher than un-inoculated control (154g at RHRS, Mashobra, 170g at Sablog, Matiana, 161g at Nanni, Matiana and 167g at Kyari, Kotkhai). A similar trend was observed for other fruit pomological traits viz. fruit breath, fruit length, fruit volume.
5. CKA1 root inoculation along with foliar spray and foliar spray alone had highest total soluble solid (7.90% at RHRS, Mashobra, 8.30% at Sablog, Matiana, 8.00%

at Nanni, Matiana and 7.90 % at Kyari, Kotkhai) which was significantly higher than control (6.50% at RHRS, Mashobra, 6.70% at Sablog, Matiana, 6.60% at Nanni, Matiana and 6.50% at Kyari, Kotkhai). Similar trend was observed in case of titrable acidity.

5.4 Evaluation of population dynamics of inoculated PGPR in the rhizosphere of apple

1. Re-isolated bacterial strains were compared with ARDRA fingerprints of *B. licheniformis* strain CKA1. Identical ARDRA finger prints of re-isolate strains with strain CKA1 presented in plate further confirmed the similar molecular identity of re-isolated stains with *B. licheniformis* strain CKA1.
2. In the present study, use of these techniques has paved the way for development of molecular marker for identification of *B. licheniformis* strain CKA1 as indicated by the presence of unique band in ARDRA finger print of strain CKA1 and re-isolates.
3. The significantly higher bacterial population of culturable microbial communities in rhizosphere soil and endophytic rhizobacteria of treated plants over the untreated control irrespective of other treatment is an indication of the aggressive nature of PGPR in colonizing all parts of root system.

In conclusion, the present has lead to better understanding the structural and functional diversity of culturable bacterial communities colonizing the rhizosphere of apple trees at ((a) healthy and root rot infected diseased apple orchards; (b) replant sites and new plantation sites) at different locations of Himachal Pradesh. Studies carried out using *B. licheniformis* strain CKA1 as model PGPR has resulted in development of a sustainably viable PGPR based technology, using single strain formulation with multifunctional PGP traits to address the long-standing desire of farmers/orchardists to ensure that their land remains productive in future. Furthermore, the evaluation of different bio-formulation application strategies, in the present studies has paved the way for enhanced apple production in Trans-Himalayan region of Himachal Pradesh, where apple productivity is limited not only by strict geo-physical conditions, but also by root rot infected diseased apple orchards and reduced survival of apple plants at replant sites. Therefore, the use of *B. licheniformis* strain CKA1 as a bioinoculant is an environmentally benign biological approach for managing fungal diseases, enhancing the plant

health and maximizing profitability over the short (minimizing the use of chemicals) and long term (increasing growth and yield). The findings in present work are of much significance considering current scenario where at present more than 30 per cent of the apple plantations in the state of Himachal have completed their productive life and apple orchard rejuvenation projects are being ambitiously launched by State agencies for restoring six decade old planted apple orchards.

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ABSTRACT

Apple (*Malus domestica* Borkh.) is one of the most important fruit crops grown and consumed in the world. In Himachal Pradesh, apple crop occupies an area of 1,07,700 hectare with a production of 7,38,700 metric tons. Attempts to enhance apple productivity, in the Trans Himalayan region has resulted in rapid expansion in area under apple cultivation. However, the strict physio-geographic conditions, pose several hurdles. High yielding modern varieties are being adopted, to increase the productivity, which are highly responsive to external inputs. This results in indiscriminate use of chemical inputs, which poses potential threat to human health and environment. The utilization of plant growth promoting rhizobacteria (PGPR) may help to develop an environmentally benign biological approach for managing fungal diseases and enhancing the plant health resulting in increase in yield. In the last decade of research, several PGPR strains that have been identified and the research on PGPR has seen a tremendous boost, mainly because the role of the rhizosphere as an ecological unit has gained importance in the functioning of the biosphere.

The present study envisages better understanding of structural and functional rhizobacterial diversity associated with apple replant sites and root rot infected diseased apple orchards. Principal component analysis revealed detailed insight into correlation between apple associated rhizobacterial population and different plant growth promoting traits in (a) healthy and root rot infected apple orchards; (b) apple replant sites and new plantation apple orchards. Molecular diversity analysis employing techniques viz. amplified ribosomal DNA restriction analysis (ARDRA) and DNA sequence analysis of 16S rRNA gene revealed comparative insight into structural variations in rhizobacterial diversity.

In planta evaluations using microbial formulations developed using PGPR strains viz. *Bacillus licheniformis* strain CKA1, *B.methylotropicus* strain CKAM, *Serratia plymuthica* strain G4(3), *B.methylotropicus* Strain RG1(3), and their consortium have been comprehensively evaluated in the present study. Bio-control potential of microbial formulations against phyto-pathogens has also been assessed using *in planta* experimentation. Strain CKA1 based bio-formulation proved to be the most effective as compared to all other PGPR strains in providing antagonism against pytopathogens as revealed by experimentations carried out under net house conditions.

Attempts have also been made, in the present study to understand the genetic basis of mineral phosphate solubilization in strain CKA1. Cloning and sequencing of *gdh* gene has paved the way for understanding molecular mechanisms involved in nutrient mobilization of essential nutrient (phosphorus) and its subsequent enhanced uptake by plants, as mediated by the rhizobacteria.

Multi-location trials were carried out with inoculation of *B. licheniformis* strain CKA1 to access its effect on early vegetative growth of apple plants under field conditions. Effect of strain CKA1 on vegetative growth of apple was also compared at fresh plantation and re-plantation sites. Results revealed significant increase in vegetative growth over un-inoculated controls at all the locations studied. Effect of liquid formulation of CKA1 on apple yield, pomological traits, and fruit content and nutrient parameters was also evaluated at different locations. Comparison of different application strategies of liquid formulation viz. soil drenching in basin of apple trees alone, foliar spray at pink bud stage and soil drenching in combination with foliar spray for three consecutive years revealed that maximum apple yield/tree (28.00kg at RHRS, Mashobra; 103.75kg at Sablog, Matiana; 20.23kg at Nanni, Matiana; 110.50kg at Kyari, Kotkhai) was observed when apple trees were treated with CKA1 using a combination of root inoculation and foliar spray.

Population dynamics studies carried out to determine the fate of inoculated strain of CKA1 in apple rhizosphere revealed successful establishment and colonization of the inoculated strain as indicated by high values Simpson's index of Dominance which ranged from 1.51 to 3.40 at different locations.

Apple orchard rejuvenation projects are being ambitiously launched by State agencies for restoring six decade old planted apple orchards. Integrated nutrient management systems comprising biological systems especially, single strain multifunctional PGPR such as strain CKA1 opens up new avenues not only for improving crop yield but also in sustaining soil health.

Signature of Major Advisor

Signature of the Student

Countersigned

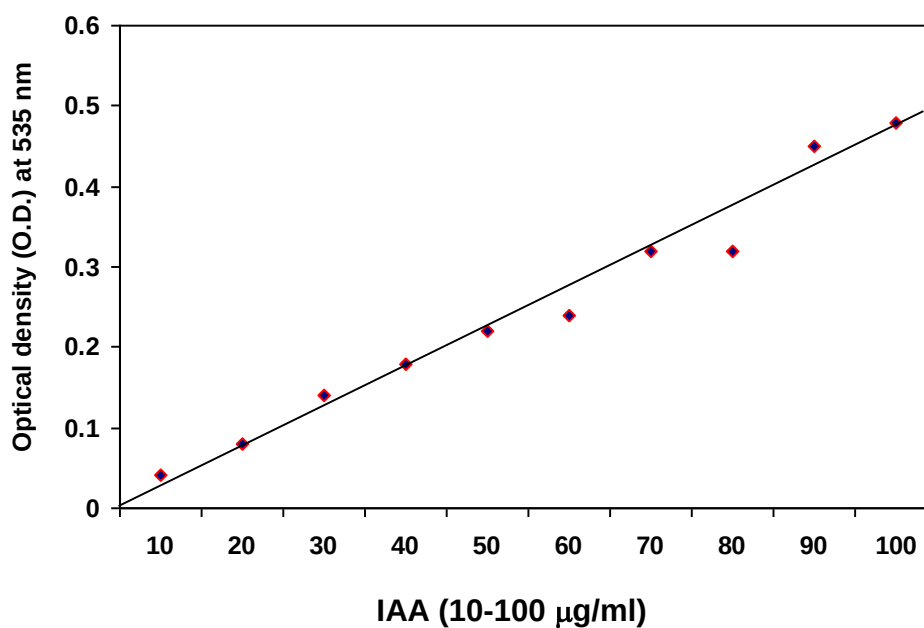
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APPENDIX I

1.1 Preparation of standard curve (10-100 µg/ml) for IAA Stock solution (100 ppm):

10 mg of IAA (99.00 %) pure was dissolved in 50 ml distilled water and the final volume was made to 100 ml in a volumetric flask.

IAA (ml)	Distilled water (ml)	Final volume (ml)	Salkowski reagent (ml)	ppm (ml)	Optical density (O.D.) at 535 nm
0.3	2.7	3	2	10	0.04
0.6	2.4	3	2	20	0.08
0.9	2.1	3	2	30	0.14
1.2	1.8	3	2	40	0.18
1.5	1.5	3	2	50	0.20
1.8	1.2	3	2	60	0.24
2.1	0.9	3	2	70	0.30
2.4	0.6	3	2	80	0.32
2.7	0.3	3	2	90	0.40
3.0	0.0	3	2	100	0.48



1.2 Preparation of the standard curve for P- estimation

i) 0.2195 g KH_2PO_4 + distilled water = 1 litre standard solution (50 ppm)

Volume of standard solution (ml)	Distilled water(ml)				concentration (ppm)	Optical density (O.D.) at 660 nm
0.0	1.0	+ 5ml Ammonium molybdate	+1 ml. working solution SnCl_2	Final volume 25ml.	0	0.00
0.1	0.9				5	0.07
0.2	0.8				10	0.13
0.3	0.7				15	0.20
0.4	0.6				20	0.39
0.5	0.5				25	0.33
0.6	0.4				30	0.46
0.7	0.3				35	0.46
0.8	0.2				40	0.47
0.9	0.1				45	0.56
1.0	0.0				50	0.66

ii) Estimation of Phosphorous in sample

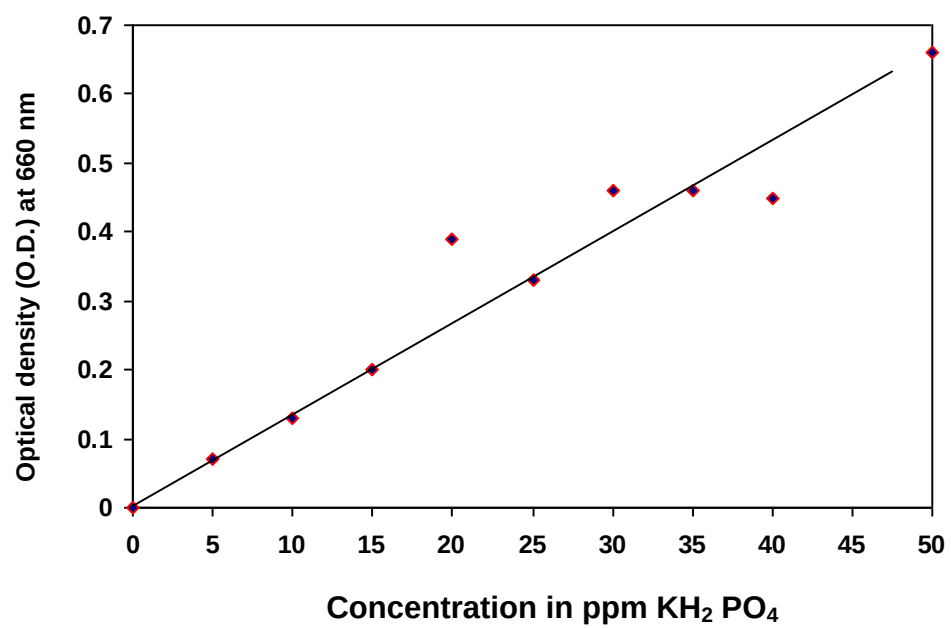
- 50 ml PVK broth + 10 per cent inoculum (1 O.D at 540nm)
- Incubation under shaking condition
- Centrifugation
- 1ml Culture filtrate + distilled water = 25 ml (dilution 25 times)
- 5ml of above CF (step d) + 5ml. Ammonium molybdate + 1ml working solution of SnCl_2 + d. w = final volume to 25ml (dilution 5 times) O.D at 660 nm.

Total dilution $25 \times 5 = 125$ times

iii) Calculations:

Concentration (ppm) from standard curve $\times 125$ (dilution factor) = P-solubilized (ppm)

Final concentration calculated after deducting from P-solubilized in control



APPENDIX-II

Anova for Table 16: Effect of selected PGPR inoculations on seed germination and growth promotion of apple seedling under net house conditions

Source	d.f.	Mean Sum of Square				Leaf surface area
		Germination (%)	Shoot length	Shoot diameter	Root length	
Treatment	5	249.374	19.314	33.595	1.475	63.974
Error	12	13.217	3.595	0.361	0.281	2.735
Total	17					

Anova for Table 17: Effect of selected PGPR inoculation on vegetative growth of one year old apple seedling under net house conditions

Source	d.f.	Mean Sum of Square			
		Shoot length	Shoot diameter	Root length	Leaf surface area
Treatment	5	585.510	1.9765	1.475	41.92
Error	12	26.983	0.579	0.281	4.30
Total	17				

Anova for Table 18: Effect of PGPR inoculation on vegetative growth of two year old grafted apple seedling under net house conditions

Red Chief					
Source	d.f.	Mean Sum of Square			
		Shoot length	Shoot diameter	Leaf surface area	
Treatment	5	30.085	14.607	16.979	
Error	12	6.204	2.260	5.887	
Total	17				

Oregon Spur					
Source	d.f.	Mean Sum of Square			
		Shoot length	Shoot diameter	Leaf surface area	
Treatment	5	18.716	23.267	9.100	
Error	12	6.127	5.530	2.601	
Total	17				

Top red					
Source of Variation	d.f.	Mean Sum of Square			
		Shoot length	Shoot diameter	Leaf surface area	
Treatment	5	29.476	17.783	15.894	
Error	12	6.981	5.198	6.670	
Total	17				

Anova for Table 19: Effect of PGPR inoculation on vegetative growth of two month old transplanted apple seedling under field conditions

Source	d.f.	Mean Sum of Square		
		Shoot length	Shoot diameter	Leaf surface area
Treatment	5	580.734	3.483	35.435
Error	12	12.820	0.510	6.523
Total	17			

Anova for Table 20: Biocontrol efficacy of bacterial antagonists toward *Dematophora nectrix* under net house conditions

Source of variation	d.f.	Mean Sum of Square				Antagonists population after trial
		Disease incidence after 2 weeks	Disease incidence after 4 weeks	Disease severity index after 2 weeks	Disease severity index after 4weeks	
Treatment (T)	5	1,734.009	1,517.898	886.325	867.554	1,976.000
Error	12	3.193	7.628	2.984	4.733	8.167
Total	17					

Anova for Table 21: Biocontrol efficacy of bacterial antagonists towards *Phytopathora cactorum* under net house conditions

Source of variation	d.f.	Mean Sum of Square				Antagonists population after trial
		Disease incidence after 2 weeks	Disease incidence after 4 weeks	Disease severity index after 2 weeks	Disease severity index after 4weeks	
Treatment (T)	5	213.989	192.335	57.603	231.186	1,821.614
Error	12	3.156	1.975	1.746	2.834	7.974
Total	17					

Anova for Table 22: Effect of *B.licheniformis* strain CKA1 inoculation on vegetative growth of apple plants (cv. Red Chief) at replant sites of apple orchard at different locations

Source	d.f.	Mean Sum of Square		
		Shoot length	Shoot diameter	Leaf surface area
Replication	2			
Factor A	1	6,813.825	36.236	27.412
Factor B	3	2,313.088	48.983	66.106
Intraction A X B	3	264.074	3.246	2.661
Error	14	3.440	0.233	0.550
Total	23			

Anova for Table 23: Effect of most efficient *B.licheniformis* strain CKA1 inoculation on vegetative growth of apple plants (cv.Red Chief) at new plantation sites at different locations

Source	d.f.	Mean Sum of Square		
		Shoot length	Shoot diameter	Leaf surface area
Replication	2			
Factor A	1	6,350.800	1.451	14.453
Factor B	3	14,266.445	86.030	2.577
Intraction A X B	3	391.928	1.002	1.126
Error	14	21.747	0.169	0.562
Total	23			

Anova for Table 24: Effect of *B. licheniformis* strain CKA1 inoculation on vegetative growth of apple seedlings (cv.Top Red) at replant sites of apple orchard at different locations

Source	d.f.	Mean Sum of Square		
		Shoot length	Shoot diameter	Leaf surface area
Replication	2			
Factor A	1	4,642.605	102.770	64.673
Factor B	2	1,622.160	42.159	28.609
Intraction A X B	2	628.289	3.489	1.181
Error	10	0.642	2.443	0.249
Total	17			

Anova for Table 25: Effect of *B. licheniformis* strain CKA1 inoculation on vegetative growth of apple seedlings (cv.Super Chief) at replant sites of apple orchard at different locations

Source	d.f.	Mean Sum of Square		
		Shoot length	Shoot diameter	Leaf surface area
Replication	2			
Factor A	1	1,598.208	15.736	13.006
Factor B	2	17.510	6.500	3.116
Intraction A X B	2	6.673	4.239	1.136
Error	10	0.191	1.416	0.946
Total	17			

CURRICULUM VITAE

Name : Shalini Chandel
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10+2	First	HP Board of School Education, Dharmshala	2006
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During the study period : University Fellowship

(SHALINI CHANDEL)