

Studies on Microbial Phosphate Solubilization and Development of Inoculum Formulations

A Thesis
Submitted in fulfillment of the requirement
for the award of the degree of

**DOCTOR OF PHILOSOPHY
IN
BIOTECHNOLOGY**

Gurdeep Kaur
(Regd. No. 900900001)



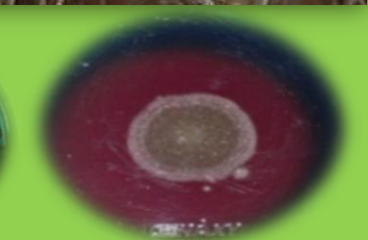
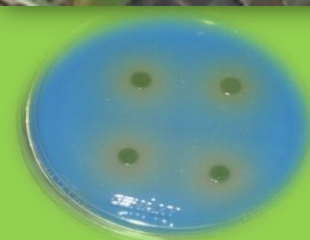
Department of Biotechnology

Thapar University

Patiala-147004

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Certificate

It is hereby certified that the thesis “**Studies on microbial phosphate solubilization and development of inoculum formulations**” which is submitted by Miss **Gurdeep kaur, (Regd. No. 900900001)**, in fulfillment of the requirement for the award of the degree of **DOCTOR OF PHILOSOPHY** in the Department of Biotechnology, Thapar University, Patiala, Punjab, India, is a record of the candidate’s own independent and original research work carried out by her under my supervision and guidance. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

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Declaration

I hereby declare that the work which is being presented in the thesis “**Studies on microbial phosphate solubilization and development of inoculum formulations**” submitted by me for the award of the degree of **DOCTOR OF PHILOSOPHY** in the Department of Biotechnology, Thapar University, Patiala, is true and original record of my own independent and original research work carried out under the kind supervision of Prof. M. Sudhakara Reddy, Department of Biotechnology, Thapar University, Patiala, India. The matter embodied in this thesis has not been submitted in part or full to any other University or Institute for the award of any degree in India or abroad.

Date: 11. Feb, 2014

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Place: Patiala.

Acknowledgement

*There are too many people I must acknowledge who have helped me generously and whole heartedly to complete my present endeavor. First and foremost, I must acknowledge and thanks to the **GOD**, the Almighty (**Waheguru Ji**), for his showers of blessings throughout my research work to complete the research successfully. I always feel their presence and who have always been there for me during my hard and happy times.*

*With due respect, I wish to express my deep sense of gratitude, acclamation and indebtedness to my supervisor **Prof. M. SUDHAKARA REDDY**, Professor, Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, India for his pertinent guidance, patronage, prudent advises, motivation, enthusiasm, immense knowledge and dedication for research rendered to me during my work, without which the present endeavor would not have achieved the same status. His guidance helped me in all the time of research and writing of this thesis. I extend my heartfelt thanks to him forever throughout my life.*

*Besides my advisor, I would like to thank the rest of my doctoral committee: **Dr. N. Tejo Prakash** (Professor, DBTES, TU) and **Dr. P.K. Bajpai**, Dean (Research and Sponsored Projects), for their encouragement, insightful comments, and relevant questions.*

*I deem it a pleasure to express my regards and gratitude to **Dr. Dinesh Goyal** (Professor and Head, DBTES, TU), for his kindness, support, professional guidance and valuable advice in many different ways.*

*I am thankful to **Dr. Anil Kumar**, **Dr. N. Das**, **Dr Anita Rajor** and **Dr. Pinaki Dey** (Assitant Professors in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala) for giving me all the valuable tips and suggestions to sort out the problems in my experimental work.*

*Sincere thanks to **Mrs. M. Vasundhara** (Assistant Professor) in the Department of Biotechnology and Environmental Sciences, Thapar University, Patiala, with whom I had worked as Teaching Associate. I wish to convey my regards and gratitude to her for her advice, sharing her knowledge, supervision and encouragement which have helped me in learning the various streams of biotechnology.*

My warm thanks are due to all faculty members of Department of Biotechnology, TU, Patiala, for their meticulous guidance, keen interest, invaluable suggestion and constant support during the course of my study. The personal and professional guidance that I received from them would be cherished lifelong.

*I wish to thank **Dr. S.C Sharma** (Nodal Officer, Assistant Economist, PAU Regional Research Station for Kandi Area, Ballawal Saunkhri), for providing me encouragement and valuable tips and suggestions for improving my experimental work.*

*I wish to convey my regards and gratitude to **Mr. Rajpaul Singh Gandhi**, who allow me to conduct field experiments in their Green Valley organic farm at Pojewal Punjab.*

*I express my regards and grateful to my colleagues **Dr. Himani, Dr. Giridhar, Dr. Santosh karan, Dr. A. Raghavendra, Dr. Varenym Achal, Dr. Diwaker Aggarwal, Dr. Vijay Rana, Dr. Richu Singla** Ph.D Scholars, for providing keen interest, unfailing support, inspiration, critical observations and ingenious suggestions for my research work.*

*I would like this opportunity to express my heartfelt thanks to my friends **Dr. Harpreet Kaur, Ms. Navdeep Kaur Dhami, Ms. Mahima Bansal, Mr. Sanjog Garyali, Mr. Balwant Verma, Ms. Poonam Bhatia, Mr. Harpreet Singh Bhangu, Dr. Sukhrishpal K. Thirunavvkkarasu, Ms. Rajinder Kaur, Ms. Mandeep, Ms. Pankil, Ms. Bhawna, Ms. Amanpreet, Ms. Seema, Ms. Gurpreet Khaira and Ms. Rabia** for their invaluable smiles and friendship during the course, which has motivated and encouraged me for my research work. I also thank those who could not find a separate name but helped me directly or indirectly.*

*I extend my thanks to our lab attendant **Mr. Baban, Mr. Lallan, Mr. Gurmeet**, staff members of TIFAC CORE- **Mr. Naminder Singh** and **Mr. Joga Singh**, staff members of tissue culture for their prompt help at different stages of the study.*

*I would like to convey my thanks to **Mr. Manmeet Singh, Mr. Ashwani Aggarwal Mr. Jain** and **Mr. Vijay** for their cooperation.*

*My sincere thanks are due to the whole **lab staff and office staff** of Department of Biotechnology and Environmental Sciences, Thapar University, who had provided me all the help and support for my research work.*

*I owe my personal reckoning of gratitude and benevolence to **my Parents** for their love, care, patience, inspiration, and constant driving force, which has enabled me to complete my task.*

*I am also indebted to my brothers **Amrit Pal Singh, Hardeep Singh and Sonu** for their love and support.*

*I wish to express my gratitude and thanks to my supervisor **Prof. M.S. Reddy** for proving me financial support in the form of fellowship by appointed me as project fellow in **UGC-project, India, and Thapar University, Patiala**, for providing me good infrastructure facilities to help accomplish my work.*

Date: 11. Feb, 2014

Place: Patiala

Gurdeep Kaur.
(Gurdeep Kaur)



*Dedicated
To
My beloved
Parents*



List of Publications

The following publications are the outcome of the present research work:

Kaur G, Reddy MS. 2013. Role of phosphate-solubilizing bacteria in improving the soil fertility and crop productivity in organic farming. Arch. Agro. Soil Sci. 60: 549-564.

Kaur G, Reddy MS. 2013. Phosphate solubilizing rhizobacteria from an organic farm and their influence on the growth and yield of maize (*Zea mays* L.). J. Gen. Appl. Microbiol. 59: 295-303

Kaur G, Reddy MS. 2013. Influence of P-solubilizing bacteria on crop yield and soil fertility at multilocational sites. Eur. J. Soil. Biol. 61: 35-40.

Kaur G, Reddy MS. 2013. Phosphate-solubilizing bacteria, rock phosphate and chemical fertilizers effects on crop and economics. Pedosphere (communicated).

Conferences:

- **Gurdeep Kaur** and M. Sudhakara Reddy (2010). Phosphate solubilization and plant growth promoting activity of bacteria isolated from organic fields National conference on **-Emerging Trends in Biopharmaceuticals: Relevance to Human Health** and 4th Annual Convention of Biotechnology and Pharmacy. Department of Biotechnology and Environmental sciences, TIFAC-CORE, Thapar University, Patiala, Punjab, India.
- **Gurdeep Kaur** and M. Sudhakara Reddy (2011). Phosphate solubilization of fungal isolates isolated from rock phosphate mined out soils. **National symposium on Fungal Biology and applications**. Department of Botany, Punjabi University, Patiala, Punjab, India.

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Abbreviations

bp	Base pair
BLAST	Basic local alignment search tool
CTAB	Cetyltrimethyl ammonium bromide
cfu	Colony forming Units
DNA	Deoxyribonucleic acid
dNTP	2'-deoxynucleoside-5'-triphosphate
EDTA	Ethylenediamine-tetra acetic acid g Gram h Hours ha Hectare
IPTG	Isopropyl—thiogalactoside
Kb	Kilo base
l	Liter
mS cm ⁻¹	Millisiemens per cm
mg	Milligram
mg/kg (ppm)	Milligram per kilogram (parts per million)
ml	Milliliter
NCBI	National centre for biotechnology and information
P	Phosphorus
PCR	Polymerase chain reaction
PSMs	Phosphate-solubilizing microorganisms
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
RRP	Rajasthan rock phosphate
TCP	Tri-calcium phosphate
Tris	Tris-(hydroxymethyl-) aminomethane
μg	Microgram
μl	Microliter
X-Gal	5-Bromo-4-chloro-3-indolyl--D-galactoside

Chapter 1

Introduction

INTRODUCTION

1.1 Phosphorus

The world population is increasing day by day (Lal 2000), hence there is need for plenty of food crops to meet the requirement of growing population. Crops need several nutrients to reach their maximum potential yield. The nutrients, which are required by the plants, occur naturally in the soil, but sometimes these are added as lime or fertilizer into the soil. After nitrogen, phosphorus (P) is one of the major essential macronutrient for plant growth and development (Bagyaraj et al. 2000). About 98 % soils have inadequate supply of available Phosphorus (Hansan 1996) and likely to induce deficiency of this mineral. Phosphorus has several roles in the plants and is involved in functioning of nucleic acids, proteins, photosynthesis and in the formation of oils, sugars and starches etc. It is helpful in the rapid growth of the roots and shoots. Most of the soils contain the substantial reserves of total P; large part of it relatively remains inert and only less than 10 % of soil P enters the plant-animal cycle (Kucey et al. 1989). When P is added as fertilizer to the soil, it gets fixed. The soil microorganisms solubilize this P and make it available to the plants (Hilda and Fraga 1999).

1.2 Phosphorus in soil: Status and availability

P is present in several hundred to several thousand grams per acre in the soil, but its large amount in soils is not available to growing plants. P forms the 0.12 % of the earth crust. The amount of phosphorus which is available in the soil is 0.05 %, out of this only 0.1 % is available for the plants (Scheffer and Schachtshabel 1988). About 50 % of the districts in India need higher levels of P in soils than are currently being used (Hasan 1996). Apatite is the largest reservoir of phosphate on Earth (Stevenson 1986) and is less soluble in water. P is not found in elemental form because this form is extremely reactive. It combines with oxygen

when exposed to air. In natural system like soil and water, P exists as phosphate, a chemical form in which phosphorus is surrounded by oxygen atoms (Hyland et al. 2005). Orthophosphate is the simplest phosphate with chemical formula PO_4^{-3} . In water, orthophosphate mostly exists as $\text{H}_2\text{PO}_4^{-1}$ in acidic condition or as HPO_4^{-2} in alkaline condition (Bushman et al. 2009). It is present in the form of $\text{H}_2\text{PO}_4^{-1}$ and HPO_4^{-2} for the uptake by the plants. The P fixation and precipitation is highly dependent on soil pH and type, thus, in acidic soils free oxides and hydroxides of Al and Fe fix P and in alkaline soils it is fixed by Ca, which causes its low efficiency (Goldstein 1986).

Agricultural soils have the large amount of organic and inorganic phosphorus, but this is unavailable for plants use. This is due to the high reactivity of P with some metal complexes such as Fe, Al and Ca, leading to precipitation and adsorption of P in soil (Fig. 1.1). About 30 to 50 % of the P in soil occurs in organic forms (Rodriguez and Fraga 1999). The organic phosphorus in the soil is largely in the form of inositol (soil phytate), synthesized by microorganisms and plants and is most stable (Anderson 1980; Harley 1983). The phosphorus in bound form is made available to the plants by soil microorganisms like bacteria and fungi, which solubilize the bound form of phosphorus and make it available to the plants (Jisha and Mathur 2006). The other common forms of organic phosphorus are phosphomonoesters, phosphodiester including nucleic acids, phospholipids, glycerophosphate, sugar phosphate and coenzymes (Martinez et al. 1968). These organic forms must be converted into inorganic phosphate or low molecular weight organic acids before they can be assimilated by plants. The organic forms are utilized by plants after mineralization and subsequent release of inorganic phosphorus (Yadav and Tarafdar 2001). Plants complete their phosphorus requirement by uptake of phosphate anions from the soil solution (Richardson et al. 2000). Many of the phosphorus compounds have high molecular weight, therefore these must first be converted to either soluble phosphate (pi, HPO_4^{-2} ,

$\text{H}_2\text{PO}_4^{-1}$), or low molecular weight organic phosphate, to be assimilated by the plant cell (Goldstein 1994).

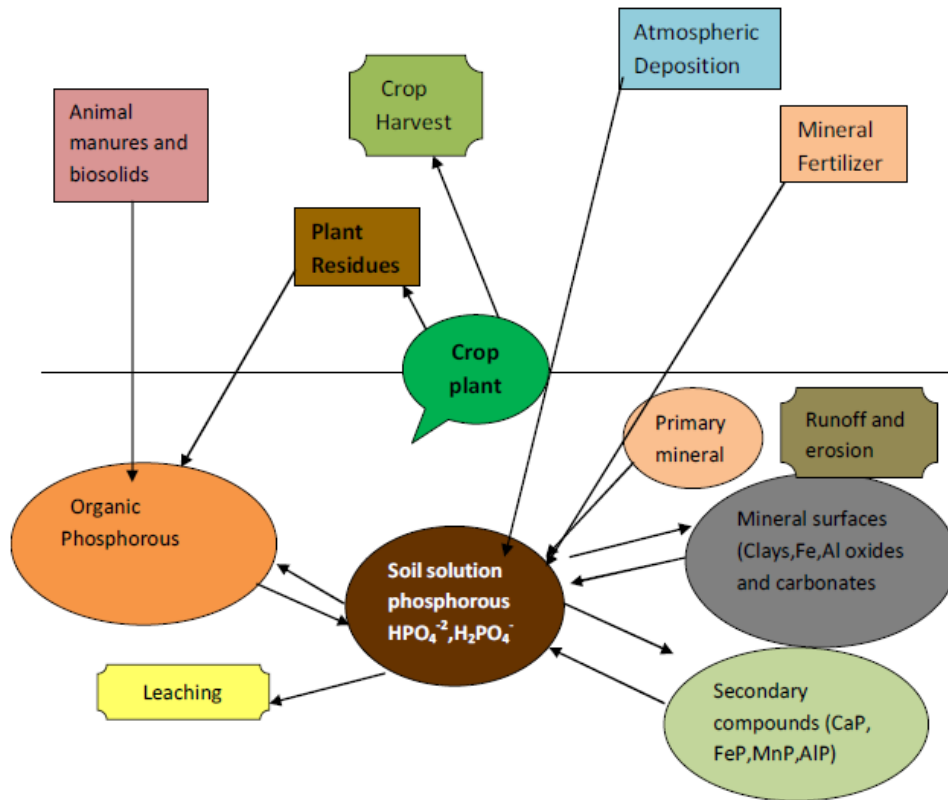


Fig. 1.1 Phosphorus cycle in nature (Anthony et al. 2009).

1.3 Phosphorus in agro ecosystem soils

In agricultural ecosystem, phosphorus constraints are much more critical, because phosphorus in the harvested crop is removed from the system, with only limited quantities being returned in crop residues and animal manures. As a result, extreme phosphorus deficiencies are quite common where no supplementary source of these elements is applied to the soils. The phosphorus cycle in soil is a system which involves soil, land and microorganisms. Major processes include the uptake of soil phosphorus by plants, recycling (the return of plant and animal residues), biological turnover (mineralization and immobilization) fixation to clay,

solubilization (Stevenson 1986). Phosphorus not supplied through biochemical fixation but, must come from other sources to meet plant requirement. These sources include commercial fertilizers, animal manures, plant residues, wastes and native compounds of phosphorus, both organic and inorganic already present in the soil. The soil P cycle is a dynamic process involving the transformation of P by geochemical and biological process (Melissa and kin 2006). Fig 1.2 depicts the general cycle of phosphorus in the soils (portion of organic and inorganic form of phosphorus) into pools based on its availability in plants.

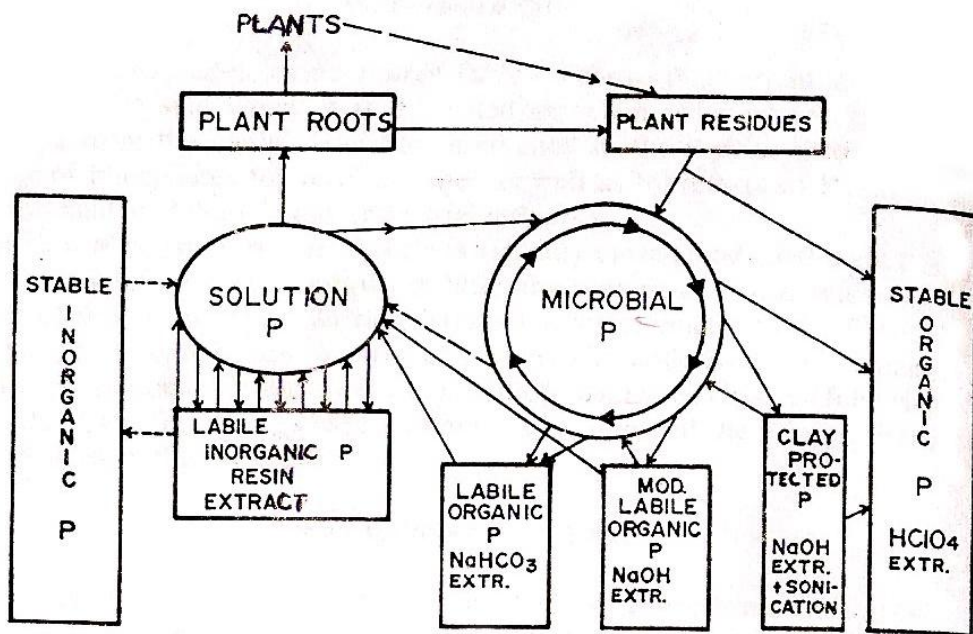


Fig. 1.2 Phosphorus cycle in soil, showing the partition of organic and inorganic forms of P into pools based on availability to plants (Stevenson 1986).

1.4 Interaction of phosphatic compounds in soil

Many soils throughout the world are P-deficient. Most mineral nutrients in soil solution are present in milli molar amount; phosphorus is only available in micro molar quantity or less (Goldstein 1994). Free phosphorus concentration even in fertile soil is generally not higher than 10 mM even at pH 6.5. The majority of applied phosphorus is rapidly fixed in soil into

fractions that are poorly available to plant roots (Fig. 1.3) (Sanyal and De Data 1991; Yadav and Dadarwal 1997). In soils, inorganic forms of phosphorus such as fluorapatites, hydroxyapatite, chloroapaties, iron and aluminium phosphate as in combination with clay fractions do occur. Inorganic phosphates in acidic soils are associated with iron (Fe) and aluminium (Al) compounds where as calcium (Ca) phosphates are predominant forms of inorganic phosphates in neutral as calcareous soils (Gyaneshwar et al. 2002).

Beside this, large quantities of xenobiotics phosphonates, which are used as pesticides, detergent additives, antibiotics and flame retardants, are released into the environment. These C-P compounds are generally resistant to chemical hydrolysis and bio-degradation, but several reports have documented microbial P release from these sources (Mc Grath et al. 1998).

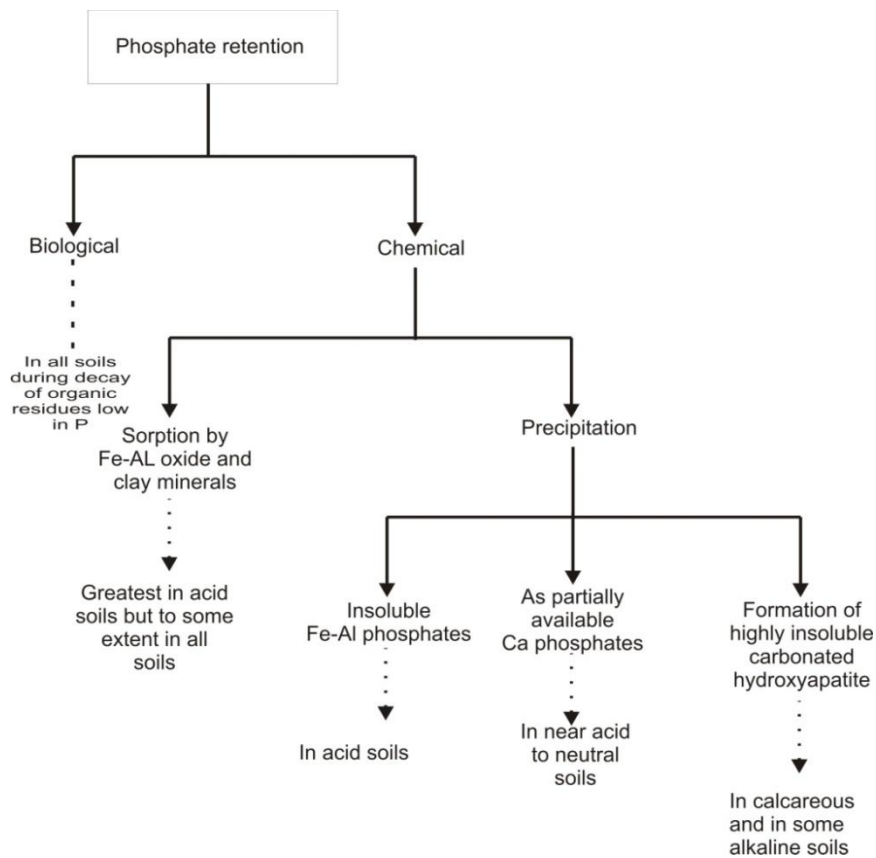


Fig. 1.3 Phosphate fixation reactions in soil (Sauchelli 1951).

1.5 Chemical phosphate fertilizers

To increase the availability of P for the plants, large amount of fertilizers are being applied to soil. But a proportion of fertilizer P after application is quickly transformed to the insoluble form (Vassilev and Vassilev 2003; Pradhan and Sukla 2005). This lead to the frequent application of phosphate fertilizers, but its use on a regular basis has become costly affair and also environmentally undesirable (Reddy et al. 2002).

Chemical fertilizers have played a significant role in the green revolution, but unbalanced use of them, lead to reduction in soil fertility and to environment degradation (Gyaneshwar et al. 2002). Traditional P fertilizer production is based on chemical processing of insoluble mineral phosphate high-grade ore, which includes an energy intensive treatment with sulphuric acid at high temperature. This process is environmentally undesirable and also is manufactured from non-renewable resources and its usage leads to air, water and land pollution and reduction of biomass production and biological diversity (Vance 1998). By seeing the harmful effects of chemical fertilizers, an integrated concept of ‘Sustainability’ in agriculture started. As it pertain to agriculture, sustainable describes farming systems that are capable of maintaining their productivity and usefulness to society indefinitely. Such systems must be resource conserving, socially supportive, commercially competitive and environmentally sound. Thus, the term sustainable agriculture means as integrated system of plant and animal production practices with a site specific application that will, over time the long term: satisfy human food and fiber need, enhance environmental quality and sustain the economic viability of farm operations. The goal of sustainable agriculture is, hence, to minimize adverse impacts to the immediate and off-farm environments while providing a sustained level of production and profit. However, the most important constraint limiting crop yields in developing nation worldwide, and especially among resource-poor farmers, is soil fertility. Unless the soil fertility is restored in these areas, farmers can get little benefits from

the use of improved varieties and more productive cultural practices. Moreover, the consistent and alarming increase in the human population has again threatened the world's food security. There is therefore, an urgent need for a second green revolution to increase the food production by around 50 % in the next year in order to sustain the population pressure (Vasil 1998; Leisinger 1999). Scientists are therefore looking vigorously for an alternative source of phosphatic fertilizers to supplement, or replace in some cases, the chemical fertilizers to ensure competitive yields of crops with sustainability. So an alternative to chemical phosphatic fertilizers is microbial inoculants (biofertilizers). The utilization of microbial products has several advantages over conventional chemicals for agricultural purposes: (i) microbial products are considered safer than many of the chemicals now in use; (ii) neither toxic substances nor microbes themselves will be accumulated in the food chain; (iii) self-replication of microbes circumvents the need for repeated application; (iv) target organism seldom develop resistance as is the case when chemical agents are used to eliminate the pests harmful to plant growth; and (v) properly developed bio-control agents are not considered harmful to ecological processes of the environment (Gould 1990; Shen 1997).

1.6 Rock phosphate (RP); economic source of phosphate fertilizers

In order to ensure food security in developing countries, there is a need for the sustainable intensification of agricultural production systems towards supporting productivity gains and income generation. In this context, novel, soil-specific technologies will have to be developed, pilot tested and transferred to farmers in a relatively short time. Phosphorus is an essential nutrient element for plants and animals. The appropriate and sound utilization of rock phosphate (RP) as P sources can contribute to sustainable agricultural intensification, particularly in developing countries endowed with RP resources. In recent years, the possibility of practical use of rock phosphate as fertilizer has received significant interest in India. It is estimated that about 260 million tons rock phosphate deposits are available and

this material should provides a cheap source of phosphate fertilizer for crop production (FAI 2002). Out of the total resources, 35 % are in Jharkhand, 31 % in Rajasthan, 17 % in Madhya Pradesh, 9 % in Utter Pradesh and 8 % in Uttarakhand.

Direct application of RP would minimize pollution and decreases the costs of chemical treatment. The use of rock phosphate as phosphate fertilizer and its solubilization by microbes (Kang et al. 2002), through the production of organic acids (Maliha et al. 2004), have become a valid alternative to chemical fertilizers. Rock phosphate is widely distributed throughout the world both geographically and geologically (Zapata and Roy 2004), in conjugation with phosphate-solubilizing microorganisms, rock phosphate provides a cheap source of P fertilizer for crop production (Zaidi 1999; Gull et al. 2004). RP may originate from igneous, sedimentary, metamorphic, and biogenic sources, with sedimentary being the most widespread forms of apatite, the primary P bearing mineral in RP, include fluorapatites, hydroxyl apatite, carbonated-hydroxyapatite, and francolite (Van Straaten 2002). Ghani et al (1994) found that lower the soil pH, more available the P from RP becomes. Even with soil acidity below pH 5.5-6.6, RP becomes as effective as super phosphate only after 4 years of annual direct application.

1.7 Phosphate-solubilizing microorganisms

From several years, great attention has been dedicated to study the role, that soil microorganisms play in the dynamics of phosphate, particularly those able to solubilize insoluble P forms (Rao 1992). These microorganisms are bacteria and fungi that inhabitant the rhizosphere (Barea and Azcon 1975, Bowen and Rovira 1999). Evidence of the involvement of microorganisms in solubilization of inorganic phosphates was reported as early as 1903 (Kucey et al. 1989; Khan et al. 2007). Since then, extensive studies on the solubilization of mineral phosphates by microorganisms have been reviewed (Goldstein

1986; Kucey et al. 1989; Reddy et al. 2002; Tarafdar et al. 2003; Achal et al. 2007; Aseri et al. 2009; Himani and Reddy 2012). Phosphate-solubilizing microorganisms (PSMs) are ubiquitous, and their numbers vary from soil to soil. In general, among the whole microbial population in soil, P-solubilizing bacteria constitute 1-50 % and P-solubilizing fungi 0.1 to 0.5 % of the total respective population (Chen et al. 2006). Phosphate-solubilizing bacteria generally out-number P-solubilizing fungi by 2-150 folds (Kucey et al. 1989; Alam et al. 2002). Some researchers prefer to use fungal P solubilizers arguing that bacterial strains can lose their ability to solubilize P after several cycles of *in vitro* culture (Whitelaw 2000), but this point is quite controversial. Most P-solubilizing bacteria (Venkateswarul et al. 1984) and fungi (Venkateswarul et al. 1984; Tarafdar et al. 2003; Tarafdar & Gharu 2005; Achal et al. 2007; Yadav and Tarafdar 2007; Aseri et al. 2009; Yadav and Tarafdar 2010) were isolated from the rhizosphere of various plants and are known to be metabolically more active than those isolated from sources other than rhizosphere. The rhizosphere is the region of soil that is immediately near to the root surface and that is affected by root exudates (Kennedy 1999). There are different types of substances that diffuse from the roots and that stimulate the microbial activity, such as carbohydrates (sugars and oligosaccharides), organic acids, vitamins, nucleotides, flavonoids, enzymes, hormones, and volatile compounds (Prescott et al. 1999). The result is a dense and active microbial population that interacts with the roots and within it.

1.8 Phosphate-solubilizing microorganisms as plant growth promoters

These organisms in addition to providing P to plants also facilitate plant growth by other mechanisms. PSMs include largely bacteria and fungi. Phosphate-solubilizing microorganisms can enhance the plant growth by a wide variety of mechanisms like:

1.8.1 Phosphate solubilization

Despite the fact that the amount of phosphorus in the soil is generally quite high (often between 400 and 1,200 mg kg⁻¹ of soil) most of this phosphorus is insoluble and therefore not available to support plant growth. The insoluble phosphorus is present either as an inorganic mineral such as apatite or as one of several organic forms including inositol phosphate (soil phytate), phosphomonesters, and phosphotriesters (Khan et al. 2007). In addition, much of the soluble inorganic phosphorus that is used as chemical fertilizer is immobilized soon after it is applied so that it then become unavailable to plants and is therefore wasted. The limited bioavailability of phosphorus from the soil, combined with the fact that this element is essential for plant growth and inability to obtain sufficient phosphorus often limits plant growth (Feng et al. 2004). Thus, solubilization and mineralization of phosphorus by phosphate-solubilizing microorganisms is an important trait in plant growth-promoting bacteria (PGPB) as well as in plant growth-promoting fungi (PGPF) (Richardson 2001; Rodríguez and Fraga 1999).

Typically, the solubilization of inorganic phosphorus occurs as a consequence of the action of low molecular weight organic acids such as gluconic acid and citric acid, both of which are synthesized by various soil bacteria (Bnayahu 1991; Rodriguez et al. 2004). On the other hand, the mineralization of organic phosphorus occurs through the synthesis of a variety of different phosphatases, catalyzing the hydrolysis of phosphoric esters (Rodríguez and Fraga 1999). Importantly, phosphate solubilization and mineralization can coexist in the same bacterial strain (Tao et al. 2008). Plant-growth-promoting rhizobacteria (PGPR) are also known to have beneficial influence on phytoremediation process (Singh and Cameotra 2013).

1.8.2 Nitrogen fixation

About 78 % of the earth atmosphere is made up of free nitrogen (N₂) produced by biological and chemical processes within the biosphere and not combined with other elements. All plants need nitrogen for their growth. However, plants cannot get the nitrogen they need, from atmospheric supply. This limitation can be compensated by moving closer to or inside the plants, viz. in diazotrophs present in rhizosphere, rhizoplane or those growing endophytically. Some important non-symbiotic nitrogen-fixing bacteria include *Gluconacetobacter diazotrophicus*, *Herbaspirillum* sp., *Azotobacter* sp. (Barriuso and Solano 2008), *Achromobacter*, *Acetobacter*, *Alcaligenes*, *Arthrobacter*, *Azospirillum*, *Azomonas*, *Bacillus*, *Beijerinckia*, *Clostridium*, *Corynebacterium*, *Derxia*, *Enterobacter*, *Klebsiella*, *Pseudomonas*, *Rhodospirillum*, *Rhodopseudomonas* and *Xanthobacter* (Saxena and Tilak 1998).

1.8.3 Indole acetic acid production (IAA)

IAA (indole-3-acetic acid) is the member of the group of phytohormones and is generally considered the most important native auxin (Ashrafuzzaman 2009). It functions as an important signal molecule in the regulation of plant development including organogenesis, tropic responses, cellular responses such as cell expansion, division, and differentiation, and gene regulation (Ryu and Patten 2008). The phytohormones synthesized by plant growth promoting rhizobacteria (PGPRs) influenced the root hair development, respiration rate, metabolism and root proliferation which in turn resulted in better mineral uptake of the inoculated plants (Bar and Okon 1993).

Diverse bacterial species possess the ability to produce the auxin phytohormone IAA. Different biosynthesis pathways have been identified and redundancy for IAA biosynthesis is widespread among plant-associated bacteria. Interactions between IAA-producing bacteria

and plants lead to diverse outcomes on the plant side, varying from pathogenesis to phytostimulation. Reviewing the role of bacterial IAA in different microorganism–plant interactions highlights the fact that bacteria use this phytohormone to interact with plants as part of their colonization strategy, including phytostimulation and circumvention of basal plant defense mechanisms. There are numerous soil microflora involved in the synthesis of auxin in pure culture and soil (Barazani and Friedman 1999). The potential for auxin biosynthesis by rhizobacteria can be used as a tool for the screening of effective strains (Khalid 2004).

1.8.4 Siderophore production

Iron, an element essential for microbial growth, is mostly unavailable because it is mainly present in soil in a hard-to-solubilize mineral form. To sequester iron from the environment, numerous soil microorganisms secrete low-molecular-weight, iron binding molecules, called siderophore, which have a high capacity for binding Fe^{3+} . The low-soluble, bound iron is transported back to the microbial cell and is available for growth. Siderophores produced by biocontrol-PGPRs have a higher affinity for iron than the siderophores produced by fungal pathogens, allowing the former microbes to scavenge most of the available iron, and thereby prevent proliferation of fungal pathogens. Depletion of iron from the rhizosphere does not affect plant growth as plants can thrive on less iron than microorganisms. Moreover, some plants can bind and release iron from bacterial iron-siderophore complexes, and use the iron for growth. Thus, the plant benefits in two ways: from the suppression of pathogens (Jagadeesh et al. 2001) and from enhanced iron nutrition, resulting in increased plant growth (Bashan and Bashan 2005) (Fig. 1.4).

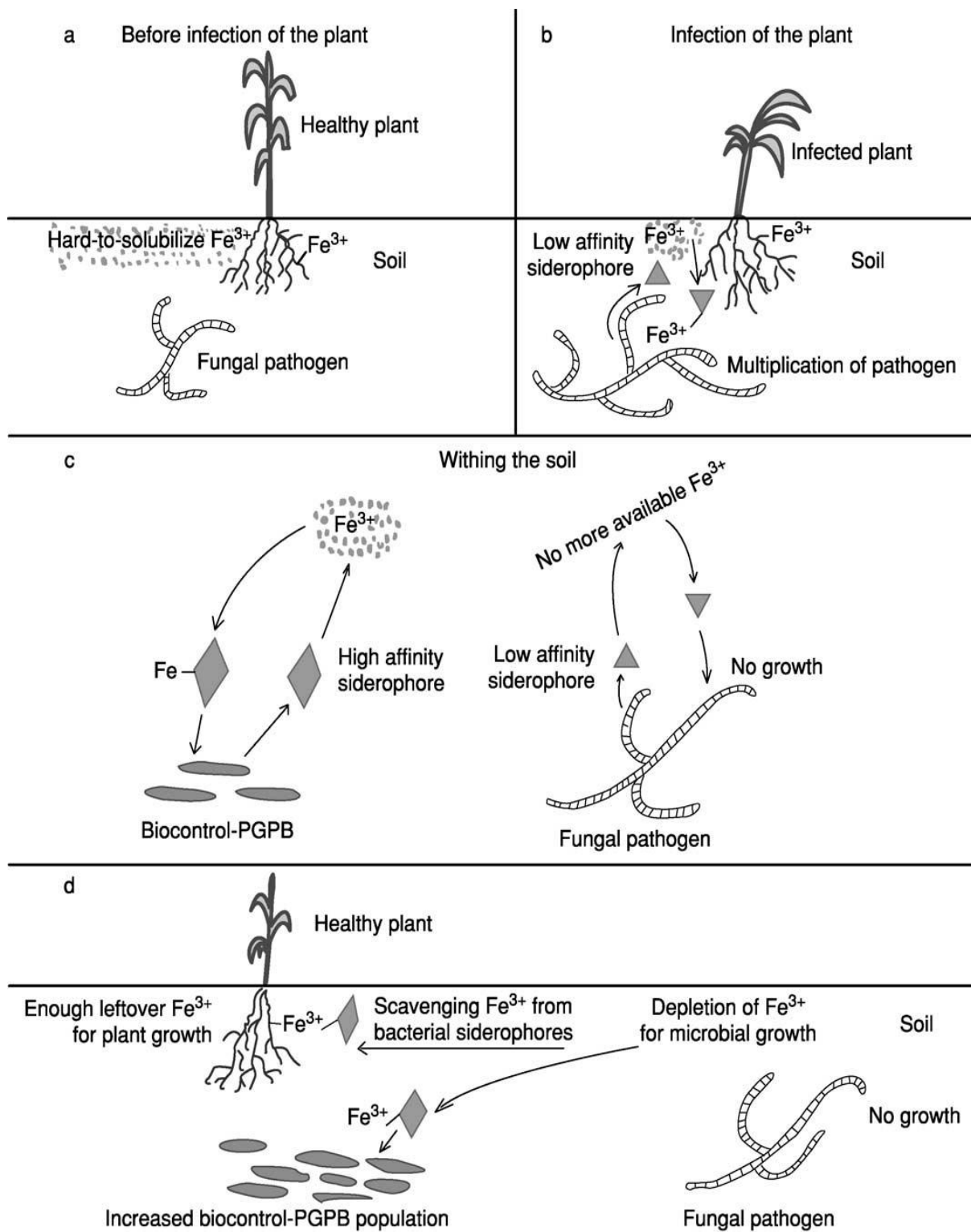


Fig. 1.4 Biological control of fungal pathogens by PGPR using siderophores (Bashan and Bashan 2005).

1.8.5 HCN production

One group of microorganisms, which acts as bio-control agents of weeds include the deleterious rhizobacteria (DRB), that can colonize plant root surfaces and able to suppress the plant growth (Suslow and Schroth 1982). Many DRB are plant specific (Schippers et al. 1987). Cyanide is a dreaded chemical, produced by them as it has toxic properties. Although cyanide acts as a general metabolic inhibitor, it is synthesized, excreted and metabolized by hundreds of organisms, including bacteria, algae, fungi, plants, and insects, as a mean to avoid predation or competition. The host plants are generally not negatively affected by inoculation with cyanide-producing bacterial strains and host-specific rhizobacteria can act as biological weed-control agents (Zeller et al. 2007). A secondary metabolite produced commonly by rhizosphere *Pseudomonads* is hydrogen cyanide (HCN), a gas known to negatively affect root metabolism and root growth (Schippers et al. 1990) and is a potential and environmentally compatible mechanism for biological control of weeds (Heydari 2008).

1.9 Phosphate-solubilizing microorganisms as bio-fertilizers

Although several phosphate-solubilizing microorganisms occur in soil, usually their numbers are not high enough to compete with other microorganisms commonly established in the rhizosphere. Thus the amount of P liberated by them is generally not sufficient for a substantial increase in, *in situ* plant growth. Therefore, inoculation of plants by a target microorganism at a much higher concentration than that normally found in soil is necessary to take advantage of the property of phosphate solubilization for plant yield enhancement.

There have been a number of reports on plant growth promotion by microorganisms that have the ability to solubilize inorganic and/or organic P from soil after their inoculation in soil or plant seeds (Klopper et al. 1989; Gaur and Ostwal 1972; Subba Rao 1982; Kucey 1989). Among the heterogeneous and naturally abundant microbes inhabiting the rhizosphere,

phosphate-solubilizing microorganisms (PSM) including bacteria and fungi have provided an alternative biotechnological solution in sustainable agriculture to meet the P demands of plants. An increase in P availability to plants through the inoculation of PSMs has been reported in pot experiments and under field conditions (Richa et al. 2007; Himani and Reddy 2012). Swarnalakshmi et al (2013) also reported that addition of inoculants along with RP significantly increased P uptake in comparison with chemical fertilizers or single inoculation of bio-inoculants in wheat. Sundra et al (2002) found that application of PSBs combine with RP is more effective than chemical phosphate fertilizers.

1.10 PSMs as bio-inoculants in organic farming

Organic farming is a method of farming system which primarily aimed at cultivating the land and raising crops in such a way, as to keep the soil alive and in good health by use of organic wastes (crop, animal and farm wastes, aquatic wastes) and other biological materials along with beneficial microorganisms (biofertilizers) to release nutrients to crops for increased sustainable production in an eco-friendly pollution free environment. To the maximum extent, feasible relies upon crop rotations, crop residues, animal manures, off-farm organic waste, and mineral grade rock additives (Lampkin 1990). The nutrient reservoirs in the soil shrink when crops are removed from the field at harvest. This nutrient export creates a P deficit, necessitating regular P addition to replace the harvested P. Several studies investigating whole form P budgets have found nutrient P deficits in many organic forms and illustrate the need for nutrient additions (Nelson and Mikkelson 2008). This leads to the need of frequent application of phosphate fertilizers, but organic farming avoids the inputs of synthetic chemicals and their consequences (Hajra 2001). An active soil microflora and a considerable pool of accessible nutrients are, therefore, important priorities in organic farming (Fliebbach and Mader 2000). Natural rock phosphate along with phosphate-

solubilizing microorganisms may be a valuable alternative for P fertilizers in an organic farming.

1.11 Inoculum formulations

For sustainable agriculture, substitution of high priced chemical fertilizers with eco-friendly biofertilizers is the most desired practice. The microbial inoculation in the form of seed bacterization has been proved beneficial for the maintenance of soil health, but the use of a suitable carrier, capable of supporting high viable microbial population for a prolonged duration is of utmost importance. Though, a number of carriers including charcoal soil mixture, wheat bran (Gaind and Gaur 1990), peat, press mud (Jauhri and Philip 1984), calcium alginate (Viveganandan and Jauhri 2000), fly ash (Gaind and Gaur 2002; Grewal et al. 2001; Kalra et al. 2000) have been found successful in maintaining high shelf life of phosphobacteria, but still there is wide scope for exploring some cheap and easily available waste material as a carrier for biological inoculants.

1.12 Aim of present study

Phosphorus is an essential macronutrient required by the plants for their growth and development. Added P fertilizers undergo fixation due to the complex exchanges within the soil (Altomare et al. 1999). This leads to the need of frequent application of phosphate fertilizers, but its use on a regular basis has become a costly affair and also environmentally undesirable (Reddy et al. 2002). Chemical fertilizers are also adversely affecting the soil microbial population (Vassilev and Vassileva 2003). Natural rock phosphates have been recognized as a valuable alternative for P fertilizers. In India, it is estimated that there are almost 260 million tons of rock phosphate deposits and this material should provide a cheap source of phosphate fertilizer for crop production (FAI 2002). Unfortunately, rock phosphate (RP) is not readily available to the plants in soils with a pH > 5.5-6.0. Because of this,

extension services are reluctant to be recommended and farmers are hesitant to utilize RP directly. Several P-solubilizing microorganisms have the ability to convert insoluble low grade rock phosphates into soluble forms available for plant growth (Vyas and Gulati 2009). Organic applications increased nutrient status, microbial activity and productive potential of soil while the use of only chemical fertilizers in the cropping system resulted in a poor microbial activity and productive potential of soil (Kang et al. 2005).

There is no doubt that bacterial inocula can increase the yield of various crops significantly, but the performance has generally been inconsistent. A key factor involved in the lack of success has been the rapid decline of the size of populations of active cells, to levels ineffective to achieve the objective, following introduction into soil. Potential of bacterial inoculums may be determined in a single experiment, but the consistent performance can only be determined in multiple trails (Kloepper et al. 1989).

Keeping in view the effect of conventional (chemical fertilization) methods and organic farming practices on diversity and productivity of microorganisms, in the present investigation, we have isolated phosphate-solubilizing microorganisms from the rhizospheric soil of an organic farm and studied their plant growth promotion activities. To check the consistent performance, selected isolates were used as bio-inoculants alone or along with RP fertilization at three different sites come under different agroclimatic regions. In the present study, we have also evaluated the comparative effect of phosphate chemical fertilizer, biological phosphate fertilizers (phosphate-solubilizing bacteria and fungi) and biological phosphate fertilizers in association with rock phosphate fertilizer on the growth, yield and nutrients uptake of maize and wheat crop. The effect of these fertilizers on physiochemical properties of rhizospheric soil of all experimental fields were also studied. We have developed the inoculum formulations of these selected isolates on the basis of their shelf life and productive potential by using different carrier materials.

OBJECTIVES

- Isolation and identification of phosphate solubilizing microorganisms for alkaline soils
- To study the physiological characteristics and elucidation of mechanism of phosphate solubilization
- To develop the inoculum formulations for field applications with respect to phosphate solubilization efficiency and shelf life

Chapter 2

Review of literature

REVIEW OF LITERATURE

2.1 Phosphorus

As the world population continues to increase at an alarming rate, the demands placed upon agriculture to supply future food will be one of the greatest challenges facing the agrarian communities. In order to meet this challenge, a great deal of efforts focusing on the soil biological system and the agro-ecosystem as a whole is needed enabling better understand the complex processes and interactions governing the stability of agricultural lands. Phosphorus, the second most important macro-nutrient required by the plants, next to nitrogen, is reported to be a critical factor of many crop production systems, due to the fact that the limited availability in soluble forms in the soils (Xiao et al. 2011). Phosphorus is going to be plant nutrient that will limit the agricultural production in the next millennium and unlike the case of nitrogen; there is no large atmospheric source that can be made biologically available (Ezawa et al. 2002). In most soils, its content is about 0.05 % of which only 0.1 % is plant available.

2.2 Role of Phosphorus in Plants

Phosphorus plays an important role in metabolism of crop plants (Vikram and Hamzehzarghani 2008). About 10-25% of fertilizer P is acquired by the plants (Saha and Biswas 2009) for promoting their functions. P is absorbed mainly during the vegetative growth; therefore most of its absorbed form is re-translocated in fruits and seeds during the reproductive stages. Phosphorus is the important nutrient for plant growth (Eftkhari et al. 2010) and it is the constituent of various cellular functions or activities such as cell division, development, photosynthesis, breakdown of sugars, nutrient uptake and transport within the plant (Griffith 1999). The plants which are deficient in P, show retarded growth and causes dark green coloration due to enhancement of anthocyanin formation (Khan et al. 2009).

2.3 Soil phosphorus

The soil P cycle is a dynamic process involving the transformation of P by geochemical and biological processes. Plant-available P occurs in the soil solution as orthophosphate anions, predominantly $\text{H}_2\text{PO}_4^{-1}$ and HPO_4^{-2} . Solid inorganic and organic forms of P are found in labile and poorly soluble forms in the soil, and as a result, can replenish plant available P with varying degrees of effectiveness.

Plant available P, or solution P, is in equilibrium with a relatively labile fraction of P that is adsorbed to aluminum or ferric hydrous oxides, clays, calcium carbonates and organic matter (i.e. is associated with the solid phase of the soil) (Whitelaw 2000). As a result, solution P is easily replenished in response to plant uptake through desorption of P from the labile solid fraction (Whitelaw 2000). However, only a small fraction of P in the solid phase remains in a labile form, as it can become strongly adsorbed to the soil or participate in precipitation reactions. P may become strongly fixed and eventually precipitate as variscite and strongite in acid soils, or with Ca^{2+} in alkaline soils. The effects of P precipitation are significant in acidic soils, where twice the amount of added P per unit surface area is fixed compared to neutral or calcareous soils (Whitelaw 2000).

The organic P pool generally constitutes 30 to 80 % of the total soil P (Oberson et al. 1996) and represents a labile P fraction that may supply P to plants through mineralization by the microbial biomass (Stewart and Tiessen 1987). The microbial biomass is a small fraction of the total soil organic P, containing anywhere between 3 to 24 % depending on cultivation (Brookes et al. 1984). However, it is significant in its role as recycler of P and as a relatively labile P source (Kwabiah et al. 2003). P loss through crop removal can significantly reduce the soluble and labile P in the soil and decrease total soil P without external inputs. Overall, soil pH is the main property controlling inorganic P forms, although Al, Fe, and Ca content

determine the amounts of these forms. In acid soils, Al and Fe dominate P fixation, while Ca compounds fix P in alkaline soils (Fig. 2.1). As a result, P availability is greatest at soil pH between 6 and 7.

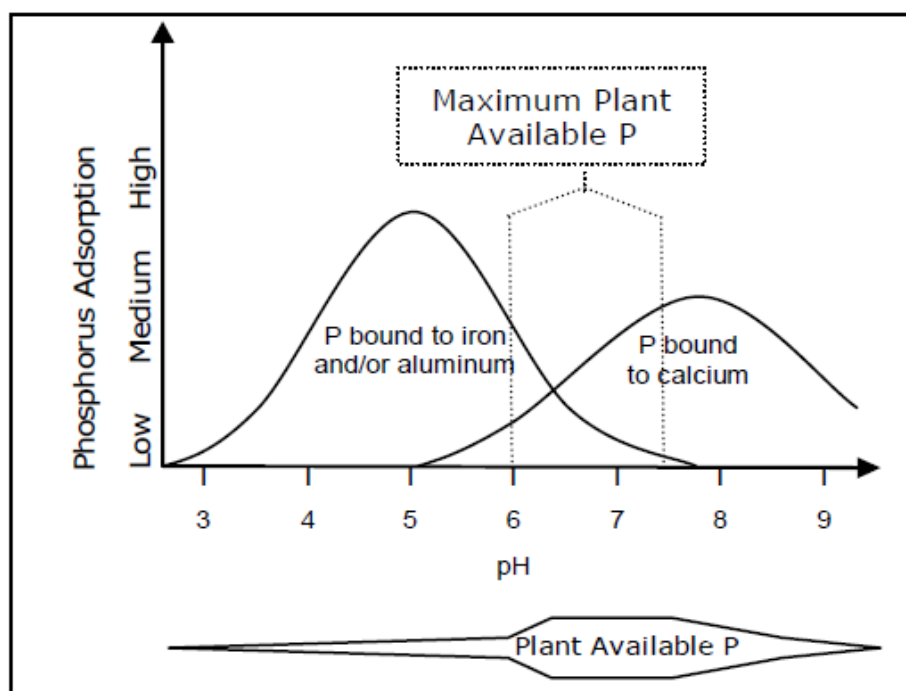


Fig. 2.1 Soil pH impacts P availability (Sauchelli 1951).

2.4 Phosphorus problem in soil

The soils being poor in organic matter ($1.5-4.2 \text{ mg kg}^{-1}$), most of the phosphorus is present in inorganic form as calcium and aluminium phosphatase. About 20-25 % of total phosphorus in arid soils of India is organic in nature and 68 % organic phosphorus in the soil is present as phytin (Yadav and Tarafdar 2007), which are not directly available to plants. Therefore, application of phosphatic fertilizers to the soil is essential to maintain adequate amount of soluble P in the soil solution for optimum plant growth as well as to maintain soils sustainability. Efficiency of P fertilizer throughout the world is around 10-25 % (Lindsay 1979), and concentration of bio-available P in the soil is very low reaching the level of 1.0 mg kg^{-1} soil (Goldstein 1994). Phosphorus is taken up from the soil in the form of soluble

orthophosphate ions; $\text{H}_2\text{PO}_4^{-1}$, HPO_4^{-2} and PO_4^{-3} and generally the availability of these ions to the plants is in the order of $\text{H}_2\text{PO}_4^{-1} > \text{HPO}_4^{-2} > \text{PO}_4^{-3}$. The type of orthophosphate ion present in the soil is depending on soil reactions. At the relatively low pH of 4 to 5, orthophosphate usually exist as $\text{H}_2\text{PO}_4^{-1}$ ions. On increasing pH, first HPO_4^{-2} ions are formed which convert to PO_4^{-3} as the soil reaction become alkaline. Large amount of P applied as fertilizer enters in to the immobile pools through precipitation reaction with highly reactive Al^{3+} and Fe^{3+} in acidic soil and Ca^{2+} in calcareous or normal soils (Fig. 2.2) (Gyaneshwar et al. 2002). Although, total P pool is high, only a part is available to plants. So, the release and mobilization of insoluble and fixed forms of P is an important aspect of increasing soil P availability.

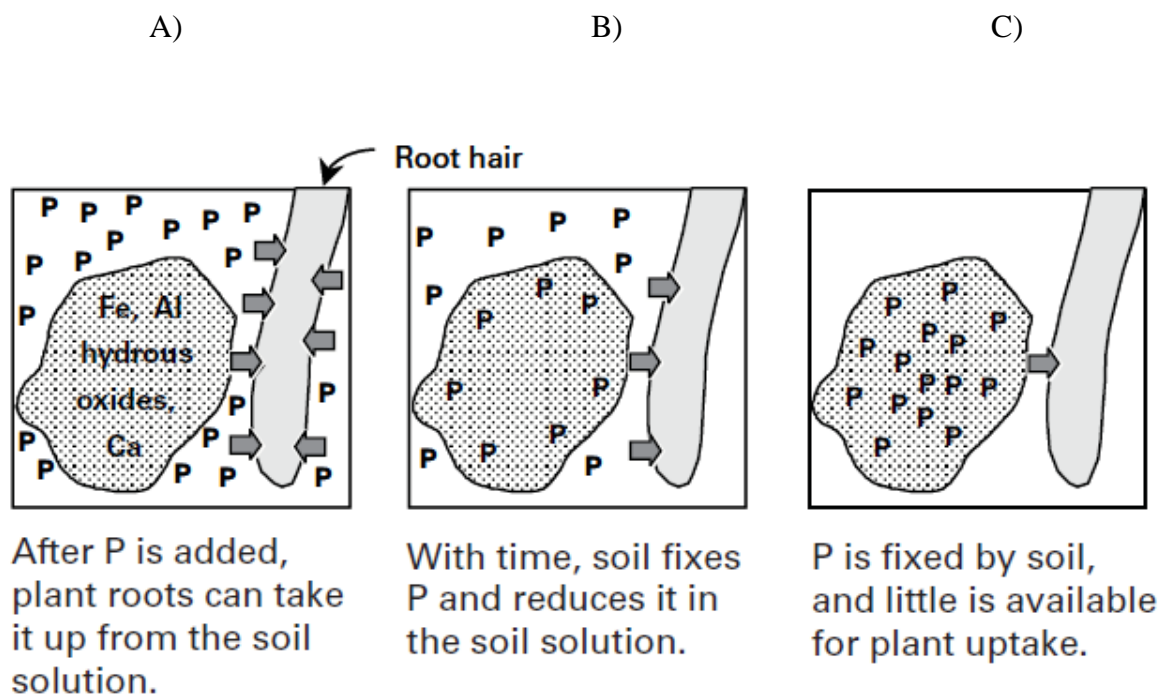


Fig. 2.2 Picture shows how phosphorus (phosphates) is tied up by soil minerals. A) A large percentage of the P is available for root uptake immediately after fertilization application. B) P in solution binds rapidly to the surface of soil minerals. Roots may still use this P. C) eventually; most of the bound P becomes part of the structure of the mineral, with its plant availability being significantly reduced (Sauchelli 1951).

2.5 Phosphate fertilizers use in agriculture and the environmental impacts

Technological advances in agriculture are helping to meet the food needs of an ever-increasing world population. Although the population has been growing and available land for agriculture has been shrinking, intensive agriculture that involves heavy and continuous use of fertilizers has ensured high crop productivity. As an example, increased use of fertilizers played an important role in the immense success in food productivity during the period of the green revolution (Tilman et al. 2002). However, reports have shown that continuous use of fertilizers is generating environmental problems. Use of chemical fertilizers on regular basis has become a costly affair and also environmentally undesirable (Reddy et al. 2002). Low efficiency in the uptake of fertilizer is a major factor that aggravates the negative environmental effects (Barlog and Grzebisz 2004). P growth-limiting nutrient, is applied in high percentage, sometimes up to 90 %, is precipitated by metal complexes in the soil (Rodriguez and Fraga 1999; Gyaneshwar et al. 2002) and can later lead to P pollution (Rodriguez and Fraga 1999; Sharpley et al. 2003). Therefore, in order to sustain the production, problems of phosphorus deficiency are needed to be arrested through the application of phosphorus fertilizers (Khan et al. 2010). The repeated and injudicious applications of these phosphorus containing fertilizers, however, lead to (1) the loss of soil fertility (Gyaneshwar et al. 2002) (2) disturbance to microbial diversity and their associated metabolic activities, and (3) reduced yield of agronomic crops (Khan et al. 2009). This has led to the search for environment-friendly and economically feasible alternative strategies for improving crop production in low or phosphorus deficient soils. In fact, most agricultural soils are obviously having large reserves of phosphorus. Phosphorus as a plant nutrient can also have impacts on the environment. Too much or too little phosphorus can have severe and widespread negative impacts on the environmental quality. The principal environmental problems related to phosphorus are land degradation caused by too little available phosphorus

and accelerated eutrophication caused by too much available phosphorus (Brady and Weil 2002). Both problems are related to the role of phosphorus as a plant nutrient. However, as the greater part of them, approximately 95 to 99 % is present in the form of insoluble phosphates; utilization of them by plants is virtually restricted (Pradhan and Sukla 2005). It has been suggested that this accumulated phosphates in agricultural soils is sufficient to sustain maximum crop yields worldwide for about 100 years. Instead of making attempts to utilize these reserves, chemical fertilizers are widely used in meeting the phosphorus need of crops. However, as the fertilizer production is dependent upon fossil energy sources, continuous use of chemical fertilizers has become a matter of great concern, not only because of the diminishing availability of costly inputs but environmental concerns also. Under this background, it has obviously brought the subject of mineral phosphate solubilization in the forefront (Khan et al. 2007).

2.6 Rock phosphate

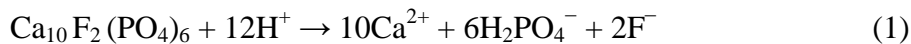
In recent years the possibility of practical use of rock phosphate as fertilizer has received significant interest in India, it is estimated that about 260 million tons of rock phosphate (RP) deposits are available and this material should provides a cheap source of phosphate fertilizer for crop production (FAI 2002).

Direct application of RP would minimize pollution and decreases the costs of chemical treatment. The use of rock phosphate as phosphate fertilizer and its solubilization by microbes (Kang et al. 2002), through the production of organic acids (Maliha et al. 2004), have become a valid alternative to chemical fertilizers. Rock phosphate is widely distributed throughout the world, both geographically and geologically (Zapata and Roy 2004) in conjugation with phosphate-solubilizing microorganisms, rock phosphate provides a cheap source of P fertilizer for crop production (Zaidi 1999; Gull et al. 2004). RP may originate

from igneous, sedimentary, metamorphic, and biogenic sources, with sedimentary being the most widespread forms of apatite, the primary P bearing mineral in RP, include fluorapatites, hydroxyl apatite, carbonated-hydroxyapatite, and francolite (Van Straaten 2002).

2.7 Solubilization of rock phosphates

RP may originate from igneous, sedimentary, metamorphic, and biogenic sources, with sedimentary being the most widespread (Van Straaten 2002). Forms of apatite, the primary P bearing mineral in RP, include fluorapatite, hydroxyapatite, carbonate hydroxyapatite, and francolite (Van Straaten 2002). In general, high carbonate-substituted forms of apatite (francolite) will solubilize more readily than pure forms of fluorapatite, releasing more P for plant use (Anderson et al. 1985). In addition to RP source, the major factors influences on RP solubility are soil properties, crop species, and management practices (Chien and Menon 1995). These factors have various influences on the equilibrium of the dissolution reaction of a given apatite mineral. A simplified dissolution equation is shown in Equation 1.



Engelstad et al (1974) found that lower the soil pH, more available the P from RP. The addition of RP will increase total soil P with the potential to replenish labile P and plant-available P. By utilizing microbial mechanisms that can effectively extract P from RP and release it into the soil solution or into labile fraction of the soil, RP resources may provide a viable alternative for P fertilization. It is well established that RP application is not economically feasible, particularly in soil conditions characterized by high P sorption capacity, low cation exchange capacity, high pH, low rainfall, low organic matter content, low microbial activity, etc. For these reasons, there is renewed and growing interest in manipulating RP i.e. bio-solubilization of RP using phosphate-solubilizing microorganisms for enhancement of the agronomic value of rock phosphate (Vassilev et al. 2003). Soil

microorganisms play an important role in mobilizing P mainly by bringing about pH changes in soil microenvironment and producing chelating substances (Rodriguez and Fraga 1999).

2.8 Phosphate-solubilizing microorganisms

Phosphorus is added to soil in the term of phosphate fertilizer, part of which is utilized by the plant and the remainder converted into fixed and insoluble forms of phosphorus (Afzal et al. 2005; Mehrvarz et al. 2008). There is nevertheless enough scope to use finely ground rock phosphate directly on the farm, especially in acidic soils. Since P availability from the phosphoric reserves; that is rock phosphate under neutral and alkaline conditions is scarce/negligible. Phosphate-solubilizing microbes dissolving imprisoned/interlocked phosphates appear to have an important implication in Indian agriculture. From time to time the ability of different microorganisms to solubilize bound phosphates incorporated in solid or liquid media has been demonstrated. These reactions take place in rhizosphere and because solubilizing microorganisms dissolve more phosphates than they require for growth and metabolism, the surplus can be absorbed by plants (Gand and Gaur 1991; Bijaya et al. 2003; Dubey 2000).

In the frame of agriculture, the micro flora is of great significance because it has both beneficial and detrimental influence upon mans ability to feed itself (Gaur 1990; Whitelaw 2000). A second property involved in the improvement of host plant nutrition is the ability of fungal hyphae to explore a larger volume of soil than can the root system, thus limiting the formation of a depletion zone and making available more nutrients to the plant. This might be particularly important for molecules, such as ammonium and phosphate ions, with low mobility in soil (Marmeisse et al. 1998). PSM are those which can convert insoluble form of mineral phosphates into primary and secondary orthophosphates. PSM include different groups of microorganisms, bacteria and fungi in particular which have been reported to

solubilize insoluble phosphatic compounds. Microbial involvement in solubilization of inorganic phosphates was early found in 1903 by incubating tri-calcium phosphate with bacteria from milk and soil infusions.

Subsequently, Sackett et al. (1908) using the agar plate technique provided conclusive evidence to show that soil bacteria dissolve di-calcium phosphate, tri-calcium phosphate, bone meal and rock phosphates. In, India, Sundara Rao et al. (1963) reported increase in yield and phosphate uptake by tomato and wheat by bacterization with phosphobacterin (i.e. *Bacillus megaterium* var. Phosphaticum) name given by Russian scientists and an Indian strain of *Bacillus megaterium*.

The most efficient P-solubilizing bacterial strains among bacteria are *Pseudomonas striata* and *Bacillus polymyxa* and among fungi are *Aspergillus awamori*, *A. niger* and *Penicillium digitatum* (Ostwal and Bhide 1972, Reddy et al. 2002).

Dave and Patel (2003) while comparing the solubilization of various insoluble inorganic phosphates by *Pseudomonas* isolates observed the following trends of solubilization of different P source :- Bone meal > TCP > DCP > Iron phosphate > Senegal rock phosphate > aluminium phosphate. Reyes et al (2006) studied the biodiversity of phosphate-solubilizing microorganisms (PSM) of rock phosphate mine in Tachira, Venezuela, a larger number of PSMs were found in the rhizosphere than in the bulk soil. A list of phosphate-solubilizing bacteria and fungi is presented in Table 2.1.

Table 2.1 List of PSMs which are reported to solubilizing insoluble form of P to soluble form.

Organisms	References
Bacteria	
<i>Azospirillum</i>	Seshadri et al. 2000.

<i>Bacillus spp.</i> , <i>Bacillus cereus</i> , <i>Bacillus megaterium</i> , <i>Bacillus subtilis</i> .	Kundu and Gaur 1982; Freitas et al. 1997; Singh and Kapoor 1998; Chatli et al. 2008.
<i>Bradyrhizobium</i>	Halder et al. 1991.
<i>Burkholderia spp.</i> <i>Cladosporium sp.</i>	Rodriguez et al. 1999; Kim et al. 2005; Singh and Kapoor 1998.
<i>Enterobacter spp.</i> , <i>Enterobacter aerogenes</i> , <i>Enterobacter agglomerans</i> .	Gyaneshwar et al. 1999; Chung et al. 2005; Sharma et al. 2005; Thakkar et al. 1993; Kim et al. 1998.
<i>Erwinia herbicola</i>	Goldstein et al. 1993.
<i>Klebsiella sp.</i>	Chung et al. 2005.
<i>Mesorhizobium sp.</i>	Peix et al. 2001.
<i>Pontoea spp.</i>	Chung et al. 2005; Son et al. 2006.
<i>Proteus mirabilis</i> <i>Citrobacter freundii</i>	Thaller et al. 1995
<i>Pseudomonas spp.</i> , <i>Pseudomonas striata</i>	Kundu and Gaur 1982; Goldstein et al. 1993; Illmer et al. 1995; Peix et al. 2004; Babana and Antoun 2006; Hameeda et al. 2008.
<i>Rhizobium meliloti</i> , <i>Rhizobium japonicum</i> , <i>Rhizobium leguminosarum</i> , <i>Rhizobium sp.</i>	Halder et al. 1991; Halder and Chakrabarty 1993.
<i>Serratia spp.</i>	Thaller et al. 1995; Hameeda et al. 2006.
<i>Xanthomonas sp.</i> , <i>Flavobacterium</i>	Freitas et al. 1997.
Fungi	
<i>Aspergillus spp.</i> , <i>Aspergillus tubingensis</i> , <i>Aspergillus niger</i>	Reddy et al. 2002; Pradhan and sukla 2006; Babana and Antoun 2006. Himani and Reddy 2012.
<i>Fromitopsis sp.</i>	Kang et al. 2002.
<i>Glomus spp.</i>	Singh and Kapoor 1998; Duponnois et al. 2005.

<i>Penicillium spp.</i>	Whitelaw et al. 1999; Reyes et al. 2002; Pradhan and Sukla 2006; Babana and Antoun 2006. Himani and Reddy 2012.
<i>Trichoderma spp.</i>	Altomare et al. 1999; Zayed and Motaal 2005.
<i>Eupenicillium parvum</i>	Vyas et al. 2007.

2.9 Mechanisms of P solubilization by PSMs

Several mechanisms have been proposed to explain the P solubilization by PSMs; they are associated with the release of organic and inorganic acids, and the excretion of protons that accompanies to the NH_4^+ assimilation (Kucey 1983; Roos and Luckner 1984; Abd-Alla 1994; Whitelaw 2000). In addition, the release of phosphatase enzymes that mineralize organic P compounds has also been suggested as another mechanism involved (Stevenson 1986). Azam and Memon 1996, affirm that *Nitrosomonas* and *Thiobacillus* mobilized inorganic phosphates by producing nitric and sulfuric acid. Equally, phosphates may be released from solid compounds by carbonic acid formed as a result of the decomposition of organic residues (Memon 1996).

2.9.1 H^+ excretion

Microbial excretion of H^+ occurs in response to the assimilation of cations, primarily related to N source. It is a well-known fungal phenomenon that H^+ is excreted in exchange for NH_4^+ (Asea et al. 1988, Banik and Dey 1982). Using laboratory media, it has been observed that more RP is solubilized when using an NH_4^+ rather than a NO_3^- source of N (Whitelaw et al. 1999). In the same study, it was found that pH was generally lower and titratable acidity higher, when NH_4^+ was used. Similarly, Vora and Shelat (1998) tested a variety of N sources and found that ammonium sulphate promoted the most RP solubilization for bacterial species, *Bacillus circulans*, *Bacillus brevis*, and *Bacillus coagulans*. For some microorganisms, the

release of H⁺ ions due to the assimilation of NH₄⁺ seems to be the sole mechanism promoting insoluble P dissolution. The solubilization of insoluble phosphate without production of acid is due to release of protons accompanying respiration or ammonium assimilation (Kucey 1983).

2.9.2 Organic acid production

Microorganisms are known to produce organic acids in varying concentrations and types. Commonly reported organic acids produced by microorganisms include gluconic acid, citric acid and oxalic acid (Richardson 2001). Many organic acids are effective in solubilizing soil phosphates, these acids are produced by rhizosphere microorganisms (Marschner 1997). Kim et al (1997) point out that the production of organic acid was the major mechanism involved in the solubilization of hydroxyapatite (rock phosphate) by the PSRB *Enterobacter agglomerans*, but other mechanisms might be involved. Under *in vitro* conditions, the pH of the growth medium has decreased as a result of the release of organic acids by PSRB. Some of the organic acids commonly found are gluconic acid (Di- Simine et al. 1998; Bar- Yosef et al. 1999), oxalic acid, citric acid (Kim et al. 1997), lactic acid, tartaric acid, aspartic acid (Venkateswarlu et al. 1984). These acids are the product of the microbial metabolism, mostly by oxidative respiration or by fermentation of organic carbon sources (e.g., glucose) (Prescott et al. 1999).

In case of gram negative bacteria, Goldstein (1995) described the pathway of solubilization of insoluble P (Fig. 2.3). His work suggested that the direct oxidation pathway for glucose dissimilation forms the metabolic basis for the strongly mineral phosphate solublizing (MPS) phenotype. The MPS function provides the reason for the expression of direct oxidation pathway in many bacteria whose bioenergetics needs are satisfied by Embden-Meyerhof,

Entner-Doudoroff or Pentose phosphate pathways. Glucose may be provided by plant or result of biodegradation act as substrate for direct oxidation pathway.

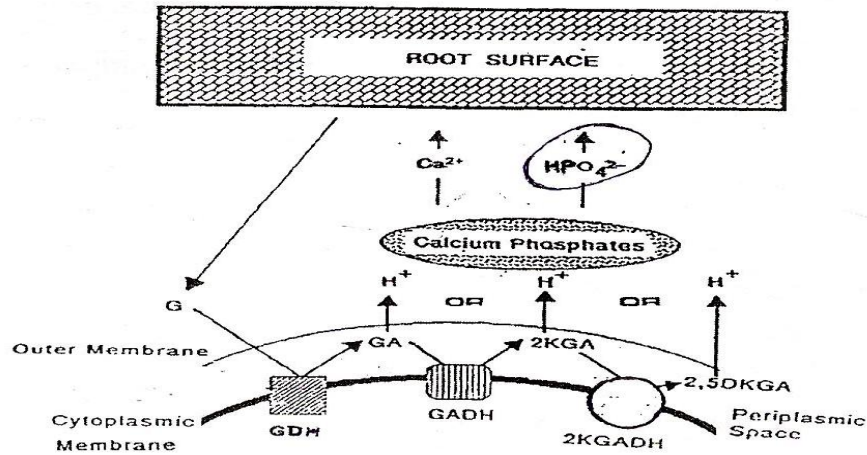


Fig. 2.3 Role of oxidative pathway in release of Pi from tri-calcium phosphate at or near rhizosphere (Goldstein, 1995). G = glucose; GA = gluconic acid; 2KGA = 2-ketogluconic acid; 2,5 DKGA = 2,5 di Ketogluconic acid; GDH = glucose dehydrogenase; GADH = glukonate dehydrogenase; 2KGADH = 2-ketogluconate dehydrogenase.

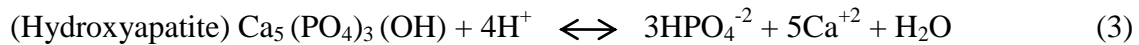
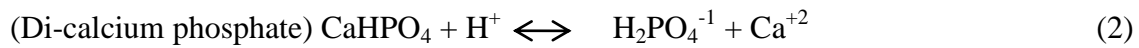
These acids are produced by the pathway depending on the physiological conditions in the periplasmic space and extra cellular environment. Solubilization of phosphate is result of acidification of the periplasmic space which in form, results in acidification of the region adjacent to the cell or colony by the direct oxidation of glucose, it results in the production of gluconic acid by quinoprotein glucose dehydrogenase. Depending upon the bacterial species gluconic acid may undergo one or two additional $2e^-/2H^+$ oxidation resulting in production of 2- Keto- gluconic acid. Organic acids produced by phosphate-solubilizing microorganisms are listed in Table 2.2.

Table 2.2 Production of organic acids by PSMs.

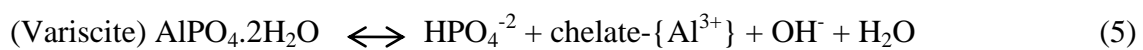
Microorganisms	Predominant acid produced	References
<i>Aspergillus fumigatus</i> , <i>Bacillus firmus</i> , <i>Micrococcus spp.</i>	Oxalic acid, Tartaric acid, Citric acid	Banik and Dey 1982
<i>Aspergillus sp.</i> , <i>Penicillium sp.</i> , <i>Bacillus spp.</i>	Oxalic acid, Succinic acid, Citric acid, 2-keto gluconic acid	Banik and Dey 1983
<i>Aspergillus niger</i>	Succinic acid	Venkateshwarlu et al. 1984
<i>Aspergillus awamori</i> , <i>Penicillium digitatum</i>	Succinic acid, Citric acid, Tartaric acid	Gaur 1990
<i>Aspergillus niger</i> , <i>Penicillium simplicissimum</i> .	Citric acid	Burgstaller et al. 1992
<i>Penicillium bilaji</i>	Citric acid, Oxalic acid	Cunningham and Kuiack 1992.
<i>Aspergillus niger</i>	Citric acid, Oxalic acid, Gluconic acid	Illmer et al. 1995
<i>Pseudomonas cepacia</i>	Gluconic acid, 2-keto-gluconic acid	Bar-Yosef et al. 1999
<i>Aspergillus sp.</i> , <i>Bacillus spp.</i> , <i>Arthrobacter sp.</i> , <i>Enterobacter spp.</i> , <i>Pseudomonas spp.</i>	Succinic acid	Vazquez et al. 2000
<i>Enterobacter intermedium</i>	2-ketogluconic acid	Hwangbo et al. 2003
<i>Burkholderia cepacia</i>	Gluconic acid	Lin et al. 2006.
<i>Aspergillus niger</i>	Citric acid, Oxalic acid, Gluconic acid	Illmer et al. 1995

<i>Pseudomonas cepacia</i>	Gluconic acid, 2-keto-gluconic acid	Bar-Yosef et al. 1999
<i>Aspergillus sp.</i> , <i>Bacillus spp.</i> , <i>Arthrobacter sp.</i> , <i>Enterobacter spp.</i> , <i>Pseudomonas spp.</i>	Succinic acid	Vazquez et al. 2000
<i>Enterobacter intermedium</i>	2-ketogluconic acid	Hwangbo et al. 2003

When PSRB are inoculated to neutral or alkaline soils, the acid production decreases the rhizosphere pH, thus favoring the solubility of calcium phosphates and apatites. If the activity of H⁺ increases in the reactants of the reactions (2) and (3), these reactions proceed. In addition, the sequestering of Ca by organic anions favors the reactions.



In acid soils, the minerals variscite and strengite control the solubility of phosphate (Lindsay 1979). The presence of organic acids propitiates the formation of complexes with Al and Fe ions, which inturn facilitates the dissolution of these minerals. If Fe³⁺ and Al³⁺ are sequestered via chelation with organic anions the reactions 4 and 5 proceed to the right. However, this point is controversial because the reduction in soil pH might also solubilize other iron and aluminum minerals that would reprecipitate again phosphates to form newly strengite and variscite (Lindsay 1979).



On the other hand, organic anions produced by PSM can also compete with phosphates for fixation sites on the surface of soil colloids. He and Zhu (1997, 1998) demonstrated that sorbed phosphates on the surfaces of kaolynite, goethite, montmorillonite and amorphous Al-oxides were displaced by microbial activity presumably using organic acids. The ability of organic acids to solubilize rock phosphate is attributed to the following mechanisms: acidification, chelation, and exchange reactions (Omar 1998).

2.9.3 Acidification

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). As discussed previously, H^+ ions favor RP solubilization by shifting the equilibrium of the dissolution equation (Equation 1), subsequently releasing more P into solution. Organic acids buffer solution pH and will continue to dissociate as protons are consumed by the dissolution reaction (Welch et al. 2002). Microorganisms often export organic acids as anions, which must be actively transported across the plasma membrane. The export of organic acid anions by fungi can occur by an H^+ -symport transport system, causing acidification of the external solution (Netik et al. 1997).

2.9.4 Chelation

In addition to pH reduction, organic acid anions can solubilize RP through chelation reactions. 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 (Kucey 1988). By complexing with cations on the mineral surface, organic acid anions loosen cation-oxygen bonds of the mineral structure and

catalyze the release of cations to solution (Welch et al. 2002). Organic acid anions continue to alter the equilibrium of the dissolution reaction by complexing with cations in solution, effectively lowering the solution saturation point (Welch et al. 2002). Results from a study by Kpombrekou-A and Tabatabai (1994) showed that the ability of organic acids to solubilize RP is affected by the chemical structure, as well as the type and position of functional groups of the organic ligand. These results were further supported by Narsian and Patel (2000), who tested the effects of known chelators including EDTA, DTPA, NTA, aluminon, and oxine, on RP solubilization. This study concluded that RP solubilization was related to the functional groups of the chelators, particularly carboxylic and phenolic hydroxyls. The formation of complexes between chelator and cations such as Al^{3+} and Ca^{2+} depends on the number and kind of functional groups involved as well as the specific cation. It has been found that acids with an increased number of carboxyl groups are more effective at solubilizing RP (Xu et al. 2004, Kpombrekou-A and Tabatabai 1994). For example, Ca^{2+} was found to form complexes more readily with tri-carboxylic acids such as citric acid, over di-carboxylic acids such as malic and tartaric acids (Whitelaw 2000). An increased number of hydroxyl (OH^-) groups have a positive effect on the ability of an organic acid anion to dissolve RP, and a-substitution seems to enhance this effect (Kpombrekou-A and Tabatabai 1994).

Organic acid anions continue to alternate equilibrium of the dissolution reaction by complexing with cations in solution, effectively lowering the solution saturation point (Melissa and Kim 2006). Solubilization was related to the functional group of the chelators, particularly carboxylic and phenolic hydroxyls. 2-keto gluconic acid produced by many PSM is a powerful chelators of calcium and very effective in solubilizing insoluble phosphates such as hydroxyapatite, fluorapatites and aluminium phosphate (Duff et al. 1994). Firsching (1969) reported that citrates, tartarates and oxalates are good chelators of calcium.

2.9.5 Ligand exchange reactions

In a study using four soils of varying pH, CaCO₃ and organic C contents, Gerke et al (2000) found that in all soils more P was mobilized when citrate was added than when the soil was subjected to a wide range of pH changes. This finding indicated that the P was mobilized due to ligand exchange between the citrate and the phosphate adsorbed to the Fe- and Al- sites rather than dissolution from Ca-P precipitates (Gerke et al. 2000). In soil, citrate may mobilize phosphate when it is adsorbed at levels greater than 10 mol g⁻¹ soil; however below this critical value, citrate will not out-compete phosphate for soil adsorption sites (Gerke et al. 2000).

2.9.6 Mineralization of organic phosphorus

Phosphate-solubilizing microorganisms produce enzymes which lead to the solubilization of phosphatic compounds (Rodriguez and Fraga 1999). Phosphorus can be released from organic compounds in soil by three groups of enzymes (1) Non specific phosphatases, which perform dephosphorylation of phosphoester or phosphoanhydride bonds in organic matter (2) Phytases, which specifically cause P release from phytic acid, and (3) phosphonates and C-P lyases, enzymes that perform C-P cleavage in organophosphonates. The main activity apparently corresponds to the work of acid phosphatase and Phytase enzymes because of predominant presence of their substrates in soil (Rodriguez et al. 2006).

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. Organic P may constitute 4-90 % of the total soil P. The degradability of organic phosphorus compounds depends 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 (Ohtake et al. 1996 and McGrath et al. 1995). The mineralization of these compounds is carried out by means of the action of several phosphatases (also called phosphohydrolases). These dephosphorylating reactions involve the hydrolysis of phosphoester or phosphoanhydride bonds. The phosphohydrolases are clustered in acid or alkaline. The acid phosphohydrolases, unlike alkaline phosphatases, show optimal catalytic activity at acidic to neutral pH values.

The major source of phosphatase activity in soil is considered to be of microbial origin (Garcia et al. 1992; Xu and Johnson 1995). In particular, phosphatase activity is substantially increased in the rhizosphere (Tarafdar and Junk 1987). The major portion of the P that is applied to soil rapidly becomes fixed into inorganic and organic fractions which are poorly available to plants (Sanyal and De Datta 1991). Phosphatases (phytase and acid phosphatase) produced by soil microorganisms play a major role in mineralization of organic forms of soil P to release phosphate (Raghothama 1999). *Aspergillus* and *Penicillium* are major genera of phosphatases and phytase producing fungi (Aseri et al. 2009). High production of acid phosphatase and phytase enzyme by filamentous fungi in culture filtrate was reported by Relwani et al (2008). Aseri et al (2009) reported that fungi execute extracellular phytase activity many times more than extracellular phosphatase activity. Richardson et al (2005) reported that decrease in pH of soils makes phytase less effective in the soil environment. Pandey et al (2008) showed higher acid phosphatase activity than alkaline phosphatase activity in all the treatments. Phytates account for a large component of the organic P, some 20-50 % of the total soil organic P (Anderson 1980); yet appear to be only poorly utilized by plants (Hayes et al. 2000; Richardson et al. 2000). Phytases have been reported in bacteria (Kim et al. 1998), yeasts (Lambrechts et al. 1992) and fungi (Shieh and Ware 1968).

2.9.7 Production of carbon dioxide

Carbon dioxide produced by plant roots and microflora is responsible for the solubilization of inorganic phosphates and thus increases the availability of phosphorus to plants by lowering the pH due to the formation of carbonic acid (Hay man 1975).

2.9.8 Production of hydrogen sulphate

It is produced by anaerobic microorganisms from sulphate reducing bacteria such as *Desulfovibrio* and *Desulfotomaculum* (Doelle 1969) which react with soil minerals and thus release phosphate. H_2S reduces ferric phosphate ($FePO_4$) to ferrous sulphate leading to release of $H_2PO_4^-$ ions.

2.9.9 Production of mineral acids

Mineral acids including nitric acid, sulphuric acids have been reported to be produced due to oxidation of nitrogenous compounds or inorganic compounds of sulphur oxidizing bacteria, which react with chemical phosphates converting them into soluble forms (Gaur 1990).

2.9.10 Production of siderophores

Siderophores are iron chelating compounds produced by certain soil bacteria and fungi (Somani and Dadhich 2005) that increases plant available phosphorus in acidic soils, where ferric phosphate occurs as the source of insoluble phosphate by forming complex with iron there by releasing phosphate, thus, the role of siderophores in P-solubilization appears to be important in acidic soils.

2.10 Factors affecting the efficiency of phosphate-solubilizing microorganisms

2.10.1 Carbon sources

Carbon source has been reported to play an important role in microbial solubilization of insoluble phosphates, as it is the key factor in determining the microbial growth and the type of extracellular metabolic products produced. Phosphate solubilization activity of *Aspergillus* sp. was evaluated in the presence of given carbon sources, by replacing glucose respectively of Pikovskaya's (PVK) medium. Production of acids was greatly affected by the nature of carbon sources. Glucose and maltose decreased the pH of the medium to maximum extent and caused highest solubilization of phosphorus, followed by sucrose, xylose and galactose. In control flask, without any addition of carbon source, some growth did occur due to presence of yeast extract in the medium, but drop in pH and P-solubilization was quite low (Pradhan and Sukla 2005). Glucose, galactose, sucrose and arabinose are found to be effective in case of bacteria (Gaur 1990).

2.10.2 Nitrogen sources

Phosphate-solubilizing microorganisms solubilize a good amount of insoluble phosphate in presence of a nitrogen source. The use of ammonium sulphate, urea, ammonium nitrate, and potassium nitrate enhance the phosphate solubilization of *Pseudomonas striata*. Similarly ammonium nitrate plays an important role in P solubilization of *Aspergillus awamori*. Ammonium sulfate was found to be best in reducing the medium pH to 3.31 and simultaneous solubilization of $411\mu\text{g ml}^{-1}$ of P, out of all the nitrogen sources used (Pradhan and Sukla 2005). Son et al (2006) studied solubilization of insoluble phosphate by *Pantoea agglomerans* and found that P-solubilization increased by adding glucose. P-solubilization in relation to nitrogen sources was in the following order: $\text{KNO}_3 > (\text{NH}_4)_2\text{SO}_4 > \text{NH}_4\text{NO}_3 > \text{asparagine} > \text{tryptophan}$ in case of *Aspergillus niger* (Relwani et al. 2008)

2.10.3 Nature of insoluble phosphate

The extent of solubilization has been reported to depend upon the nature of insoluble phosphate as tri-calcium phosphate and hydroxyapatite solubilize more easily than the rock phosphate. Also tri-calcium phosphates solubilizes in great extent than aluminium phosphate and ferric phosphate. It was reported that di-calcium phosphate could be solubilized more readily than tri-calcium phosphate by some bacteria (Sujatha et al. 2004). Microbial solubilization of rock phosphate is influenced by the physical and chemical properties of the rock phosphate and the microorganisms involved.

2.10.4 Particle size of rock phosphate

Particle size of rock phosphate has an important bearing on the degree of solubilization. Finer particle sized rock phosphate are more easily solubilized compared to coarse particles. Gaur (1990) reported maximum solubilization of rock phosphate by *Aspergillus awamori* and *Pseudomonas striata* when the particle size ranged between 30-99 mesh.

2.10.5 pH

Optimum pH for maximum solubilization of inorganic phosphate is neutral or slightly acidic in case of bacteria. Whereas an acidic medium (pH 4.5) is favorable for fungal phosphate solubilizers. In general, phosphate-solubilization was associated to the pH decrease (Fankem et al. 2006).

2.10.6 Temperature

The optimum phosphate-solubilizing activity of fungi is at the temperature range of 30-35 °C, whereas for *Pseudomonas striata* it ranges between 25 °C to 30 °C, higher temperature (More than 40 °C) greatly hamper the activity of phosphate-solubilizing microorganisms (Gaur 1990).

2.10.7 Aeration

Aeration has an additive influence on growth and P-solubilization by bacteria as well as by fungi. Most of these organisms are heterotrophic and aerobic, aeration improves their growth and their efficiency to solubilize rock phosphate. A significant increase in rock phosphate solubilization was observed in shake culture than static culture in case of studies conducted with *Pseudomonas striata* and *Aspergillus awamori*.

2.11 Effects of phosphate-solubilizing bacteria on physiology of crop plants

PSBs have the various beneficial effects on the plants. These bacteria exert the direct or indirect effects on the plants. Direct effects include the increased solubilization and uptake of nutrients or production of plant growth regulators, while the indirect effects include suppression of pathogens and producing metal binding molecules, known as siderophores (Hayat et al. 2010). In most bacteria, mineral phosphate dissolving capacity has been shown to be due the production of organic acids (Rodriguez and Fraga 1999). These bacteria can directly or indirectly affect the plant growth (Mantelin and Touraine 2004). They act as the chemical messengers by producing hormones, which are effective at very low concentration. They are synthesized in one part of the plant and are transported to another location and affect a plants ability to respond to its environment (Sahran 2011). Phosphate-solubilizing bacteria assist in good supply of nutrients to plants, improve soil structure and also help in the bio-accumulation or microbial leaching of inorganic compounds (Brierley 1985). The plant microbe interactions by PSBs such as *Azotobacter*, *Azospirillum*, *Bacillus*, *Klebsiella*, *Pseudomonas etc.* in the rhizosphere play a vital role in transformation pathways, mobilization of nutrients and solubilization processes of nutrients from limited nutrient pool and subsequently uptake of essential nutrients by plants to realize their genetic potential (Hayat et al. 2010).

PGPR influence other physiological processes of the plants through production of hormones (Dobbelaere et al. 2003). Out of these hormones, IAA is the predominant and most active that is known to stimulate both rapid (e.g. increase in cell elongation) and long term (e.g. cell division and differentiation) responses in plants (Hagen 1990; Cleland 1990).

2.12 Application of PSMs on Crops

Phosphate-solubilizing bacteria play an important role in enhancement of growth and yield of crop plants by providing them phosphorus, which is otherwise unavailable to plants (Gyaneshwar et al. 2002). The effective strains of PSM are used to increase the level of available P in the soil. With increase in the level of available P, there is overall increase in the plant growth. Symbiotic relationship was observed between the PSM and crop plants, as soluble phosphorus was provided by bacteria and fungi for the plants that in turn provide carbon (Rodriguez and Fraga 1999). Tomar et al (1996) tested the efficiency of a PSB *Pseudomonas* sp. on the growth and yield of gram (*Cicer arietinum*) that resulted in increase in its growth and grain yield. In soybean, the application of PSB *Pseudomonas* sp. enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability and uptake in soybean crop (*Glycine max*) (Son et al. 2006). In green gram (*Vigna radiata*), the inoculation with different PSB isolates like *Pseudomonas*, *Bacillus*, *Xanthomonas*, *Serratia* and *Enterobacter* resulted in higher nodule number, nodule dry weight, shoot dry matter and total dry matter. Majority of PSB were able to improve growth parameter of green gram significantly compared to rock phosphate control and single superphosphate control (Vikram and Hamzehzarghani 2008).

Twenty seven PSBs including seventeen bacteria and ten fungal isolates were isolated from the rhizosphere soil of crop plants. Out of these, *Aspergillus niger* and *Penicillium vermiculosum* were found to be the most efficient strains and the four bacteria *Bacillus* sp.

and *Pseudomonas stutzeri* were selected to test their ability to solubilize phosphates in liquid media. These were tested on wheat which showed great yield and nutrient (*Triticum aestivum*) (Jisha and Mathur 2005). Gram positive *Bacillus* showed significant effects on winter wheat, total phosphorus and plant biomass, both under pot and field conditions. It was observed that these bacteria had the capability to convert the non available forms of phosphorus into plant available forms (Chen et al. 2006). Inoculation of maize (*Zea mays*) with two efficient screened strains i.e. *Serratia marcescens* and *Pseudomonas sp.*, both under greenhouse and field conditions, showed the increased plant biomass. These both strains survived up to 96 days after sowing (Hameeda et al. 2006). A field experiment was conducted for three years to evaluate the performance of groundnut (*Arachis hypogea*) under alluvial soils of eastern India with different types of inoculants such as *Rhizobium*, PSB (*Bacillus polymyxa*), no inoculants and different levels of cobalt. Higher yield and nutrient uptake was observed with inoculation of *Rhizobium* and *Bacillus polymyxa*. Also, the kernel yield was recorded to be highest, which was 16.50 % higher over no inoculants, respectively (Basu and Bhadoria 2008). Ekin (2010) investigated the efficiency of PSB, *Bacillus M-13* on the growth and productivity of sunflower (*Helianthus annuus*) which resulted in the improved seed quality and oil yield. An increase in head diameter, 1,000 seed weight, kernel ratio and oil content was observed which led to the seed and oil yield increase of 15 and 24.7 % over no application, respectively. A field experiment was conducted on cotton crop with *Bacillus sp.* and results showed that *Bacillus sp.* significantly increased the seed cotton yield, number of bolls/plant, boll weight, plant height, staple length, plant phosphorus and available phosphorus in the soil (Akhtar et al. 2010). It was concluded that PSB not only exert beneficial effects on crop, but also enhance the phosphate concentration in the soil.

The effects of PSB (*Bacillus FS-3*) application were studied on phosphorus content of tomato (*Lycopersicon esculentum*) under green house conditions with five different fertilizer

treatments. A greater increase was noticed in plant root and shoot weight and phosphorus uptake in treatments with PSB application than without PSB in all of fertilizer treatments (Turan et al. 2007. Phosphorus is the key nutrient required by the sugarcane (*Saccharum officinarum*) for the higher productivity of sugar. Application of phosphate-solubilizing bacteria, *Bacillus megaterium* var. *phosphaticum*, with varying amounts of phosphorus fertilizer, increased the sugarcane growth and yield and the status of available phosphorus in the soil. Enhanced tillering, stalk population and stalk weight was observed, which led to increase in cane yield (Sundara et al. 2002). A list of phosphate-solubilizing microorganisms and their effects on different crops is presented in Table 2.3.

Table 2.3 Agronomic response of phosphate-solubilizing microorganisms.

Bacteria	Crop	Response	Reference
<i>Glomus</i> sp., <i>Bacillus circulans</i> , <i>Cladosporium herbarum</i>	<i>Triticum aestivum</i>	Improved growth and yield	Singh and Kapoor 1998
<i>Rhizobacteria</i>	Wheat and Rice	Increased yield, nutrient uptake and IAA production	Khalid et al. 2004
<i>Bacillus subtilis</i> , <i>Aspergillus awamori</i> , <i>Aspergillus niger</i> , <i>Pseudomonas fluorescens</i>	<i>Solanum lycopersicum</i>	Increased nutrient uptake and P bioavailability	Khan et al. 2002
<i>Bacillus cereus</i> MJ-1	Red Pepper	Increased P uptake, and plant biomass	Joo et al. 2005
<i>Pseudomonas</i> sp., <i>Bacillus</i> sp., <i>Aspergillus</i> sp.	<i>Gossypium</i> sp.	Increased P uptake	Narula et al. 2005.

<i>Enterobacterium</i>	<i>Pisum sativum</i> and <i>Cicer arietinum</i>	Increased P uptake and biomass	Hynes et al. 2008.
<i>Thiobacillus</i>	<i>Brassica napus</i>	Increased yield	Salimpour et al. 2010
<i>Aspergillus niger</i> , <i>Aspergillus tubingensis</i>	Wheat, Maize	Increased yield and total P uptake	Richa et al. 2007; Himani and Reddy 2011, 2012.
<i>Pantoea agglomerans</i>	Rice	Increased in growth and yield	Khalimi et al. 2012.

2.13 Application of PSM on crop along with rock phosphate

The fact that certain soil microbes are capable of dissolving relatively insoluble phosphatic compounds has opened the possibility of inducing microbial solubilization of phosphates in the soil (Gaur 1990; Nahas 1996; Bojinova et al. 1997). Inoculation with biofertilizers (*Rhizobium* and AM) was impressive in improving the growth and biomass of Shisham under normal soil whereas in alkaline soil, blending of micronutrients with biofertilizers (*Rhizobium* + AM) had better growth and biomass (Revathi et al 2013). When compared with chemical treatments, microbial solubilization of rock phosphate is an environmentally mild approach (Vassilev and Vassileva 2003). Among the sources of P, rock phosphate and pyrite proved to be best to enhance the grain yield. This basic idea has forced the scientists to see the effect of phosphate-solubilizing microorganisms, when applied in conjugation with low grade rock phosphates and the results obtained were overwhelming. Kaushik et al (2004) reported that 30 kg P₂O₅ as SSP can safely be replaced with 30 kg P₂O₅ as Udaipur Rock Phosphate in the presence of phosphorus solubilizing bacteria as inoculants. It not only increased paddy and wheat yield significantly, but also improved soil health in terms of carbon buildup and available phosphorus. Similarly, Asewar et al (2003) reported that application of 20 kg P₂O₅

as RP in combination with PSB (*Bacillus magaterium*) was superior to 40 kg P₂O₅ as RP alone. In spite of increasing P availability and P uptake, use of PSB also increases plant biomass and N accumulation in the plant biomass (Dubey 2001). It can finally be inferred that 50 % of the costly super phosphate could be replaced by rock phosphate, a cheap source of P, when applied in conjunction with PSB (Sundara et al. 2002).

Traditionally, most experiments using PSM have employed two major strategies for increasing P availability from RP: the management of existing soil microbial populations to optimize their capacity to mobilize P, and the development of specific microbial inoculants (Richardson 2001). Although, there have been some successful results from soil inoculations of PSM (Kucey and Leggett 1989; Omar 1998; Asea et al.1988), in general, the results in terms of plant growth and crop yields, have been highly variable (Gyaneshwar et al. 2002). Organisms that can effectively solubilize RP in the laboratory, under controlled conditions, may be unable to do so in the field (Richardson 2001). These variations in effectiveness may be due to the reduced ability of inoculated PSM to survive and colonize the rhizosphere, coupled with competition for resources with native soil microorganisms (Gyaneshwar et al. 2002). Sharma and Prasad (2003) conducted a field experiment at Indian Agricultural Research Institute, New Delhi during 1996 to 1997 to 1998 to 1999 to study the effect of phosphate-solubilizing bacteria (PSB) and incorporation of wheat and rice residue on the relative efficiency of di-ammonium phosphate (DAP) and Mussoorie rock phosphate (MRP) in three cycles of rice-wheat cropping system and results showed that low grade rock phosphate such as MRP can be advantageously utilized in rice-wheat cropping system when applied with PSB inoculation and incorporation of rice and wheat residues.

Jana and Das (1992a, 1992b) have shown that, rock phosphate may be a suitable substitute for chemical phosphatic fertilizer. inoculating wheat seeds with rock phosphate-solubilizing microorganisms under field conditions, it is possible to obtain the same amount of wheat

grain yields compared to those produced by the expensive di-ammonium phosphate (DAP) fertilizer (Babana and Antoun 2005). *Bacillus* sp. has been identified to be the most effective agent in the process (Banik and Ninawe 1988), which has been clearly demonstrated in aquatic systems with rock phosphate as an insoluble source of P (Sahu and Jana 2000). Sundara et al. 2002 found that application of PSB combined with apatite is more effective than phosphorus fertilizer. Comparing dry weight of plants treated with PSBs and bio-phosphate, the results showed that inoculated plants with bio-phosphate increased more greatly dry weight than plants inoculated only by phosphate-solubilizing bacteria. Medina and Probanza (2003) found that *Bacillus pumillus* and *Glomus deserticola* have higher efficiency than chemical phosphorus fertilizer in production of dry weight. Phosphorus is one of the necessary elements (Trollove 2003), if the rate of soil phosphorus is declined, the yield also will decrease. Inoculated plants with PSB in both pot and field experiments had desirable yield. In the field conditions, when PSB was combined with rock phosphate (apatite), yield was more greatly increased (Akbari et al. 2010). Sundara et al (2002), who found that application of rock phosphate with phosphate-solubilizing bacteria, enriched the rhizosphere more than the other treatments. Himani and Reddy (2012) suggest that there was a significant increase in yield and total P in maize and wheat crop when *Aspergillus niger* and *Aspergillus tubingensis* were used as bio-inoculants along with rock phosphate solubilization.

Since not many reports are available on the use of phosphate-solubilizing microorganisms as bio-inoculants along with RP fertilization in organic farming and comparison of PSM as bio-inoculants at different agroclimatic regions, this is an attempt to isolate the PSM from rhizospheric soil of organic field and studying their effects as bio-inoculant along with RP fertilization on crop yield and soil fertility in organic field and also at multilocational sites at different agroclimatic regions.

Chapter 3

Materials and methods

MATERIALS AND METHODS

3.1 Isolation and identification of phosphate-solubilizing microorganisms for alkaline soils

3.1.1 Collection of soil samples

To isolate the phosphate-solubilizing microorganisms, soil samples were collected from different agricultural soil niches. These niches were rhizosphere of bamboo (*Bambusa vulgaris*) plants (multipurpose agroforestry crop) grown in experimental field of CORE, Thapar university, Patiala (30.30° N, 76.38° E); surface soil of potato (*Solanum tuberosum*) (tuberous crop) field of CORE, Thapar University, Patiala; surface soil of CORE (no vegetation, open area); rhizosphere soil of mustard (oil-seed crop) (*Brassica campestris*); rhizosphere of mature maize (cereal crop) (*Zea mays*) plants, rhizosphere soil of small maize plants, surface soil of berseem crop (fodder crop) (*Trifolium alexandrinum*) field; rhizosphere of sunflower (oil-seed crop) (*Helianthus annuus*) grown in agriculture field of Balachaur (31.07° N, 76.32° E), Punjab; and rhizosphere of *Stevia rebaudiana* (medicinal plant) grown in organic field of Pojewal (31.65° N, 76.26° E), Punjab, India. Soil samples to the depth of 5-10 cm were drawn carefully and were collected in sterile plastic bags and brought to the laboratory and stored at 4 °C until used for the isolation of phosphate-solubilizing microorganisms.

3.1.2 Isolation of phosphate-solubilizing microorganisms

Isolation of phosphate-solubilizing microorganisms was done by serial dilution method on Pikovskaya's (PKV) agar plates (Pikovskaya 1948).

Composition of Pikovskaya's agar medium (Pikovskaya 1948)

Ingredients	gms/liter
Yeast Extract	0.50
Dextrose	10.0
Tri-calcium phosphate	5.00
Ammonium Sulphate	0.50
Potassium Chloride	0.20
Magnesium Sulphate	0.10
Manganese Sulphate	0.001
Ferrous Sulphate	0.001
Agar	15.00
Distilled Water	1000 ml
pH	7.2 ± 0.2

All ingredients except tri-calcium phosphate $\text{Ca}_3(\text{PO}_4)_2$ were dissolved in 1000 ml of distilled water, pH adjusted to 7.2 and autoclaved at 15 Psi for 15 minutes. Tri-calcium phosphate was autoclaved separately at 15 Psi for 15 minutes. Later these were mixed together and poured in to sterilized Petri plates. After solidification of medium, these Petri-plates were incubated at 30 °C for overnight to check contamination.

3.1.2.1 Phosphate-solubilizing bacteria

For the isolation of phosphate-solubilizing bacteria 10 gram of soil sample was transferred to 90 ml of sterile physiological saline solution (0.85 % NaCl in distilled water) and mixed thoroughly. Serial dilutions were made up to 10^{-7} and from each dilution 100 µl were spread on Pikovskaya's agar plates supplemented with 0.5 % tri-calcium phosphate (TCP) and incubated at 30 °C. Colonies showing the zone of solubilization were streaked on PKV agar plates to check their purity and stored in 40 % glycerol at -80 °C for further use.

3.1.2.2 Phosphate-solubilizing fungi

For the isolation of phosphate-solubilizing fungi, soil samples were serially diluted up to 10^{-5} in sterile physiological saline solution (0.85 % NaCl in distilled water) and pour plating was done on PKV agar plates supplemented with 0.5 % TCP, having 0.003 % w/v of Rose Bengal powder to selectively inhibit the bacterial growth and restrict the size and height of colonies of more rapidly growing molds. The plates were incubated at 30 °C. Distinct colonies showing halo zone were selected, purified by repeated culturing and maintained on PKV agar slants at 4 °C.

3.1.3 Qualitative screening of phosphate-solubilizing bacteria and fungi

Pure culture of phosphate-solubilizing bacteria and fungi were spot inoculated on PVK agar plates and incubated at 30 °C. For this PKV agar plates were formally divided into four parts. Ten µl suspensions of two days grown culture of bacterial isolates was used to inoculate centre of each quarter part of the Petri plate. Each isolate was inoculated in duplicate. In case of fungal isolates, 5 mm diameter disc, cut from the periphery of the actively growing colonies of fungal isolates, and were inoculated on PKV agar plates. The halo zone of phosphate solubilization around growth was recorded (in mm) after every 24 hours of incubation. The colonies forming more than 5.0 mm zone of solubilization were selected as efficient strains. Results were recorded up to five days. Solubilization index was evaluated according to the ratio of the total diameter (colony + halo zone) and the colony diameter (Edi-Premono et al. 1996).

3.1.4 Test of purity and stability (Fankem 2006)

To estimate the capability of the obtained isolates in solubilizing sparingly soluble phosphate ($\text{Ca}_3(\text{PO}_4)_2$), PVK agar medium was used along with Bromo Cresol Green (BCG) dye as an

indicator. 5.0 ml of 0.5 % Bromo Cresol Green was added to 1000 ml of PVK agar medium. In this medium the BCG was used as pH indicator dye which, in pH greater than 6 is green but changes into yellow color when the pH falls within 3.8-5.4.

Preparation of Bromo Cresol Green (Gadagi and Tongmin 2002)

A stock solution of 0.5% dye was prepared by dissolving 0.5 g BCG in 100 ml 70 % ethanol. The final pH was adjusted to 6.5 with KOH. BCG was sterilized by Glass Fibric Circle filter. Five ml of this stock solution was added aseptically to 1000 ml of autoclaved PVK agar medium.

Procedure

A five ml aliquot of the sterilized stock solution of BCG was added to 1000 ml of autoclaved PVK agar medium, and were mixed together, and poured in Petri plates. After solidification, these Petri plates were incubated at 30 °C for overnight to check the contamination. Phosphate-solubilizing bacterial culture of 0.8 O.D._{600 nm} was point inoculated on the Petri plates. In case of fungal isolates 5 mm diameter disc, cut from the periphery of the actively growing colonies of fungal isolates were inoculated on PKV agar plates supplemented with BCG dye. Plates were then incubated at 30 °C. Results were recorded after three to five days of incubation.

3.1.5 Quantitative Assay for P-solubilization in liquid medium (Jackson 1973)

These bacterial and fungal isolates were further tested for solubilization of tri-calcium phosphate and Rajasthan rock phosphate (RP) in quantitative terms.

Reagents

1. **Chloromolybdic acid:** 15.0 g ammonium molybdate was dissolved in about 400 ml of distilled water. Filtered and then 400 ml of 10 N HCl was added slowly with rapid stirring. Volume was made to 1000 ml with distilled water and stored in amber glass bottle.

2. **Chlorostannous acid:**

Stock Solution:

SnCl ₂ .2H ₂ O	10.0 g
Conc. HCl	25.0 ml

SnCl₂ crystals were dissolved in conc. HCl and solution was kept in glass under airtight stopper.

Working Solution: Fresh working solution was prepared by adding 1.0 ml of the above solution to 132.0 ml of distilled water.

Assay procedure for quantitative P solubilization in liquid medium

The PSB were grown in 50 ml nutrient broth for 24 hours at 30 °C in incubator shaker. 0.1 ml of each PSB was aseptically transferred to 100.0 ml PVK broth contained in 250 ml conical flasks. Similarly 5 mm diameter disc, cut from the periphery of the actively growing colonies of fungal isolates were inoculated in 100 ml PKV broth in 250 ml conical flask. The flasks were incubated at 30 °C in a rotary shaker at 130 rpm. Five ml of culture suspension was taken out in sterile conditions at regular interval of 2 days from third day onward and centrifuged at 10,000 rpm for 10 min. Then 500 µl aliquot of each supernatant was transferred to 50.0 ml volumetric flask. This was followed by addition of 10.0 ml chloromolybdic acid. The contents of the flasks were diluted to 40.0 ml with distilled water. Then 1 ml of chlorostannous acid was added. After mixing, the volume was made up to 50.0

ml with distilled water. The blue color intensity of the solution was measured in a spectrophotometer at O.D. 660 nm. The soluble 'P' was estimated from standard curve of KH_2PO_4 (100 ppm) drawn against O.D. 600 nm. In case of fungi at the end of incubation, the contents of the flasks were filtered through Whatman no. 42 filter paper, washed repeatedly with distilled water and the mycelial mass was dried at 70 °C for 48 hours. Dry weight of the mycelium represents growth.

pH of the culture filtrates was also recorded. Isolates showed maximum P-solubilization in the TCP were tested for acid phosphatase and alkaline phosphatase enzyme production (Tabatabai and Bremner 1969), phytase enzyme production (Heinonen and Lahti 1981) and for organic acid exudation in culture supernatant by using an HPLC method as described by Relwani et al (2008).

Both fungal and bacterial isolates showed maximum P-solubilization in TCP were further screened for rock phosphate solubilization by supplementing RP equivalent to 100 mg P_2O_5 100 ml⁻¹. Rock phosphate used in this study was obtained from Rajasthan State Mines and Minerals, Ltd., Udaipur, India. The chemical constituents of the RP were: 31.5 % P_2O_5 , 45.4 % CaO, 3.4 % MgO, 8.4 % Al_2O_3 , 3.1 % fluoride, 0.044 % organic carbon and 0.003 % available P. Acid phosphatase and alkaline phosphatase activities, phytase activity and organic acids production was determined in culture supernatant.

3.1.6 Acid and alkaline phosphatase activity (Tabatabai and Bremner 1969)

Reagents

1. 5X modified universal buffer (MUB) (Skujins et al. 1962)

Tris (hydroxyl methyl) amino methane	12.10 g
Maleic acid	11.60 g
Citric acid	14.00 g
Boric acid	6.28 g
1 N NaOH	488 ml

Final volume was made up to 1000 ml with distilled water and stored at 4 °C.

Before use, the pH of 200 ml of MUB was adjusted to 5.5 with 0.5 N HCl for the assay of acid phosphatase or with 0.5 N NaOH to pH 9.0 for the alkaline phosphatase.

The volume of pH modified buffer was made up to 1 liter with distilled water.

2. p-nitrophenyl phosphate solution (0.115 M): Dissolved 4.268 g disodium p-nitrophenyl phosphate hexahydrate in 100 ml of appropriate pH adjusted, diluted MUB (pH 5.5) for acid phosphatase and (pH 9.0) for alkaline phosphatase. The p-nitrophenyl phosphate substrate was stored at 4 °C but no longer than 10 days.

3. NaOH (0.5N): 20 g of NaOH was dissolved in 70 ml distilled water and the volume was made up to 1 litre.

4. p-nitrophenol standard: 1 mg per 1 ml p-nitrophenol solution was prepared in modified universal buffer. The solution was stored in dark bottle at 4 °C for no longer than 21 days.

Procedure

1. 0.5 ml aliquots of enzyme sample was taken (here culture filtrate).
2. Added 4.0 ml of the diluted MUB (pH 5.5 for acid phosphatase and pH 9.0 for alkaline phosphatase).
3. 1 ml of filter sterilized 0.115 M disodium p-nitro phenyl phosphate solution was added (of the same pH as of MUB).
4. The content was incubated at 37 °C for one hour in dark, after one hour of incubation add 5 ml of 0.5 N NaOH solution to stop the reaction.
5. The content was then checked for phosphatase activity by measured the yellow color intensity with UV-Vis spectrophotometer at 410 nm.
6. Phosphatase enzyme activity was indicated as the amount of p-nitro phenol released in the filtrate from the p-nitro phenyl phosphate substrate as per ml of supernatant. The p-nitro phenol content was calculated with reference to a calibration graph plotted from the results obtained by standards containing 10-100 $\mu\text{g ml}^{-1}$ of p-nitro phenol.
7. Controls were analyzed in the similar manner, except the p-nitro phenyl phosphate substrate was added after the NaOH solution.

3.1.7 Phytase enzyme activity (Heinonen and Lahti 1981)

Reagents

1. **Substrate solution:** Dissolved 2.5 mM dodecasodium phytate in 0.2 M sodium acetate buffer of pH 5.5.
2. **Color stop solution:** (10 mM ammonium molybdate : 5 N sulphuric acid: acetone, 1:1:2 ratio)
3. **1 M citric acid**
4. **Standard KH_2PO_4 solution:** mg/ml stock.

Procedure

1. 0.5 ml aliquots of enzyme sample was taken (here culture filtrate).
2. Added 0.5 ml substrate solution (2.5 mM dodecasodium phytate in 0.2 M sodium acetate buffer of pH 5.5) and incubated for 10 min at 37 °C.
3. The reaction was stopped by addition of 2 ml ice cold color stop solution (10 mM ammonium molybdate : 5 N sulphuric acid: acetone, 1:1:2 ratio).
4. Added 100 µl 1 M citric acid and O.D. was taken at 380 nm.
5. Un-inoculated medium of experiment was taken as control.
6. Calculated the phytase activity per ml of sample. Enzyme activity was expressed as micromoles of inorganic P released per hour per milliliter of culture filtrate from sodium phytate at 37 °C.

3.1.8 Organic acid estimation

Organic acids produced by P-solubilizing microorganisms were determined using HPLC method. The HPLC equipment was series 200 of Perkin Elmer, USA, equipped with Polypore-H column (Brownlee Column, Perkin Elmer, USA) and a Micro-Guard column (Perkin Elmer, USA). After 5 days of incubation, the culture filtrates were passed through a 0.22 µm filter and subjected to HPLC with a polypore H column (Perkin Elmer, USA). The mobile phase consisted of 0.008 N H₂SO₄ at a flow rate of 0.3 ml min⁻¹. Detection was performed by a UV-VIS detector at 210 nm (Relwani et al. 2008). HPLC profiles of the culture filtrates were analyzed by comparison with the elution profiles of pure organic acids (Bio-Rad Standard containing oxalic acid, succinic acid, acetic acid, citric acid, malic acid, and formic acid whereas gluconic acid (Sigma, USA) was injected separately).

3.1.9 Biochemical characterization of P solubilizing microorganisms

3.1.9.1 Morphological and biochemical studies of P solubilizing bacteria

The bacterial isolates solubilizing TCP as well as rock phosphate (RRP) to greater extent were further characterized.

3.1.9.1.1 Gram staining

A thin smear of actively growing bacterial cells was made on separate glass slides, air dried and heat fixed the smears. Hold the slides using slide racks. Cover each smear with crystal violet for 30 sec and washed each slide with distilled water for a few seconds, using wash bottle. Each smear was covered with Grams iodine solution for 60 seconds. Iodine solution was washed off with 95% ethyl alcohol. Ethyl alcohol was added drop by drop, until no more colour flows from the smear. Slides were washed with distilled water and Safranin was applied to smear for 30 seconds. Slides were washed with distilled water and blot dried with absorbent paper. Let the stained slides air dry. Slides were examined microscopically using oil-immersion objective. Those bacteria that appeared purple were referred to as Gram positive and those appeared pink were described as Gram negative bacteria.

3.1.9.1.2 Hanging drop method

Hanging drop preparation is useful for microscopic examination of living microorganisms, especially bacteria without staining them and to see their motility due to flagella.

Cleaned and flamed a hanging drop slide and placed it on the table with the depression uppermost. A little Vaseline was spread around the cavity of the slides. A cover slip was cleaned and Vaseline was applied on each of the four corners of the cover slip. One loopful of culture was transferred in the centre of the cover slip. Depression slide was placed on the cover slip, with the cavity facing down so that the depression covers the suspension.

Preparation was examined under low-power objective with reduced light and a drop of oil was placed on the cover slip and the preparation was examined under oil-immersion objective. True motility was shown by bacteria that were moved swiftly across the microscope.

3.1.9.1.3 Catalase test

1. Small amount of bacterial cells were placed onto a clean microscope slide.
2. A few drops of H₂O₂ (3%) was added onto the smear.
3. A positive result was the rapid evolution of O₂ as evidenced by bubbling.
4. A negative result was no bubbles or only a few scattered bubbles.

3.1.9.1.4 Oxidase test

1. Small amount of organism from an agar slant/ plate was obtained with a sterile swab.
2. One drop of reagent (N,N,N',N'-tetramethyl phenylenediamine dihydrochloride) was placed onto the culture on the swab.
3. Positive reactions turned the bacteria violet to purple immediately or within 10 to 30 seconds. Delayed reactions were ignored.

3.1.9.1.5 Nitrate reduction test

Nitrate broth composition

Ingredients	gms / liter
Peptic digest of animal tissue	5.000
Meat extract	3.000
Potassium nitrate	1.000
Sodium chloride	30.000
Final pH	7.0 ± 0.2

Distilled water 1000 ml

Dispensed the medium in tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Preparation of Nitrate test reagents:

- 1. Sulfanilic Acid:** Dissolved 8 grams of sulfanilic acid in 1 liter 5 N acetic acid.
- 2. Alpha-Naphthylamine reagent:** Dissolved 5 grams of alpha-naphthylamine in 1 liter 5 N acetic acid.

Procedure

Nitrate media was used to determine the ability of an organism to reduce nitrate (NO_3) to nitrite (NO_2) using the enzyme nitrate reductase. It also tests the ability of organisms to perform nitrification on nitrate and nitrite to produce molecular nitrogen. Nitrate broth contained nutrients and potassium nitrate as a source of nitrate. After incubating the nitrate broth, 2-3 drops of sulfanilic acid and α -naphthylamine were added. If the organism had reduced nitrate to nitrite, the nitrites in the medium will form nitrous acid. Sulfanilic acid was added; which reacted with the nitrous acid to produce diazotized sulfanilic acid. This reacts with the α -naphthylamine to form a red colored compound. Therefore, if the medium turns red after the addition of the nitrate reagents, it was considered a positive result for nitrate reduction. If the nitrate has not been reduced or if it has been reduced to free nitrogen gas the broth remains colourless. To differentiate between these two, zink dust was added. This catalysis the reduction of nitrate in the broth to nitrite which then allows the formation of diazonium compound which resulting red color. If the organism has reduced the nitrate to free nitrogen gas then the medium will remain colorless. There are positive results, if nitrate reduced either to nitrite or nitrogen gas. Negative results were obtained if nitrate has not been reduced.

3.1.9.1.6 Starch hydrolysis test

Requirements

1. Starch agar

Ingredients	g/l
Starch (soluble)	20.0
Peptone	5.0
Beef extract	3.0
Agar	15.0
pH	7.0
Distilled water	1000.0 ml

2. Iodine solution

Iodine	1.0 g
Potassium iodide	2.9 g
Distilled water	300.0 ml

A homogeneous preparation of the iodine and iodide was made by using mortar and pestle. Transferred the content to a reagent bottle and water was added to make a total volume of 300 ml. store the solution in a glass-stopper brown bottle.

Procedure

1. Using sterile technique, a single streak inoculation of each test organism was made into the centre of its appropriately labeled starch agar plate.
2. Incubated bacterial inoculated plates for 48 hours at 37 °C and fungal inoculated plates for 72-96 hours at 25 °C in an inverted position.
3. Surface of the plates was flooded with iodine solution with a dropper for 30 seconds.

4. Poured off the excess iodine solution.
5. The plates were examined for the starch hydrolysis around the line of growth of each organism, i.e. the color change of the medium.
6. Starch in the presence of iodine produced a dark blue coloration of the medium, and a yellow zone around the colony in an otherwise blue medium indicated amyolytic activity.

3.1.9.1.7 Cellulose production test

Evidence for the microbial utilization of cellulose can be detected using hexadecyltrimethyl ammonium bromide. This reagent precipitates intact carboxymethyl cellulose (CMC) in the medium and thus clear zones around a colony in an otherwise opaque medium indicating degradation of CMC.

Requirements

1. Modified Czapek-mineral salt medium

Ingredients	g/l
Sodium nitrate	2.0
Potassium phosphate	1.0
Magnesium sulphate	0.5
Potassium chloride	0.5
Carboxymethyl cellulose	5.0
Peptone	2.0
Agar	20.0
Distilled water	1000 ml

2. Carboxymethyl cellulose (CMC)

3. Hexadecyltrimethyl ammonium bromide (1 % solution)

Procedure

1. Dissolved the agar in 400 ml of hot distilled water by added in small amounts and stirring with a glass rod.
2. Dissolved the magnesium sulphate, potassium chloride, peptone, sodium nitrate in 200 ml of water.
3. Dissolved potassium phosphate in 100 ml of water.
4. Dissolved CMC in 200 ml of water with heat and mix.
5. Mixed all the solutions and made up to 1000 ml volume.
6. The pH of the medium was adjusted to 6.5 with the addition of acid or alkali and autoclaved at 15 lb/in² pressure (121 °C) for 15 minutes.
7. Poured the autoclaved medium cooled to 45-50 °C into sterile Petri plates and allow the media to solidify.
8. The appropriately labeled plates were inoculated with the respective organism.
9. Incubated the inoculated plates at 35 °C for 2-5 days.
10. The plates were flooded with 1 % aqueous solution of hexadecyltrimethyl ammonium bromide and the plates were observed for the formation of a zone around the growth.

3.1.9.1.8 Urease test

Requirements

1. Urea agar medium

Ingredients	gms/litre
Peptic digest of animal tissue	1.000
Dextrose	1.000
Sodium chloride	5.000

Disodium phosphate	1.200
Monopotassium phosphate	0.800
Phenol red	0.012
Agar	15.000
Final pH	6.8 ± 0.2

Medium was sterilized by autoclaving at 10 lbs pressure (115 °C) for 20 minutes. Allowed to cool to 50 °C and aseptically added 50 ml of sterile 40 % urea solution (FD048) and mix well. Dispensed into sterile tubes and allowed to set in the slanting position.

Procedure

1. Inoculated the urea agar slants with test organism.
2. Incubated, inoculated slants for 24-48 hours at 37 °C.
3. Examined the slants as to their color for the presence of urease (red or cerise color) and for no urease (yellow color).

3.1.9.1.9 Fermentation of carbohydrates

The fermentation broth contains ingredients of nutrient broth, a specific carbohydrate and a pH indicator (phenol red), which is red at a neutral pH-7 and turns yellow at or below a pH of 6.8 due to the production of an organic acid.

Procedure

1. Preparation of fermentation medium

Peptone	10.0 g
Carbohydrates	5.0 g
Sodium chloride	15.0 g

Phenol red	0.018 g
Distilled water	1000.0 ml
pH	7.3

(Carbohydrates used were glucose, fructose, lactose and sucrose)

2. Broth was taken into fermentation tubes and Durham tubes were added in inverted position, was autoclaved at 12 lb pressure for 15 minutes.
3. Inoculated the four types of sugar fermentation broth with tested organism and kept one uninoculated tube of each fermentation broth as a comparative control. Incubated all the inoculated and uninoculated tubes at 35 °C for 24-48 hours.
4. Observed the reaction that developed in the inoculated tubes compared to uninoculated tubes i.e. changed in color (due to production of acid) or changed in color and appearance of bubbles (due to production of acid and gas).

3.1.9.1.10 IMViC tests

The IMViC test consists of four different tests (1) indole production (2) methyl-red (3) Voges-Proskauer and (4) citrate utilization.

3.1.9.1.10.1 Indole production test

1. Preparation of (1 %) tryptone broth: Dissolved 10 g of tryptophan in one litre of distilled water. Sterilized in the autoclave at 15 psi (121 °C) for 15 minutes. Inoculated the broth with test organism and incubated at 35 °C for 48 hours.
2. After 48 hours incubation, 1 ml of Kovacs reagent was added to each tube including control.
3. The tubes were shaken gently after intervals of 10-15 minutes.
4. The tubes were allowed to stand to permit the reagent to come to the top.

5. Development of a cherry (deep) red color in the top layer of the tube was a positive test for indole production. Absence of red coloration was indole negative.

3.1.9.1.10.2 Methyl-Red and Voges-Proskauer testes

Requirements

1. **MR-VP broth**
2. **Methyl red pH indicator**
3. **VP reagent I (naphthol solution)**
4. **VP reagent II (40% potassium hydroxide)**

Procedure

1. Preparation of MR-VP broth (pH 6.9) tubes

Peptone	7.0 g
Dextrose	5.0 g
Potassium phosphate	5.0 g
Distilled water	1000.0 ml

Poured the 5 ml broth in each tube and sterilized by autoclaving at 15 lb pressure for 15 minutes.

2. Inoculated the MRVP tubes with test organism and kept one tube as uninoculated comparative control.
3. Incubated all the tubes at 35 °C for 48 hours.
4. Added 5 drops of methyl red indicator to the tubes of each set.
5. Observed the change in color of methyl red for MR test.
6. Added 12 drops of V-P reagent and 2-3 drops of V-P reagent II to the other set of tubes as well as to un-inoculated control tubes.

7. The tubes were shaken gently for 30 seconds with the caps off to expose the media to oxygen.
8. The reaction was allowed to complete for 15-30 minutes. Observed the tubes for changes in color for the VP test.
9. In MR test, the methyl red indicator in the pH range of 4 was remained red which was indicative of positive test, while turning of methyl red to yellow was a negative test.
10. In VP test, the development of crimson to ruby pink color was indicative of positive VP test while no change in coloration was a negative test.

3.1.9.1.10.3 Citrate utilization test

1. Preparation of Simmons citrate agar (pH 6.9) slants

Ammonium dihydrogen phosphate	1.0 g
Dipotassium phosphate	1.0 g
Sodium chloride	5.0 g
Sodium citrate	2.0 g
Magnesium sulphate	0.2 g
Agar	15.0 g
Bromothymol blue	0.8 g
Distilled water	1000.0 ml

Poured the medium in culture tubes and sterilized by autoclaving at 15 lb pressure for 15 minutes and the slants were prepared.

2. Inoculated the Simmons citrate agar slants, with test organisms, and incubated at 37 °C for 48 hours.
3. After incubation, growth was visible on the surface and the medium color was blue showed citrate positive results, there was no growth and no change in the color of the medium showed citrate negative results.

3.1.9.1.11 Antibiotic profiling of bacterial isolate

Bacterial isolates were grown in nutrient broth until the absorbance reached to 1.0. The grown bacterial cells were spread on nutrient agar and antibiotic discs were kept on it. These plates were incubated at 37 °C and the inhibition zones were noted. Ready precoated 12 antibiotic discs (HK001-1PK, Himedia, Mumbai, India) were used to test the sensitivity of the bacterial isolates. These were: Penicillin G (10 U), Cephalothin (30 mcg), Clindamycin (2 mcg), Erythromycin (15 mcg), Amoxycylav (30 mcg), Vancomycin (30 mcg), Ofloxacin (5 mcg), Teicoplanin (30 mcg), Ceftazidime (30 mcg), Gentamicin (10 mcg), Cephoxithin (30 mcg), Oxacillin (1 mcg).

3.1.9.2 Morphological studies of P-solubilizing fungi

To study the morphology, the fungal isolates were grown on PKV agar media at 30 °C for three days. The fungal spores were placed on the slides and stained with Lactophenol cotton blue. Identification was done through visual inspection of morphology by bright field microscopy under 40x magnifications (Aneja 2006) and further confirmed by ITS region sequence analysis of the ribosomal RNA gene.

3.1.10 Molecular methods for identification of P-solubilizing microorganisms

3.1.10.1 Isolation of genomic DNA from P-solubilizing bacteria

1. A single colony of P-solubilizing bacterial isolate was picked from a freshly grown plate and transferred into 20 ml of Nutrient broth in a 250 ml conical flask. The culture was incubated for 16-24 h at 37 °C with vigorous shaking (120 rpm).
2. Cells were harvested in 2.0 ml sterile microfuge tube after centrifuging at 13000 rpm for 5 minutes at 4 °C. Media was decanted from the microfuge tubes and the tubes

were kept in an inverted position for 1 min to allow the last traces of media to drain away.

3. Resuspended each cell pellet in approximately 0.8 ml saline-EDTA buffer thoroughly. 50 µl of freshly prepared lysozyme solution was added and mixed well. Incubated at 37 °C for 20 minutes.
4. Added 0.2 ml 10 % SDS, mixed well by inversion, and incubated in water bath at 60 °C for 15 minutes.
5. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added, mixed well by inversion, and centrifuged at 10000 rpm at 4 °C for 10 min.
6. Upper aqueous phase was transferred to the sterile microfuge tube. 0.6 volume of ice cold isopropanol was added and gently mixed to precipitate DNA.
7. After centrifugation at 13000 rpm for 10 min., the supernatant was discarded and the pellet was washed twice with 70 % ethanol to remove salts.
8. The pellet was air dried and dissolved in 50 µl Tris-EDTA (pH 8.0) buffer. Stored at 4 °C (or -20 °C) for further use.

3.1.10.2 Isolation of genomic DNA from P-solubilizing fungi (Moller et al. 1992)

1. All the fungal isolates were grown in the Pikovskaya's broth at 30 °C using incubator shaker at 130 rpm.
2. After 5 days of growth, the culture was washed with sterile distilled water through Whatman filter paper no. 42 up to the medium was removed from the mycelium.
3. The mycelia culture was pressed with the help of filter paper to remove the water and kept it at -80 °C for further use.
4. Mycelium was taken into the mortar, added liquid nitrogen and crushed with the help of pestle.

5. Transferred the powder in sterilized eppendorf. Kept it at -20 °C or -80 °C.
6. A 1.5 ml of centrifuge tube was filled 1/3rd with freeze-dried mycelium powder.
7. Then 0.5 ml extraction buffer (Appendix I) (preheated at 65 °C) was added and mixed well and let stand for 15-20 minutes at 65 °C in water bath. (Vortex, if necessary added 4 µl of RNase (200 µg/ml).
8. Then 0.5 ml of equilibrated phenol was added and mixed well and left for 15 minutes at room temperature.
9. It was added with 0.5 ml of Sevag (chloroform: isoamyl alcohol; 24:1) followed by mixing. Then centrifuged tubes were incubated at room temperature for 15 minutes and centrifuged at 10,000 rpm for 10 minutes.
10. The upper aqueous layer was removed and transferred to new centrifuged tube.
11. Then 400 µl of Sevag was added and mixed by inverting gently and centrifuged for 10 minutes at 10,000 rpm and transferred supernatant to a new centrifuged tube.
12. Then 0.54 volumes of isopropanol was added to precipitate DNA and incubated at -20 °C for 20 minutes and centrifuged at 10,000 rpm for 10 minutes.
13. The pellet was washed with 100 µl of 70 % ethanol.
14. Added 225 µl of ammonium acetate (5 M) mixed gently and placed the tubes on ice for 30 minutes or longer (better to leave at 4 °C for overnight).Centrifuged at 10000 rpm at 4 °C for 10 minutes and transferred the supernatant to fresh eppendorf.
15. Added 0.55 volume of isopropanol to precipitate the DNA. Immediately centrifuged at 10,000 rpm for 5minutes at 4 °C.
16. Decanted the supernatant and washed the pallet twice with ice cold 70% ethanol to remove salts. Air dried the pellet and dissolved in 30 µl of Tris-EDTA (pH 8.0) buffer. Stored at 4°C (or -20°C) for further use.

3.1.10.3 Electrophoresis of DNA on agarose gels

DNA was loaded on 0.7 % (w/v) agarose gel prepared in 0.5x TBE, pH 8.0 (Appendix I) using a 6x loading dye (Appendix I). Ethidium bromide (0.5 µg/ml) was added to stain the gel prior to pouring. The nucleic acids were then electrophoresed at 50 volts (3 volts/cm) for 45-60 min and visualized on a U.V. transilluminator.

The concentration of DNA was determined using a Nano Drop 1000 spectrophotometer (Thermo scientific, Wilmington, DE) and quality of DNA was evaluated by measurement of the A260 and A280 and the A230/A260 ratios. Ideally, the A260/A280 ratio should be 1.8-2.0. Ratios less than 1.8 indicate protein or phenol contamination, while ratios greater than 2.0 indicate the presence of RNA.

3.1.10.4 Amplification of 16S rRNA

For amplification of 1.5 kb gene of 16S rRNA, the primers used were: (Forward primer 5'-AGA GTT TGA TCC TGG CTC AG-3' and reverse primer 5'-ACG GGC GGT GTG TTC-3' (Weisberg et al., 1991). DNA amplification was performed with GenAmp 2700 thermocycler (Applied Biosystem, USA). Reaction mixture for the PCR contained 1x PCR buffer (Fermentas, USA), each dNTPs at a concentration of 200 µM, 1.5 mM MgCl₂, each primer at a concentration of 0.1 µM and 2.5 U of Taq DNA polymerase (Fermentas, USA) in a final volume of 50 µl. PCR conditions for amplification of 1.5 kb fragment of 16S rRNA were as follows: Preheating at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 0.30 min and 72 °C for 0.30 min and final extension 72 °C for 7 min. Amplified DNA was verified by electrophoresis of aliquots of PCR product (5µl) on a 1.2% agarose gel in 0.5 % TBE buffer.

3.1.10.5 Amplification of ITS region

Fragments of the ITS1-5.8S-ITS2 of the rRNA from genomic DNA were amplified by the PCR using the primers ITS1 5'-TCC GTA GGT GAA CCT GCG G-3' and ITS4 5'-TCC TCC GCT TAT TGA TAT GC-3' as described by White et al. (1990). The 50 µl reaction mixture for PCR amplification contained the following: 40 ng DNA, 1x PCR buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTPs, 0.5 µM of each primer and 2.5 units of Taq polymerase (Fermentas, USA). Amplifications were performed in GenAmp 2700 thermocycler (Applied Biosystem, USA) with an initial denaturation step of 94 °C for 5 minutes followed by 35 cycles of 94 °C for 1 minute, 50 °C for 1 minute and 72 °C for 1:30 minutes. Final extension at 72 °C for 8 minutes was performed to ensure completion of all reactions. Controls containing no DNA template were included in every of amplification to test for the presence of contamination of reagents and reaction buffer. Aliquots (5µl) of amplification products were electrophoresed in 1.2% agarose gel and visualized on a UV transilluminator.

3.1.10.6 Restriction analysis of DNA samples by agarose gel electrophoresis

1. The 10 µl DNA solution (0.2-1.0 µg of given DNA in a reaction volume of 20 µl) was taken in a sterile microfuge tube and added sterile MQ water to make up the final volume of 17 µl.
2. The 2 µl of appropriate 10x restriction enzyme assay buffer was added and mixed thoroughly by tapping the tube.
3. 1 µl (2-5 units) of the restriction enzyme was added, mixed by tapping the tube.
4. The mixture was incubated at the appropriate temperature for 2-3 h.
5. To stop the reaction, 4-5 µl gel-loading buffer was added, mixed by vortexing briefly (as the DNA samples need to be analyzed directly on agarose gel).

6. ITS-PCR products were digested with the restriction enzyme *RsaI* and 16S rRNA products were digested with the restriction enzymes *AluI*, *MboI*, *RsaI* and *TaqI* as per manufacturer's instructions.
7. The digested DNA was run through 1.5 % (w/v) agarose gels containing ethidium bromide and visualized and photographed using Gel Doc system.

3.1.10.7 Purification of PCR products

PCR products were purified by agarose gel (0.8%) electrophoresis prior to cloning. After staining with ethidium bromide, a defined band was visualized under UV irradiation and excised. Besides removing surplus primers, nucleotides, and salts, this method possessed the advantage that incomplete (shorter) amplification fragments are also removed prior to cloning. Subsequently, the DNA was excised from the gel matrix material, using the QIAquick gel extraction kit (Qiagen Inc., USA) as per manufacturer's instructions. Purified PCR products were eluted with 30 μ l TE buffer (pH 8.0) and used for the cloning

3.1.10.8 Ligation of 16S rRNA and ITS in TA cloning vector pTZ57R/T

The 16S rRNA and ITS PCR products were cloned using the restriction independent InsTA Cloning Kit, following the manufacturer's protocol (Fermentas, USA). The 16S rRNA or ITS amplicon was ligated into pTZ57R/T vector. The reaction mixture was prepared as described below and incubated overnight at 4 °C.

Plasmid pTZ57R/T (55 ng/ μ l)	3 μ l
Insert (75 ng/ μ l)	4 μ l
Buffer (5x)	6 μ l
T4 Ligase	1 μ l
MQ water	16 μ l

3.1.10.9 Genetic transformation using CaCl₂ method

1. A single colony of *E. coli* DH5 α was picked from a freshly grown plate and transferred into 20 ml of Luria broth in a 250 ml flask. The culture was incubated for 16-20 h at 37 °C with vigorous shaking (200-250 cycles/min in a rotary shaker).
2. Aseptically transferred 200 μ l of the above-saturated culture into 20 ml of fresh LB broth in a 250 ml flask. The culture was incubated with vigorous shaking at 37 °C for 2-3 h. To monitor the growth of the culture, determined the OD_{590 nm} every one-hour (The OD_{590 nm} should be ~ 0.5).
3. The above culture was transferred to sterile, disposable, ice-cold 50 ml polypropylene tubes. Cooled the cultures to 0 °C by storing the tubes on ice for 10 min.
4. The cells were recovered by centrifugation at 5000 rpm for 10 min at 4 °C. Decant the media from the cell pellet. The tubes were kept in an inverted position for 1 min to allow the last traces of media to drain away. Pellet was resuspended in 10 ml of ice-cold 0.1 M CaCl₂ and stored on ice for 10-15 minutes. Cells were recovered by centrifugation at 5000 rpm for 10 min at 4 °C. Decant the fluid from the cell pellet, the tube was kept in an inverted position for 1 min to allow the last traces of fluid to drain away.
5. Cell pellet was resuspended the in 1 ml of ice-cold 0.1 M CaCl₂. The cells in this stage may be stored on ice for 12-24 hours. CaCl₂ was mixed very gently with the pellet, do not vortex and placed in the ice for 10-15 min, centrifuged at 5000 rpm for 10 minutes, discard the supernatant.
6. One ml of ice chilled CaCl₂ was added to the pellet, mixed properly and gently. CaCl₂ treatment for 2½ hours induced considerably a transient state of competence in the *E. coli* cells.

7. Transferred 100 μ l of the suspension of competent cells to a sterile and prechilled microfuge tube (1.5 ml capacity). Plasmid DNA sample (~100 ng in a volume of 5 μ l or less) was added to each tube. (In control experiment, competent bacteria could receive no plasmid DNA at all.). The content of the tubes were mixed gently and stored in ice for 30 minutes.
8. Transferred the tubes to a floater and were placed in a water bath that was preheated to 42 °C. The tubes were left for exactly 2 min without shaking. Rapidly transferred the tubes to an ice bath and cells were chilled for 2 minutes.
9. Now added 1 ml of LB broth to each tube and incubated the cultures for 45 minutes in incubator shaker at 37 °C. This will allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.
10. Transferred the appropriate volume (100 μ l) of transformed competent cells onto LB supplemented ampicillin (50 μ g/ml) agar plates, which were spread with 20 μ l of 100 mM IPTG (isopropyl beta-D-thiogalactopyranoside) and 40 μ l of 20 mg/ml X-gal (5-bromo-4-chloro-3-indoyl β -D galactoside). Plates were incubated at 37 °C. Transformed colonies were appeared in 12-16 h.

3.1.10.10 Blue/white screening for recombinant plasmids

After transformation of the ligated product, the *E. coli* DH5 α (LacZ) bacterial host cells were plated on Luria agar medium containing 50 μ g/ml ampicillin, for selection of transformants. X-Gal and IPTG were used to screen for colonies containing a recombinant plasmid. The cloning site in the pTZ57R/T easy vector is located in the multiple cloning site (MCS) of the plasmid's lacZ α gene; if insert was present, non-functional β -galactosidase is produced, and the transformed bacterial colony is white. White colonies were picked and grown in 2 ml LB containing ampicillin (50 μ g/ml) and simultaneously patching of these cultures was done on

LA containing ampicillin. Plasmid was isolated (described in the proceeding section) and re-amplification of the insert was done using vector's promoter specific sequences.

3.1.10.11 Isolation and purification of plasmid DNA from recombinant bacteria by alkaline lysis method

1. A single transformed *E. coli* white colony was transferred into 2 ml of LB medium containing appropriate antibiotic (ampicillin used in a final concentration of 50 µg/ml) in a capped 15-ml tube. The culture was incubated overnight at 37 °C with vigorous shaking.
2. 1.5-2.0 ml of the above-saturated culture was transferred into a microfuge tube and cells were harvested by centrifugation at 8000 rpm for 1 min in a microfuge.
3. The medium was removed, leaving the bacterial pellet as dry as possible.
4. The bacterial pellet was resuspended in 200 µl of ice-cold Solution I (Appendix I) by vortexing to ensure that the bacterial pellet is completely dispersed in this solution. Placed as such at room temperature for 3 min.
5. Further 200 µl of freshly prepared solution II (Appendix I) was added. Tubes were closed tightly and mixed the contents by gently inverting the tubes ten to twenty times for 30 seconds. Store the tubes on ice for 3 minutes.
6. Finally 300 µl of ice cold Solution III (Appendix I) added and mixed by inversion to disperse Solution III through the viscous bacterial lysate. The tubes were stored on ice for 10-15 min and centrifuged at 12,000 rpm for 10 min at 4 °C in a microfuge. Carefully transferred the supernatant to a fresh tube.
7. Added 400 µl chilled phenol:chloroform:isoamylalcohol mixture (25:24:1) for protein denaturation and centrifuged it for 10 minutes at 10000 rpm.

8. Took the supernatant in another tube and DNA was precipitated by adding equal volume of isopropanol.
9. Mixed well and allowed the mixture to stand at room temperature for 5-10 min. Centrifuged at 10,000 rpm for 10 min at 4 °C in a microfuge.
10. Supernatant was removed. The tube was kept in an inverted position on a paper towel to allow all of the fluid to drain away. Any adhering drops of fluid on the walls of the tube were removed.
11. The pellet was washed with 70 % ethanol, centrifuged at 8000 rpm for 5 min.
12. Air-dried the pellet and dissolved in 30 µl of TE buffer (pH 8.0). The DNA was stored at -20 °C for further use.

3.1.10.12 Size screening for recombinant plasmids

Clones containing approximately 1.5 kb 16S rRNA or 600 bp ITS inserts were identified by PCR screening using the rapid protocol for preparation of template DNA from single bacterial colonies and PCR amplification was done by using 16S rRNA and ITS primers. The amplification products were checked by agarose gel (1.0 % w/v) electrophoresis.

3.1.10.13 Sequencing

The fragments of 16S rRNA and ITS1-5.8S-ITS2 amplified by PCR were sequenced according Sanger et al (1977) using an automated DNA sequencer (DNA Sequencing Facility, Department of Biochemistry, South Campus, Delhi University, New Delhi, India).

3.1.10.14 DNA sequence analysis

The bacterial 16S rRNA sequences were compared against the available DNA sequences in Ez Taxon-e database (<http://eztaxon-e-ezbiocloud.net>) (Kim et al. 2012). In case of bacteria, the phylogenetic tree (Neighbor Joining) was constructed using the MEGA 5.1 (Tamura et al.

2011) software and the bootstrap values were inferred from 1000 replicates. In fungi, Nucleotide sequence comparisons were performed using the BLAST database (Altschul et al. 1997) and phylogenetic tree (Maximum Parsimony analysis) was constructed using the MEGA 5.1.

3.2 Physiological characterization phosphate-solubilizing microorganisms

3.2.1 Effect of carbon and nitrogen source on solubilization of TCP

To study the effect of different carbon sources on the growth and phosphate solubilizing activity of bacterial as well as fungal isolates, glucose was replaced with an equal amount (10 g l⁻¹) of fructose or arabinose or galactose or mannitol or maltose or lactose or sucrose or xylose sterilized separately and added to the Pikovskaya's broth [C source, 10.0 g; (NH)₂SO₄, 0.5 g; NaCl, 0.2 g; MgSO₄.7H₂O, 0.1 g; KCl, 0.2 g; Yeast extract, 0.5 g; MnSO₄, 0.1 mg; FeSO₄.7H₂O, 0.1 mg; tri-calcium phosphate (TCP), 2.19 g (equivalent to 100 mg P₂O₅/100 ml); water, 1000 ml; pH 7.2 ± 0.2]. Nitrogen sources were evaluated similarly by replacing ammonium sulfate with 0.5 g l⁻¹; NaNO₃ or NH₄NO₃ or NaNO₂ or KNO₃ or tryptophan or NH₄Cl or Urea. The flasks were incubated at 30 °C under shaking conditions for fifteen days for bacteria and for seven days for fungi. The media was analyzed for soluble P and pH reduction. In case of fungi the mycelium was washed repeatedly with distilled water and dried at 70 °C for 48 h. The fungal growth was expressed as biomass produced per flask containing 100 ml of medium. Acid phosphatase, alkaline phosphatase, phytase enzyme activity and organic acid were also estimated in the culture supernatant of bacteria and fungi.

3.2.2 Plant growth promotion activities

3.2.2.1 Indole acetic acid production

All the selected bacterial and fungal isolates were screened for indole acetic acid (IAA) production. Gordon and Paleg (1957) colorimetric method was used for quantitative measurement of IAA.

Reagents

1. **0.5 M FeCl₃** : Dissolved 810 mg FeCl₃ in 10 ml distilled water
2. **35 % HClO₄**: 50 ml HClO₄ (70%) was mixed with 50 ml of distilled water
3. **Tryptophan**: 0.1 % w/v
4. **Salpar's reagent**: Mixed 1 ml of 0.5 M FeCl₃ with 50 ml of 35 % v/v HClO₄. This reagent should be freshly prepared.
5. **Czapek's Dox broth**

Components	g/l
NaNO ₃	3.0
KH ₂ PO ₄	1.0
MgSO ₄ .7H ₂ O	0.5
KCl	0.5
FeCl ₂	Trace
Sucrose	30.0
pH	7.3

Tryptophan medium was prepared by omission of sodium nitrate from the Czapek Dox broth and substitution of 0.1 % L. tryptophan as a sole source of nitrogen.

Procedure

1. 100 µl of the bacterial inoculum (O.D.₆₀₀ = 0.8) was inoculated in 20 ml nutrient broth, both with and without tryptophan (0.1%) and incubated at 30 °C for 5 days at 130 rpm.
2. 2 ml of the culture was taken and centrifuged at 10,000 rpm for 10 min.
3. In case of fungi 5 mm mycelia disc of each fungal isolate was cut aseptically and transferred to 50 ml of Czapek Dox broth contained in 250 ml conical flask. Flasks were incubated at 30 °C for 5 days on incubator shaker at 130 rpm.
4. Culture was centrifuged at 10000 rpm for 10 min and supernatant was filtered through whatman no. 1 filter paper.
5. 1 ml of supernatant of each isolate was taken in separate test tubes and 2 ml salper's reagent was added drop wise but rapidly with continuous mixing in each tube.
6. The samples were placed in dark for 30 minutes.
7. Development of pink color was assayed with spectrophotometer at 535 nm.
8. The un-inoculated medium was used as blank and amount of IAA production was estimated from the standard curve of IAA (0-50 ppm) drawn against O.D.

3.2.2.2 HCN production activity

HCN production was tested by the method of Bakker and Schippers (1987).

Procedure

1. Bacterial cultures were inoculated on Nutrient agar plates and fungal cultures were inoculated on Czapek Dox agar plates supplemented with 4.4 g/l Glycine.
2. A whatman filter paper soaked in 2 % w/v sodium carbonate in 0.5% (w/v) picric acid solution was plated inside the lid of a petriplates.
3. The plates were then sealed with parafilm and incubated at 30 °C for 3 days.

4. A change in the filter paper color from yellow to reddish brown was considered to be an indicator of HCN production.

3.2.2.3 Siderophore production

Siderophore production ability of isolates was detected by using Chrome-Azurol 'S' (CAS) Agar medium (Schwyn and Neilands 1987).

Reagents

1. Preparation of CAS Dye

- Dissolved 60.5 mg of Chrome-Azurol 'S' dye in 50 ml distilled water and constantly mix 1mM FeCl₃.6H₂O solution prepared in 10 ml 10 mM HCl. It results dark reddish color.
- Dissolved 72.9 mg of Cetyl trimethyl ammonium bromide into 40 ml of distilled water and mix slowly into CAS dye solution under constant stirring before autoclave. This results in dark blue color.

2. Preparation of CAS Agar Medium

- Mix 100 ml CAS dye in 300 ml sterile nutrient agar before pouring check pH, if below 7.0 maintain with NaOH (autoclaved).

Procedure

1. PSB isolates and fungal isolates were spot inculcated on CAS agar plates.
2. Incubated the plates at 28 °C for 3 days.
3. Isolates exhibiting an orange/yellow halo zone were considered as siderophore producers.
4. Their diameter of zone was measured.

3.2.2.4 Detection of type of siderophore (Neilands 1981)

Reagents

1. FeCl₂ (2 %)

2. CAS broth:

Components	g/l
Succinic acid	4.0
K ₂ HPO ₄	3.0
(NH ₄) ₂ SO ₄ .H ₂ O	0.2
CAS dye	100 ml
pH	7.0

Procedure

1. 100 µl of the bacterial culture (O.D._{600 nm} = 0.8) was inoculated in 20 ml CAS both and incubated at 30 °C for 5 days at 130 rpm.
2. In case of fungi 5 mm mycelia disc of each fungal isolate was cut aseptically and transferred to 20 ml CAS broth contained in 250 ml conical flask. Flasks were incubated at 30 °C for 5 days on incubator shaker at 130 rpm.
3. After five days of incubation culture was centrifuged at 10,000 rpm for 10 min.
4. I ml of culture supernatant was added to 3 ml freshly prepared 2 % aqueous FeCl₃ solution and absorbance between 200 – 600 nm was recorded.
5. A peak at 420-450 nm indicated the presence of ferrate hydroxamate siderophores and a peak at 495 nm indicated the catecholate nature of siderophores.

3.2.2.5 Nitrogen fixation ability

The ability of the bacteria to fix di-nitrogen was measured based on acetylene reduction activity as described in Hardy et al (1973).

Jensen broth (Jensen 1942)

Ingredients	gms/litre
Sucrose	20.00
Dipotassium phosphate	1.000
Magnesium sulphate	0.500
Sodium chloride	0.500
Ferrous sulphate	0.100
Sodium molybdate	0.005
Calcium carbonate	2.000
Distilled water	1000 ml

Procedure

1. The bacterial culture (100 μ l of O.D._{600 nm} = 1.0) was inoculated in 10 ml of Jensen broth in test tubes and incubated at 30 °C at 130 rpm on rotary shaker.
2. After three days of incubation, the cotton plugs were replaced with a rubber stopper.
3. One ml of air was removed from the tube with a syringe and one ml of acetylene (0.1 atm) was injected into the tubes which were then incubated for 24 hours.
4. One ml of the gas was sampled and the ethylene concentration was measured by gas chromatography.
5. Ethylene analysis was accomplished by gas chromatography (Nucon GC-FID, model no 5900) equipped with a hydrogen flame ionization detector (FID). Instrument

operating conditions were as follows: a stainless-steel column 150×0.2 cm packed with Porapak N, 80/100, a column temperature of 60 °C, an injector temperature of 50 °C, a detector temperature of 200 °C, N₂ carrier gas and H₂ at a flow rate of 25 ml/min, and air flow rate of 300 ml/min.

3.2.3 Effect of buffering of media on phosphate solubilization

To study the effect of buffering condition on P-solubilization by phosphate-solubilizing bacteria, bacterial isolates were grown in PKV broth supplemented with 100 mg P₂O₅ per 100 ml of medium. For the buffering the media 100 mM Tris-HCl of pH 7.0, 8.0 and 9.0 was used. In another set pH of this medium was adjusted to 7.0, 8.0 and 9.0 with 100 mM NaOH. The flasks were incubated at 30 °C under shaking conditions for fifteen days. The media was analyzed for soluble P and pH reduction. Acid phosphatase, alkaline phosphatase and phytase enzyme activities were also estimated in the culture supernatant.

3.3 Inoculum formulations for field applications with respect to phosphate solubilization efficiency and shelf life

3.3.1 Field experiment

To check the effect of selected isolates on crop production and soil fertility, field experiments of maize and wheat crop were conducted at three different agroclimatic regions of Punjab, India.

One experiment was conducted at Thapar University Patiala, Punjab. This site was situated at 30.30° N latitude and 76.38° E longitude. The site falls under central plain agroclimatic region of Punjab. Soil was low in organic carbon level and in available P level. The region possessed tropical hot and dry climatic conditions characterized by very hot and dry in summer and very cold in winter. The soil in experimental site belongs to typic Ustifluvents,

sandy loam in texture with the following characteristics: pH - 8.27, electric conductivity - 0.17 mScm^{-1} , organic carbon - 0.33%, available P - 3.90 mg kg^{-1} , total P - 251 mg kg^{-1} , organic P - 138 mg kg^{-1} and total nitrogen - 0.020 %.

Second site was an agricultural field of Balachaur, Punjab, situated at 31.07° N latitude and 76.32° E longitude. The region falls under sub-mountain undulating central agroclimatic region of Punjab state and possessed dry sub humid type of climate. Soil was low in organic carbon level and in available P level. The soil in experimental site belongs to typic Ustifluvents, sandy loam in texture with the following characteristics: pH - 8.18, electric conductivity - 0.14 mScm^{-1} , organic carbon - 0.31 %, available P - 3.6 mg kg^{-1} , total P - 237 mg kg^{-1} , organic P - 124 mg kg^{-1} and total nitrogen - 0.019 %.

Third site an organic field located at Pojewal, Punjab. Organic field used in this study was a field where from last ten years no chemical fertilizer was used. Mainly animal manure, vermi-compost and green manure were used to maintain the soil fertility. The site is situated at 31.65° N latitude and 76.26° E longitude and falls under sub-mountain undulating agroclimatic region of Punjab state. The region possessed dry sub humid type of climate. Soil was medium in organic carbon level and low in available P level. The soil in experimental site belongs to typic Ustorthents, loamy sand in texture with the following characteristics: pH - 8.37, electric conductivity - 0.18 mScm^{-1} , organic carbon - 0.42 %, available P - 4.3 mg kg^{-1} , total P - 245 mg kg^{-1} , organic P - 207 mg kg^{-1} and total nitrogen - 0.035 %. All three sites used in this study were comes under different agroclimatic regions and were different in physiochemical properties of soil like in comparison to soil fertility Balachaur come under less fertile soil in comparison to Patiala and Pojewal (organic farm). Bio inoculation was done as seed inoculation.

3.3.2 Seed inoculation

Seed inoculation of phosphate-solubilizing bacteria and fungi was done by mixing the bacterial culture and fungal spores in 10 percent sugar and 40 percent gum arabic to form slurry to which seeds were added. A uniform coat of inoculum was formed around the seeds. The inoculated seeds were dried in shade and used for sowing.

3.3.2.1 Methodology for preparation of inoculum

1. Phosphate-solubilizing bacterial culture was grown in 500 ml PKV broth and incubated at 30 °C for three days at 130 rpm.
2. Centrifuged the culture (O.D._{600 nm} 1.0) at 8000 rpm for 10 min and pellet was washed three time with sterile distilled water and finally suspended in sterile distilled water to make the suspension.
3. Fungal culture was grown in PKV agar plates for five days at 30 °C.
4. Using a scalpel fungal growth was scraped from the plates and suspended in sterile distilled water.
5. For fungal consortium (FC) the scraped spores of both the fungi which were of equal quantity, were mixed in sterile distilled water. To prepare bacterial consortium (BC) both the selected bacterial isolates were grown separately to a 1.0 OD_{600 nm}, centrifuged at 8000 rpm for 10 minutes and cell pellets were mixed in sterile distilled water.
6. 50 g sugar was taken in 500 ml water and heat the sugar solution for 15 min.
7. 200 g gum arabic was added to hot sugar solution and the solution was allowed to cool at room temperature.
8. Mix the fungal and bacterial inoculum separately in above solution to form a slurry.

9. For seed inoculation, surface sterilization of seeds was performed by dipping them in 95 % ethanol for 3 min followed by 3 % sodium hypochlorite for five min and subsequently washing with sterile distilled water followed by treatment with slurry.
10. Seeds were added to the inoculums slurry and mixed properly to make a uniform coat of inoculums on the seeds.
11. Surface sterilized seeds treated with 40 % gum arabic and 10 % sugar solution that did not contain inoculum served as a control.
12. Seeds were dried in shade for two hours and were sown in the field.
13. Check the seeds for inoculation density by serial dilution method on PKV agar plates.

3.3.3 Experimental field preparation and cultivation practices

Field trials were conducted at three different sites comes under different agroclimatic regions. Field trials were conducted in a completely randomized block design; each plot size was 4 m × 4 m (16 m²) and included 15 treatments each with three replicates.

Treatments consisted of:

Soil;

Soil + *Pantoea cypripedii* (PSB-3);

Soil + *Pseudomonas plecoglossicida* (PSB-5);

Soil + *Aspergillus tubingensis* (PSF-4);

Soil + *Aspergillus niger* (PSF-7);

Soil + BC (*Pantoea cypripedii* + *Pseudomonas plecoglossicida*);

Soil + FC (*Aspergillus tubingensis* + *Aspergillus niger*);

Soil + RP;

Soil + RP + *Pantoea cypripedii* (PSB-3);

Soil + RP + *Pseudomonas plecoglossicida* (PSB-5);

Soil + RP + *Aspergillus tubingensis* (PSF-4);

Soil + RP + *Aspergillus niger* (PSF-7);

Soil + RP + BC (*Pantoea cyripedii* + *Pseudomonas plecoglossicida*);

Soil + RP + FC (*Aspergillus tubingensis* + *Aspergillus niger*);

Soil + Di-ammonium phosphate (DAP) (chemical P fertilizer).

During first year of field study, maize variety DKC-9106 (20 kg/ha) was cultivated in the rainy season. Rock phosphate was amended in respective plots at the rate of 59 kg P₂O₅ ha⁻¹ before seeding only once during maize cropping. Di-ammonium phosphate (DAP) at a rate of 59 kg P₂O₅ ha⁻¹ was added in chemical fertilizer treatment at the time of sowing of each crop. Inoculum was added as a seed treatment. At the time of seeding, size of bacterial inoculum per maize seed was 2.5- 3.0×10⁵ cfu per seed and size of fungal inoculums per maize seed was 2.3-2.9 ×10⁵ cfu per seed. All the plots were irrigated once before the sowing to ensure proper germination of seeds and then regularly during crop growth as per agronomic practices. Nitrogen fertilizer of 272 kg/ha was used in the form of urea as per agronomic practices. No other chemical fertilizer was applied to the crop.

Wheat variety PBW-621 (99 kg/ha) was sown in the same field after one year to check the effect of inoculum on wheat crop in second year. The field was tilled without disturbing the experimental design. At the time of sowing a booster dose of bio-inoculant was added as seed treatment, size of the bacterial inoculum per wheat seed was 1.7-2.0×10⁵ cfu and size of fungal inoculums per seed was 1.5-2.0 cfu. Urea was added at the rate of 272 kg/ha as per agronomic practices. DAP was added at a rate of 59 kg P₂O₅/ha in chemical fertilizer treatment at the time of sowing and no rock phosphate was added in this crop to check the effects of previously added (in maize field) RP on wheat crop growth in second year of field study. In the both maize and wheat crops field trails in chemical fertilizer treatments where

DAP was used at the rate of 129 kg/ha (59 kg P₂O₅/ha) reduced the urea dose by 47 kg/ha because DAP already contained 18 % nitrogen (N).

3.3.4 Plant and soil analysis after harvesting

At the time of harvesting of both the crops, from each plot, 10 randomly selected plants were uprooted and the root surface was cleaned several times with distilled water. Growth parameters such as shoot height, shoot and root dry biomass were measured and recorded. During each crop harvest, entire observation plot (4 m × 4 m (16 m²)) of each treatment was harvested to record the grain yield. After harvesting root, shoot and seed samples of ten randomly selected plants of each plot were oven dried at 65 °C for 72 hours and were grounded to pass through a 0.5 mm sieve and analyzed for total P content. Total phosphorus content in plant roots, shoots and seeds were determined by vanado-molybdophosphoric yellow color method described by Kitson and Mellon (1944). After harvesting in order to assess the effect of different treatments on physiochemical properties of rhizospheric soil, soil samples to the depth of 5-10 cm were drawn carefully from rhizosphere of 10 randomly selected plants from each plot and homogeneous composite sample was prepared for each plot. Rhizospheric soil samples were stored at 4 °C and analyzed within week for soil enzyme activities and for phosphate solubilizing bacterial and fungal population. For physiochemical analysis soil samples were air dried under shade and then passed through 2.0 mm sieve and stored in labeled polythene bag before analysis. Soil samples of each plot were analyzed for its pH, electric conductivity, TDS, organic carbon (Walkley and Black 1934), available P (Olsen 1954), total phosphorus (Kitson and Mellon 1944), Total nitrogen (Piper 1966), acid and alkaline phosphatase activity (Tabatabai and Bremner 1969), phytase activity (Heinonen and Lahti 1981), dehydrogenase enzyme activity (Casida 1977) and phosphate-solubilizing bacterial and fungal population in respective plots by serial dilution method on Pikovskaya's agar plates.

3.3.5 Plant and soil analytical procedures

3.3.5.1 Determination of soil pH

Procedure

1. 25g of air dried soil samples were weighed and taken in a 100 ml beaker.
2. Added 50 ml of distilled water and thoroughly stirred for 2-3 min using a glass rod.
3. Further, it was kept in shaking condition (130 rpm) for 2 hours.
4. Suspension was allowed to settle down for 30 min.
5. Mean while, pH meter was switched on and checked with two buffer solutions of known pH (pH 9.0 and pH 4.0) with the help of standardization knob.
6. The pH of sample was measured by immersing the electrode in supernatant solution and recorded when the reading was stabilized (usually after 30 sec).
7. The electrode was rinsed with distilled water and carefully wiped with filter paper for every sample.

3.3.5.2 Determination of soil electrical conductivity (EC) and total dissolved salts (TDS)

The TDS and electric conductivity (EC) was determined in 1:2 (w/v) soil: water suspension using Deluxe Water and Soil Analysis Kit (Model 191 E).

Reagent

1. **KCl solution:** Dissolved 0.5232 g dry KCl in distilled water and make up the final volume 1 liter. This solution has an electrical conductivity of 1.0 mScm^{-1} at $25 \text{ }^\circ\text{C}$. (mScm^{-1} , which is equal to mmho cm^{-1} (1 mho = 1 Siemen). The TDS of this solution is 650 ppm.

Procedure

1. Weighed 25 g soil and transferred it to a 100 ml beaker.
2. Added 50 ml distilled water to it.
3. Shook intermittently with glass rod for one hour and allowed to stand. (Alternatively, the clear extract after pH determination can also be used for EC/TDS measurement).
4. In the meanwhile, switch on the EC/TDS meter and allowed it to warm for 20 minutes.
5. KCl solution ($EC=1\text{mS cm}^{-1}$) was used to calibrate the meter.
6. The electrode was dipped in the supernatant solution and recorded the reading displayed for EC and TDS.

3.3.5.3 Available phosphorus (P)

Available phosphorus in the alkaline soil was estimated as per the method given by Olsen et al. 1954.

Reagents

1. **0.5 M NaHCO₃ extracting solution:** 84 g of sodium bicarbonate was added in distilled water and volume was made up to 2 liter. The pH was adjusted to 8.5 with 1M or 1N NaOH.
2. **Reagent A:** 12.0 g ammonium molybdate in 250 ml distilled water and 0.2908 g antimony potassium tartarate in 100 ml distilled water was added to 1000 ml of 2.5 M H₂SO₄, mixed thoroughly and volume was made up to 2 liter with distilled water.
3. **Reagent B (freshly prepared):** 1.058 g of ascorbic acid was added in 200 ml of reagent A and mixed.
4. **Sulphuric acid (2.5 M):** 140 ml of conc. H₂SO₄ was diluted to 1 liter.

- 5. Stock standard P solution (50 ppm):** 0.2917 KH_2PO_4 was dissolved in distilled water to a final volume of 1 liter.
- 6. Working standard P solution (1 ppm):** 20 ml of 50 ppm solution was diluted to 1 liter.

Procedure

- 2.5 g soil was weighed and 50 ml of extracting solution was added to it.
- Kept on a shaker for 30 minutes and was filtered through whatman filter paper no. 42.
- 10 ml aliquot of filtrate was transferred to a 100 ml beaker.
- 1 ml of 2.5 M H_2SO_4 , 15.5 ml distilled water, 8 ml reagent B and again 15.5 ml of distilled water was added.
- After 10 minutes, the intensity of the color was measured at 882 nm against blank.
- Blank was prepared as above without the soil.
- To prepare standard curve, 0, 2, 5, 10, 15 and 20 ml of 1 ppm working standard solution was added in 50 ml volumetric flasks separately. Added 10 ml of extracting solution, 1.0 ml of 2.5 ml H_2SO_4 , 8 ml Reagent B and final volume was made up to 50 ml. The P concentrations of these solutions were 0.04 ppm, 0.1 ppm, 0.2 ppm, 0.3 ppm and 0.4 ppm respectively. After 10 min read the P concentration at 882 nm.

Calculation

Available P in soil (ppm): P in extract (ppm) \times 20 (standard soil to solution ratio)

3.3.5.4 Sample preparation for elemental analysis

For the release of mineral elements from soil and sediments, di acid (HNO_3 - HClO_4) wet oxidation of sample was carried out.

3.3.5.4.1 HNO₃/ HClO₄ digestion

1. 1 g sample of air dried soil was weighed in digestion tube and added 10 ml concentrated HNO₃ digest on electric heater for 1hr at 145 °C in acid proof digestion chamber having fume exhaust system.
2. Allowed to cool it and 10 ml concentrated HNO₃ and 5 ml HClO₄ was added and heated at about 100 °C for the first one hour and then raised the temperature to about 200 °C.
3. Continued the digestion until the contents become colorless and only white fumes appeared.
4. Reduced the acid contents till white matter remains left in the digestion tube.
5. After it removed from the heating mental and cooled and added 50 % diluted HCl and filtered through whatman filter paper no. 42.
6. 2 or 3 washings with 50 % diluted HCl was given and final volume made was 50 ml with diluted 50 % HCl.
7. This was used to determine total phosphorus.

3.3.5.4.2 Total phosphorus in soil and plant samples (Kitson and Mellon 1944)

(Vanadomolybdophosphoric Yellow color Method)

Ammonium molybdate reacts under acidic conditions to form a heteropoly acid and molybdophosphoric acid. In presence of vanadium, yellow vanadomolybdophosphoric acid is formed. The intensity of the yellow color is proportional to phosphate concentration.

Reagents

1. Vanadomolybdate solution:

Solution A - 25 g ammonium molybdate [(NH₄)₆ Mo₇O₂₄.4H₂O] was dissolved in 300 ml water in a 500 ml beaker.

Solution B - Dissolved 1.25 g ammonium (meta) vanadate (NH_4VO_3) in 300 ml boiling water. Cooled, added 250 ml concentrated HNO_3 and cooled again. Solution A was added to solution B and was made up to 1000 ml in a volumetric flask.

- 2. Phosphorus stock standard solution (50 mg/l P):** Dissolved 0.2195 g of dried KH_2PO_4 in distilled water and mixed thoroughly. Acidified with 25 ml of 7 N H_2SO_4 and made the volume up to 1 liter to get 50 mg/ml P solution. 4 to 5 drops of toluene were added to prevent microbial activity. (KH_2PO_4 was dried to 100 °C for 1 hour and cooled in desiccators before weighing)

Procedure

1. 10 ml of acid digests of soil or plant sample was placed in 50 ml volumetric flask, 10 ml of the vanado molybdate reagent was added and diluted to 50 ml.
2. Mixed well and read the phosphorus concentration after 10 minutes using spectrophotometer at 420 nm.
3. Took 0, 1, 2, 3, 4 and 5 ml of 50 mg per liter stock phosphorus solution in 50 ml volumetric flasks and developed the color as mentioned above.
4. Calibrated the spectrophotometer with known phosphorus concentration and read the concentration of the sample.

Calculation

$$P \text{ (mg/kg)} = \frac{\text{vol. make up after digestion (ml)}}{\text{weight of sample (g)}} \times \frac{50}{\text{vol. of the digest used to develop color (ml)}} \times P \text{ (mg/l)}$$

3.3.5.5 Organic carbon

The total organic carbon in (%) was estimated as per the method given by Walkley and Black 1934.

Reagents

1. **1 N potassium dichromate:** Dissolved 49.04 g $K_2Cr_2O_7$ in distilled water and volume was made up to 1 liter.
2. **0.5 N ferrous ammonium sulphate:** Dissolved 198 g ferrous ammonium sulphate in distilled water and volume was made up to 1 liter.
3. **Di phenyl amine indicator:** 0.5 g of di phenyl amine indicator (DPA) was dissolved in a mixture of 20 ml water and 100 ml concentrated H_2SO_4 .
4. **Concentrated Sulphuric acid**
5. **Orthophosphoric acid (85 %)**
6. **Sodium fluoride**

Procedure

1. 1 g of soil was taken in 500 ml conical flask and 10 ml of 1 N $K_2Cr_2O_7$ was added.
2. The flask was swirled for mixing the soil and reagent.
3. 20 ml of concentrated H_2SO_4 was added and the flask was allowed to stand undisturbed for 30 minutes after which 200 ml of distilled water was added.
4. 1 ml of diphenylamine indicator was then added.
5. Ultimately the contents were titrated with freshly prepared 0.5 N ferrous ammonium sulphate till the end point is observed from blue violet to green.
6. A blank was run without soil samples.

Calculation

$$\text{Organic carbon (\%)} = \frac{10(B - T) \times 0.003 \times 100}{B \times \text{weight of soil (g)}}$$

Where,

B is volume of ferrous ammonium sulphate solution required for blank titration

T is volume of ferrous ammonium sulphate solution required for soil sample

Because organic matter contains 58 % carbon, so

Organic matter (%): Organic carbon (%) \times 1.724 (van Bemmelen factor)

3.3.5.6 Total nitrogen

Total nitrogen was estimated as per the Kjeldahl method given by Piper (1960).

Reagents

1. **Concentrated H₂SO₄**
2. **0.02 N H₂SO₄**
3. **Sulphuric salicylic acid:** 1g salicylic acid was mixed with 30 ml sulphuric acid.
4. **Sodium thiosulphate**
5. **4% boric acid:** 4 g of boric acid was dissolved in 100 ml of distilled water.
6. **Mixed indicator:** 0.066 g of methyl red and 0.099 g of bromo cresol green was dissolved in 100 ml of ethyl alcohol.
7. **50 % NaOH**
8. **Digestion mixture:** 10 g HgO , 5 g CuSO₄ and 100 g K₂SO₄ (2:1:20).

Procedure

1. 5 g soil was mixed thoroughly with sulphuric salicylic acid and followed by 5 g of sodium thiosulphate. Heating was carried out for 5 minutes followed by cooling and addition of 10 g of digestion mixture. The contents were mixed well in a kjeldahl flask.
2. The flask was kept in a digestion chamber at 100°C for two hours.
3. The color change was monitored from dark brown to greenish white after that the contents were cooled and 300 ml distilled water was added.
4. 20 ml of the digested sample, 15-20 ml NaOH and glass beads were added to the distillation flasks through the open end of the condenser attachment and stoppered. Water flow was maintained through the condenser.
5. The distillate was collected through a receiver tube in a beaker containing 15 ml boric acid and 2 drops of mixed indicator was added till the end point color changes from pink to green.
6. The distillate was titrated against 0.02 N H₂SO₄ until the color changed from green to pink.

Calculation

$$\text{Total N (\%)}: \frac{(T - B) \times \text{normality of } H_2SO_4 \times 1.4 \times 300}{\text{Weight of sample(g)}}$$

T is titer value for sample, B is for blank

3.3.5.7 Enzyme activities of soil

3.3.5.7.1 Acid and phosphatase activity in soil (Tabatabai and Bremner 1969)

Reagents

1. 5X modified universal buffer (MUB) (Skujins et al. 1962)

Tris (hydroxyl methyl) amino methane	12.10 g
Maleic acid	11.60 g
Citric acid	14.00 g
Boric acid	6.28 g
1 N NaOH	488 ml

Final volume was made up to 1000 ml with distilled water and stored at 4 °C.

Before use adjust the pH of 200 ml of solution with 0.5 N HCl to pH 5.5 for the assay of acid phosphatase or with 0.5 N NaOH to pH 9.0 for the alkaline phosphatase. Adjust the volume of the pH modified buffer to 1 liter with distilled water.

- 2. p-nitrophenyl phosphate solution (0.115 M):** Dissolve 4.268 gm disodium p-nitrophenyl phosphate hexahydrate in 100 ml of appropriate pH adjusted, diluted MUB (pH 5.5) for acid phosphatase and (pH 9.0) for alkaline phosphatase. Store the p-nitrophenyl phosphate substrate at 4 °C for no longer than 10 days.
- 3. NaOH (0.5N):** 20 g of NaOH was dissolved in distilled water and the volume was made up to 1 litre.
- 4. p-nitrophenol:** 1 mg per ml solution in modified universal buffer (pH 5.5) for acid phosphatase and (pH 9.0) for alkaline phosphatase.

Procedure

1. Weighed 1 g of soil sample (air dry) and transferred to flask.
2. Added 4.0 ml of the diluted MUB (pH 5.5 for acid phosphatase and pH 9.0 for alkaline phosphatase).
3. 1 ml of filter sterilized 0.115M p- nitrophenyl phosphate solution was added to the flask.
4. The flasks were swirled for few seconds to mix the contents.
5. The flasks were stoppered and incubated at 37 °C for 1hour in dark.
6. 4 ml of 0.5M NaOH was added to stop the reaction.
7. Mixture was swirled and filtered through whatman filter paper no.2.
8. Transferred the filtrate to glass cuvette and measured the yellow color at 410 nm.
9. Phosphatase activity was indicated as the amount of p-nitrophenol released in the filtrate from the p-nitrophenyl phosphate substrate per gram of soil. The p-nitrophenol content was calculated with reference to a calibration graph plotted from the results obtained by standard containing 0, 10, 20, 30, 40, 50 µg of p-nitrophenol.
10. To perform control, followed the procedure described for the assay but made the addition of 1 ml of p-nitrophenyl phosphate after the addition of 0.5 N NaOH (i.e. immediately before filtration).

3.3.5.7.2 Phytase activity (Heinonen and Lahti 1981)

Reagents

1. **Substrate solution:** 2.5 mM dodecasodium phytate in 0.2 M sodium acetate buffer of pH 5.5.
2. **Color stop solution:** (10 mM ammonium molybdate : 5 N sulphuric acid: acetone, 1:1:2 ratio).

3. **0.5 M NaHCO₃ extracting solution:** 84 g of sodium bicarbonate was added in distilled water and volume was made up to 2 liter. The pH was adjusted to 8.5 with 1M or 1N NaOH.
4. **1 M citric acid**
5. **Standard KH₂PO₄ solution:** mg/ml stock.

Procedure

A. Extraction

1. Weighed 5 g of soil sample into a 250 ml shaking bottle and 10 ml of sodium bicarbonate (0.5 M) extractant was added.
2. Shook for 30 minutes and centrifuged for 5 min at 12000 rpm.
3. Took supernatant in aliquots of 0.2 ml and determined the phytase activity by the method given by (Heinonen and Lahti 1981).

B. Determination

1. 0.5 ml aliquots of enzyme sample was taken (here soil extract).
2. 0.5 ml substrate solution (2.5 mM dodecasodium phytate in 0.2 M sodium acetate buffer of pH 5.5) was added and incubated for 10 min at 37 °C.
3. The reaction was stopped by addition of 2 ml ice cold color stop solution (10 mM ammonium molybdate : 5 N sulphuric acid: acetone, 1:1:2 ratio).
4. Added 100 µl 1 M citric acid.
5. O.D. was taken at 380 nm.
6. In the case of blank substrate solution was added after adding the coloring reagent.
7. Calculated the phytase activity per g of soil sample. Enzyme activity was expressed as micromoles of inorganic P released per hour per milliliter of culture filtrate from sodium phytate at 37 °C.

3.3.5.7.3 Soil dehydrogenase activity (Cassida 1977)

Reagents

1. **1, 3, 5 triphenyl tetrazolium formazan stock solution (1mg/ml):** 1 mg of tetraphenyl tetrazolium formazan (TPF) was dissolved in 1 ml of methanol.
2. **1, 3, 5 triphenyl tetrazolium formazan working solution (100 µg/ml):** added 2 ml of stock solution and volume was made up to 20 ml with methanol.
3. **2, 3, 5 triphenyl tetrazolium chloride (3 %):** 3 g of triphenyl tetrazolium chloride was dissolved in 100 ml of distilled water.
4. **0.1 % and 0.2 % yeast extract:** 0.1 g and 0.2 g of yeast extract was dissolved in 100 ml distilled water respectively.

Procedure

1. 10 g of soil sample was weighed and calcium carbonate was mixed in the ratio of 100:1.
2. 3 g of each sample was dispensed in screw cap glass vials and 0.5 ml sterile water was added followed by 1 hour incubation at 28 °C.
3. 0.25 ml of single strength substrate solution was added (0.1 % yeast extract) followed by 0.25 ml distilled water. The vials were incubated for 8 hours at 28 °C.
4. 0.5 ml of 3 % aqueous TTC (2, 3, 5 triphenyl tetrazolium chloride) and 0.25 ml double strength (0.2 %) yeast extract was added and mixed thoroughly with sterile glass rod.
5. This was followed by 6 hours incubation at 37 °C followed by immediate extraction with 25 ml methanol and subsequent filtration through whatman no. 1 filter paper.
6. The methanol extract containing red coloured formazan was read at 480 nm.
7. In 100 ml volumetric flasks 2, 5, 10, 15 and 20 ml of working standard of 100 µg ml⁻¹ 1, 3, 5 triphenyl tetrazolium formazan was added (diluted from stock solution of 1mg/ml TPF) and final volume was adjusted with methanol. The absorbance was read at 480 nm.

3.3.5.8 Population density of phosphate-solubilizing bacteria and fungi

At the end of each field trail, rhizospheric soil samples were tested for phosphate-solubilizing bacterial and fungal population in each respective plot. The rhizosphere soil adhere to the roots of harvested plants was separated by gentle tapping and stored in sterilized Petri plates at 4 °C. One gram soil of each replicate soil samples were serially diluted and plated on Pikovskaya's agar plates. The plates were incubated at 30 °C and, bacterial and fungal colonies showing a clear zone of phosphate solubilization were counted at the end of incubation.

3.3.6 Formulation development of phosphate-solubilizing bacteria and fungi

To develop the inoculum formulations rock phosphate, fly ash, charcoal and vermiculite were used as carrier materials in the present study. Rock phosphate used in this study was obtained from Rajasthan state mines and minerals limited, Udaipur, India. The chemical constituents of the RP were: 31.5% P₂O₅, 45.4% CaO, 3.4% MgO, 4.3% aluminium, 3.1% fluoride, 0.044% organic carbon and 0.003% available P, pH-7.04. Fly ash used in the present study was procured from Orissa, India. The chemical constituents of fly ash were: 0.58 % organic carbon, 2.30 mg kg⁻¹ P, 53.8% SiO₂, 31.3% Al₂O₃, 6.4% CaO, 0.52% MgO, 5.8% Fe₂O₃, 0.13% MnO, 0.96% TiO₂, 0.12% Na₂O, 0.21% K₂O, pH-7.24. Vermiculite used in present study was procured from Trade Link Engineering Services, Sirhind Road Patiala. The chemical constituents of vermiculite were: 14.39% MgO, 43.48% Al₂O₃, 12.82 % FeO, 11.92% SiO₂, H₂O 17.87%, 0.57% organic carbon, pH-8.78. Charcoal was procured from E. Merck (India) Limited, Mumbai. The chemical constituents of charcoal were: 0.2% chloride, 0.2% sulfate, 0.1% iron, 32% CO₂, 1% Al₂O₃, 17% SiO₂, 0.1% CaO, 50% O, pH-3.69. Phosphate-solubilizing bacterial culture were grown in PKV broth and incubated at 30 °C for five days at 130 rpm. Centrifuged the culture (O.D.₆₀₀ 1.0) at 8000 rpm for 10 min and pellet

was washed three time with sterile distilled water and made a suspension in sterile distilled water. Fungal cultures were grown in PKV agar plates for five days at 30 °C. Using a scalpel fungal growth was scraped from the plates and suspended in sterile distilled water. 100 g of each carrier material was transferred in poly-propylene bags and sterilized at 121°C for three hours on three alternate days. After cooling the contents to the bags were inoculated separately with 20 ml suspension of five day cultures of bacteria and fungi. The final moisture content in each bag was adjusted to 30 % with sterile distilled water. The content of the bags were mixed properly by shaking the bags and were sealed. The packets were incubated at 4 °C and 37 °C. Both the phosphate-solubilizing bacteria and fungi were enumerated periodically on 0th, 15th and 30th day and after that on regular interval of 30 days by serial dilution method on Pikovskaya's agar plates. Quality of the final product was determined by check the viable count of each formulation on PKV agar plates by serial dilution method, inoculum formulations showed maximum viable count after 270 days of incubation were tested for P-solubilization, acid phosphatase, alkaline phosphatase, phytase enzyme activity and plant growth promotion activities and compared with fresh culture.

3.3.7 Analysis of maize for aflatoxins

Monitoring grain for the presence of aflatoxins is important to ensuring consumer safety. Maize samples from the plots inoculated with P-solubilizing fungi were analyzed for B1, B2, G1 and G2 aflatoxins. Extraction method was based on AOAC-990.33 (2005).

1. 50 g of maize sample was mixed with 200 ml methanol and 50 ml 0.1N HCl and homogenized. After homogenization 125 ml the sample was filtered through filter paper in separatory funnel.

2. 50 ml of 10 % NaCl and 50 ml of hexane was added to the filtrate and shaken for 1 min. After layer separation lower layer was collected in another separatory funnel (upper layer was discarded).
3. 50 ml di-chloromethane (DCM) was added and shaken for 1 minute. After separation lower layer of DCM was collected in a beaker. The extraction was repeated for two more times with 25 ml DCM and all the extracts were collected. The extracts were filtered through sodium sulphate to remove water. The filtered extract was evaporated to 2 ml on rotavapour.
4. Slurry of 2 gm of silica gel was made with 10 ml of ether-hexane (3+1) and transferred to a cleanup column.
5. After silica gel was settled column was washed with 5 ml ether-hexane (3+1). Stopcock was opened and 1g of sodium sulphate was added on top.
6. The DCM extract was added on top. 25 ml of benzene-acetic acid (9+1) was added to column with open stopcock. 30 ml of ether-hexane (3+1) was added. Drained each wash on top of sodium sulphate. The washes were discarded.
7. Aflatoxin was eluted with 100 ml of DCM-acetone (90+10). Elute was evaporated to 5 ml on rotavapour then elute was transferred to test tube and was evaporated to dryness on water bath in presence of nitrogen stream.
8. 200 μ l of hexane was added to dried extract followed by 50 μ l of trifluoroacetic acid (TFA). Mixed on vortex for 30 seconds. After 5 minutes 0.950 ml of water-acetonitrile (9+1). Mixed on vortex for 30 seconds and kept for 10 minutes.
9. After the layers were separated the lower layer was kept for HPLC.
10. HPLC was used for separating these potentially carcinogenic aflatoxins using Zorbax-ODS (Agilent). The mobile phase consisted of solvent mixture of deionized water, methanol, and acetonitrile at a flow rate of 0.8 ml min⁻¹.

11. The concentration of aflatoxins B1, B2, G1 and G2 were calculated by comparing the peaks obtained in case of reference standards.

3.4 Statistical analysis

Three replicates were used for each experiment. In field experiments, plots with different treatments were arranged in a randomized complete block design with three replicates per treatment. The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey's test at $p < 0.05$. All the analysis was performed by using Graph Pad Prism 5.0 software.

Results

Chapter 4

Isolation and identification of phosphate-solubilizing microorganisms

4.1 Isolation and identification of phosphate-solubilizing bacteria

4.1.1 Isolation of phosphate-solubilizing bacteria

To isolate the phosphate-solubilizing bacteria, soil samples were collected from different agricultural soil niches. These niches were rhizosphere of bamboo plants (*Bambusa vulgaris*) (multipurpose agroforestry crop) grown in experimental field of CORE, Thapar university, Patiala; surface soil of potato (*Solanum tuberosum*) (tuberous crop) field of CORE, Thapar University, Patiala; surface soil of CORE (no vegetation, open area); rhizosphere soil of mustard (oil-seed crop) (*Brassica campestris*); rhizosphere of mature maize (cereal crop) (*Zea mays*) plants, rhizosphere soil of small maize plants, surface soil of berseem crop (fodder crop) (*Trifolium alexandrinum*) field; rhizosphere of sunflower (oil-seed crop) (*Helianthus annuus*) grown in agriculture field of Balachaur, Punjab; and rhizosphere of *Stevia rebaudiana* (medicinal plant) grown in organic field of Pojewal, Punjab, India (Table 4.1).

These different niches had three types of fields one was conventional agriculture field in which all the chemical fertilizers were used by farmers for the crop production and soil fertility as per agronomic practices, these conventional agriculture fields were, bamboo plants (*Bambusa vulgaris*) (multipurpose agroforestry crop) experimental field of CORE, Thapar university, Patiala; potato (*Solanum tuberosum*) (tuberous crop) field of CORE, Thapar University, Patiala; mustard (*Brassica campestris*) crop field (oil-seed crop), mature maize (cereal crop) (*Zea mays*) crop field, small maize plants crop field, berseem (*Trifolium alexandrinum*) crop field (fodder crop); sunflower crop (*Helianthus annuus*) (oil-seed crop) agriculture field of Balachaur, Punjab. Second type of crop field was organic farm of *Stevia rebaudiana* (medicinal plant). Organic field used in this study was a field where from last ten years no chemical fertilizer was used. Mainly animal manure, vermi-compost and green

manure were used to maintain the soil fertility. And third one was the surface soil of CORE, Thapar University, Patiala that is open land area, without any vegetation.

Use of Pikovskaya's medium for isolation of phosphate-solubilizing bacteria (PSB) was a simple way to detect PSB through the formation of halo (zone) on agar plates containing tri-calcium phosphate as a sole P source. Glucose was used as the preferred organic source for the isolation of PSB from the rhizosphere.

From these various soil niches a total of 1270 bacteria were isolated on Pikovskaya's agar plates by serial dilution method at 10^{-5} dilution (Table 4.1). Out of these 1270 bacterial isolates, only 169 bacterial isolates were observed to be formed a halo (zone) surrounding the colonies that were the 13 % of total bacterial population isolated (Table 4.1). Out of these 13 % phosphate-solubilizing bacterial population, maximum phosphate-solubilizing bacterial population was came from rhizosphere soil of *Stevia rebaudiana* grown in organic farm (25 %) followed by surface soil of potato (*Solanum tuberosum*) field (22 %), rhizosphere soil of sunflower (*Helianthus annuus*) (13 %), small maize (*Zea mays*) rhizosphere soil (12 %), mustard (*Brassica campestris*) rhizosphere soil (10 %), mature maize (*Zea mays*) rhizosphere (9 %), rhizosphere soil of bamboo (*Bambusa vulgaris*) (9 %), surface soil of barseem crop field (*Trifolium alexandrinum*) (8 %) and surface soil of CORE field does not contain any phosphate-solubilizing bacterial population (Table 4.1).

4.1.2 Qualitative screening of phosphate-solubilizing bacteria

Qualitative screening of phosphate-solubilizing bacterial isolates revealed variations in phosphate solubilization efficiency of selected isolates. Table 4.2 shows the selection of efficient phosphate-solubilizing bacterial isolates on qualitative basis. In total of 169 phosphate-solubilizing bacterial isolates from different niches, 32 isolates (two from rhizosphere soil of bamboo, three from surface soil of potato field, five from mustard soil,

three from mature maize, four from small maize soil, three from soil of sunflower and 12 from rhizosphere soil of *Stevia rebaudiana* grown in organic farm) were found to form more than 5 mm zone of solubilization on Pikovskaya's agar plates (Table 4.3) and thus were selected as efficient phosphate-solubilizing bacteria. The data in Table 4.3 indicates the values of diameter of the colony + halo zone (Z), diameter of colony (C) and the ratio Z/C of the different isolates obtained on PKV agar plates showed more than 5 mm zone of solubilization. The use of the ratio Z/C helps to evaluate the activity of a given microorganism. The phosphate solubilization activity of these selected isolates on PKV agar plates was ranged from 1.36 to 3.17 (Table 4.3). The phosphate solubilization activity of selected phosphate-solubilizing bacterial isolates is shown in Plate 4.1.

4.1.3 Test of purity and stability

A direct devise was worked out to test the efficiency of these strains in solubilizing sparingly soluble phosphate. Point inoculation was done on the PKV agar plates containing Bromo Creasol Green as an indicator Change in color of medium from blue to yellow was observed surrounding the phosphate-solubilizing bacterial colonies Plate 4.2). The change in color of the zone surrounding the colonies into yellow clearly indicates the drop of pH at that place, and that acidification of the medium seems to be directly associated to the process of phosphate solubilization. All the tested isolates were able to show halo zone on Bromo Creasol Green PKV agar plates (Plate 4.2).

Table 4.1 Population density (P.D.) of phosphate-solubilizing bacteria in different crop fields.

Serial No.	Soil samples of different crop fields	pH of soil	P.D. of bacteria $\times 10^5$ /g of soil	P.D. of PSB $\times 10^5$ /g of soil	% age of PSB
1.	Rhizospheric soil of bamboo (<i>Bambusa vulgaris</i>)	7.71 \pm 0.02	91	8	9 %
2.	Surface soil of potato (<i>Solanum tuberosum</i>) field	7.93 \pm 0.04	60	13	22 %
3.	Surface soil of CORE field	7.81 \pm 0.03	52	0	0 %
4.	Mustard (<i>Brassica campestris</i>) rhizospheric soil	7.82 \pm 0.03	192	19	10 %
5.	Mature maize (<i>Zea mays</i>) rhizosphere	8.09 \pm 0.03	159	15	9 %
6.	Small maize (<i>Zea mays</i>) rhizosphere	8.32 \pm 0.03	173	20	12 %
7.	Surface soil of berseem (<i>Trifolium alexandrinum</i>) field	8.18 \pm 0.03	132	10	8 %
8.	Sunflower (<i>Helianthus annuus</i>) rhizosphere	8.29 \pm 0.02	163	22	13 %
9.	Rhizosphere soil of <i>Stevia rebaudiana</i> grown in organic farm	8.37 \pm 0.02	248	62	25 %

Pronounced results are represented in bold.

Table 4.2 Qualitative screening of phosphate-solubilizing bacteria

Serial No.	Soil samples of different crop fields	Total No. of P-solubilizing Bacteria	No. of less efficient bacteria (> 5 mm zone of solubilization)	No. of efficient bacteria (<5 mm zone of solubilization)
1.	Rhizospheric soil of bamboo (<i>Bambusa vulgaris</i>)	8	6	2
2.	Surface soil of potato (<i>Solanum tuberosum</i>) field	13	10	3
3.	Surface soil of CORE field	0	0	0
4.	Mustard (<i>Brassica campestris</i>) rhizospheric soil	19	14	5
5.	Mature maize (<i>Zea mays</i>) rhizosphere	15	12	3
6.	Small maize (<i>Zea mays</i>) rhizosphere	20	16	4
7.	Surface soil of berseem (<i>Trifolium alexandrinum</i>) field	10	10	0
8.	Sunflower (<i>Helianthus annuus</i>) rhizosphere	22	19	3
9.	Rhizosphere soil of <i>Stevia rebaudiana</i> grown in organic farm	62	50	12

Pronounced results are represented in bold.

Table 4.3 Phosphate solubilization index of selected bacterial isolates.

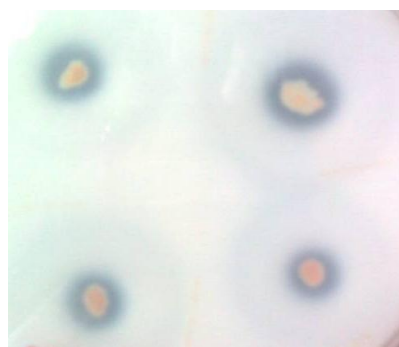
Isolates	Dia. of (colony + halo zone) (Z)	Dia. of colony (C)	Dia. of halo zone (H)	Solubilization Activity (Z/C)
Rhizospheric soil of bamboo (<i>Bambusa vulgaris</i>)				
PSB-B6	9	4	5	2.25
PSB-B8	13	8	5	1.63
Surface soil of potato (<i>Solanum tuberosum</i>) field				
PSB-P3	19	13	6	1.46
PSB-P7	17	10	7	1.70
PSB-P9	14	9	5	1.56
Mustard (<i>Brassica campestris</i>) rhizospheric soil				
PSB-S1	13	6	7	2.17
PSB-S2	12	7	5	1.71
PSB-S8	17	11	6	1.55
PSB-S14	15	9	6	1.67
PSB-S15	13	8	5	1.63
Mature maize (<i>Zea mays</i>) rhizosphere				
PSB-MM10	16	11	5	1.45
PSB-MM11	15	10	5	1.50
PSB-MM16	13	7	6	1.86
Small maize (<i>Zea mays</i>) rhizosphere				
PSB-SM4	15	10	5	1.50
PSB-SM5	14	9	5	1.56
PSB-SM9	12	6	6	2.00
PSB-SM10	15	10	5	1.50
Sunflower (<i>Helianthus annuus</i>) rhizosphere				
PSB-SF12	10	5	5	2.00
PSB-SF15	19	14	5	1.36
PSB-SF16	20	14	6	1.43
Rhizosphere soil of <i>Stevia rebaudiana</i> grown in organic farm				
PSB-1	12	7	5	1.71
PSB-3	17	12	5	1.42

PSB-4	16	11	5	1.45
PSB-5	21	9	12	2.30
PSB-6	20	13	13	1.54
PSB-7	20	14	6	1.43
PSB-8	15	9	6	1.67
PSB-9	13	7	6	1.86
PSB-10	20	8	12	2.50
PSB-11	22	10	12	2.20
PSB-12	19	6	11	3.17
PSB-13	23	9	14	2.56

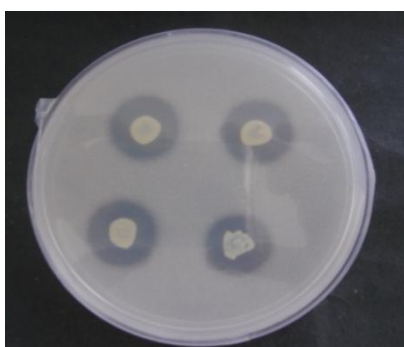
Pronounced results are represented in bold.



a)



b)



c)

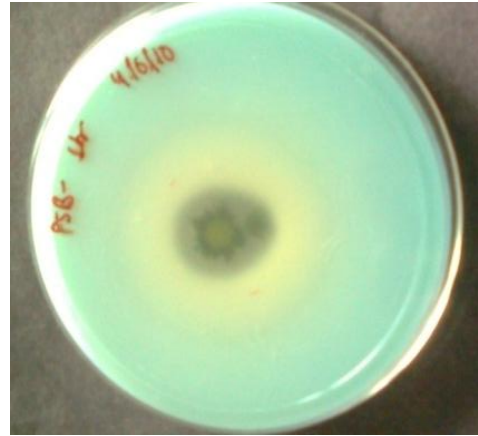


d)

Plate 4.1 a) Control (Pikovskaya's agar plate). b), c), and d) Point inoculation of isolated P-solubilizing bacteria, showing zone of solubilization on Pikovskaya's agar plates.



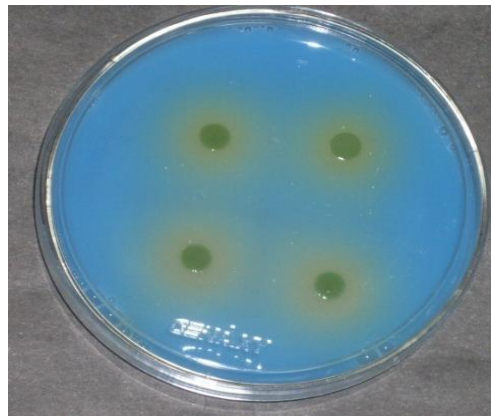
a)



b)



c)



d)

Plate 4.2 a) Control (Pikovskaya's agar plate with Bromo Cresol Green). b) - d) (Point inoculation of isolated P-solubilizing bacteria to check the stability on PKV medium containing BCG as an indicator.

4.1.4 Quantitative estimation of bacterial solubilization of tri-calcium phosphate (TCP)

Phosphate-solubilizing bacteria selected on the basis of qualitative screening were further tested for tri-calcium phosphate solubilization in PKV broth medium. For this, selected bacterial isolates were grown in PKV broth supplemented with tri-calcium phosphate (equivalent to 100 mg P_2O_5 100 ml⁻¹) and P solubilization was estimated at regular time intervals of two days from third day onward. Results represented in Table 4.4 showed that P solubilization was increased up to day five of incubation and maximum solubilization was observed on day five of incubation in most of the isolates, after day five it started decreased. With increase in P-solubilization, pH of the medium was decreased from its initial value of 7.2 to 3.61 (Table 4.4). Out of these 32 selected bacterial isolates, six bacterial isolates showed maximum P-solubilization in PKV broth supplemented with tri-calcium phosphate. These six isolates were designated as PSB-3, PSB-5, PSB-6, PSB-7, PSB-12 and PSB-13. These isolates showed P-solubilization in range of 401 to 429 $\mu\text{g ml}^{-1}$, respectively (Table 4.4).

These selected bacterial isolates were also tested for their acid phosphatase enzyme, alkaline phosphatase enzyme, phytase enzyme and organic acid production in PKV broth supplemented with tri-calcium phosphate (equivalent to 100 mg P_2O_5 100 ml⁻¹). Results represented in Fig. 4.1 showed that all the selected isolates were able to produce acid phosphatase, alkaline phosphatase and phytase enzymes in TCP supplemented PKV broth. All selected isolates were exudated organic acids in cultural medium (Table 4.5). The main organic acids exudated by these isolates were oxalic acid, citric acid, acetic acid and gluconic acid. Exudation of gluconic acid was found to be higher than that of other acids (Table 4.5). These selected bacterial isolates were further tested for rock phosphate solubilization.

Table 4.4 Release of soluble P and pH reduction at different time intervals (days) by selected bacterial isolates in Pikovskaya's broth supplemented with tri-calcium phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Isolates	3 rd day		5 th day		7 th day		9 th day	
	pH	Soluble 'P' (µg ml ⁻¹)	pH	soluble 'P' (µg ml ⁻¹)	pH	Soluble 'P' (µg ml ⁻¹)	pH	Soluble 'P' (µg ml ⁻¹)
PSB-B6	5.13 ± 0.10h	173 ± 3klm	5.19 ± 0.10cdefg	163 ± 3lm	5.69 ± 0.04bcde	119 ± 2mnop	5.93 ± 0.05bc	106 ± 7jkl
PSB-B8	5.54 ± 0.06df	62 ± 6r	5.50 ± 0.04bc	73 ± 7q	5.52 ± 0.06bcdefg	114 ± 9nopq	5.68 ± 0.03bcde	93 ± 9l
PSB-P3	5.87 ± 0.05b	139 ± 4o	5.51 ± 0.05bc	221 ± 12gh	4.67 ± 0.60jkl	345 ± 11d	5.06 ± 0.61fghi	323 ± 7b
PSB-P7	5.59 ± 0.02cde	129 ± 3op	5.57 ± 0.09bc	180 ± 4ijkl	4.51 ± 0.03klm	296 ± 4e	4.73 ± 0.10ghij	267 ± 6d
PSB-P9	5.45 ± 0.06ef	182 ± 3klm	5.55 ± 0.04bc	144 ± 11mno	5.79 ± 0.05abcd	132 ± 11lmno	5.92 ± 0.03bc	110 ± 3jkl
PSB-S1	4.81 ± 0.06i	212 ± 4j	4.73 ± 0.57g	194 ± 12ij	4.29 ± 0.03lmn	333 ± 13d	4.59 ± 0.04ijk	309 ± 4b
PSB-S2	4.60 ± 0.03j	392 ± 4a	5.13 ± 0.59cdefg	290 ± 4f	5.19 ± 0.07efghij	286 ± 6e	5.25 ± 0.04defg	258 ± 3d
PSB-S8	5.63 ± 0.02cde	117 ± 3p	5.52 ± 0.06bc	121 ± 3op	5.43 ± 0.07bcdefghi	122 ± 4mno	5.63 ± 0.07bcde	111 ± 5ijk
PSB-S14	5.36 ± 0.06fg	159 ± 7n	5.29 ± 0.02bcdef	166 ± 11klm	5.64 ± 0.07bcdef	140 ± 3klm	5.78 ± 0.06bcd	110 ± 5jkl
PSB-S15	5.10 ± 0.06h	238 ± 4h	5.24 ± 0.03cdef	146 ± 7mn	5.24 ± 0.05cdefghij	131 ± 12lmno	5.63 ± 0.05bcde	108 ± 5jkl
PSB-MM10	5.19 ± 0.08gh	190 ± 4klm	5.35 ± 0.13bcd	182 ± 7ijkl	5.39 ± 0.02bcdefghi	109 ± 3opq	5.40 ± 0.04cdef	97 ± 8kl
PSB-MM11	5.10 ± 0.04h	208 ± 7ij	5.31 ± 0.04bcde	188 ± 3ijk	5.51 ± 0.07bcdefgh	161 ± 7ijk	5.85 ± 0.03bc	130 ± 6h
PSB-MM16	5.77 ± 0.13bc	192 ± 3jk	5.79 ± 0.07b	171 ± 15jkl	5.82 ± 0.03abc	169 ± 8hij	5.85 ± 0.06bc	119 ± 8hij
PSB-SM4	5.09 ± 0.04h	177 ± 6klm	5.36 ± 0.16bc	163 ± 6lm	5.46 ± 0.06bcdefghi	138 ± 3klmn	5.66 ± 0.05bcde	112 ± 3ijk
PSB-SM5	4.56 ± 0.04j	307 ± 9f	4.82 ± 0.04efg	291 ± 9f	5.74 ± 0.10bcde	90 ± 12qr	5.97 ± 0.12b	56 ± 4m

PSB-SM9	5.76 ± 0.03bcd	165 ± 3mn	5.29 ± 0.03bcdef	328 ± 3e	5.81 ± 0.05abc	261 ± 3f	5.92 ± 0.06bc	234 ± 4f
PSB-SM10	6.30 ± 0.11a	73 ± 3r	6.38 ± 0.11a	70 ± 9q	6.36 ± 0.17a	67 ± 4r	6.59 ± 0.07a	65 ± 4m
PSB-SF12	4.8 ± 0.05i	270 ± 6g	4.85 ± 0.02defg	226 ± 6g	4.92 ± 0.04hijk	148 ± 6jkl	5.05 ± 0.06fghi	128 ± 5hi
PSB-SF15	5.16 ± 0.03h	222 ± 8hi	4.79 ± 0.03fg	285 ± 3f	5.07 ± 0.06fghijk	190 ± 7h	5.18 ± 0.06efgh	149 ± 4g
PSB-SF16	5.11 ± 0.06h	171 ± 16lmn	5.15 ± 0.04cdefg	139 ± 4no	5.20 ± 0.08defghij	110 ± 2opq	5.43 ± 0.06bcdef	99 ± 4kl
PSB-1	4.53 ± 0.05j	307 ± 8f	4.12 ± 0.05hi	365 ± 11d	4.62 ± 0.04ghijk	353 ± 6cd	4.65 ± 0.07hijk	322 ± 3b
PSB-3	3.92 ± 0.06k	369 ± 5bcd	3.89 ± 0.04hi	422 ± 4ab	3.98 ± 0.03mn	406 ± 6a	4.16 ± 0.16kl	366 ± 6a
PSB-4	5.21 ± 0.01gh	324 ± 6ef	5.38 ± 0.07bc	296 ± 11f	5.56 ± 0.07bcdef	252 ± 9f	5.88 ± 0.04bc	228 ± 4f
PSB-5	3.72 ± 0.06l	372 ± 2bc	3.61 ± 0.06i	429 ± 4a	3.76 ± 0.13n	385 ± 7ab	3.96 ± 0.12l	236 ± 4ef
PSB-6	4.45 ± 0.06j	387 ± 3ab	4.18 ± 0.06h	401 ± 5bc	4.67 ± 0.04jkl	384 ± 10ab	4.82 ± 0.06ghi	323 ± 5b
PSB-7	4.86 ± 0.03i	366 ± 2cd	4.21 ± 0.06h	410 ± 11abc	4.91 ± 0.03ijk	349 ± 7d	5.05 ± 0.10fghi	290 ± 8c
PSB-8	4.88 ± 0.04i	378 ± 4abc	4.72 ± 0.07g	398 ± 6c	4.73 ± 0.54jkl	374 ± 9bc	5.19 ± 0.04efgh	288 ± 7c
PSB-9	5.57 ± 0.04de	211 ± 9ij	5.13 ± 0.03cdefg	330 ± 9e	5.36 ± 0.10bcdefghi	220 ± 10g	5.55 ± 0.04bcdef	165 ± 6g
PSB-10	5.86 ± 0.07b	97 ± 6q	5.79 ± 0.05bc	113 ± 3p	5.86 ± 0.05ab	96 ± 3pq	5.93 ± 0.08bc	54 ± 7m
PSB-11	5.46 ± 0.07ef	189 ± 2kl	5.39 ± 0.08bc	199 ± 2hi	5.67 ± 0.04bcdef	176 ± 5hi	5.84 ± 0.13bc	111 ± 4ijk
PSB-12	3.96 ± 0.08k	350 ± 5d	3.94 ± 0.01hi	415 ± 6abc	4.16 ± 0.12lmn	335 ± 6d	4.21 ± 0.05ijk	253 ± 5de
PSB-13	4.04 ± 0.06k	330 ± 5e	3.97 ± 0.03hi	413 ± 3abc	4.07 ± 0.05mn	330 ± 15d	4.25 ± 0.07jkl	268 ± 5d
LSD (<i>P</i><0.05)	.096	9.78	0.258	12.08	0.300	12.35	0.274	8.66

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

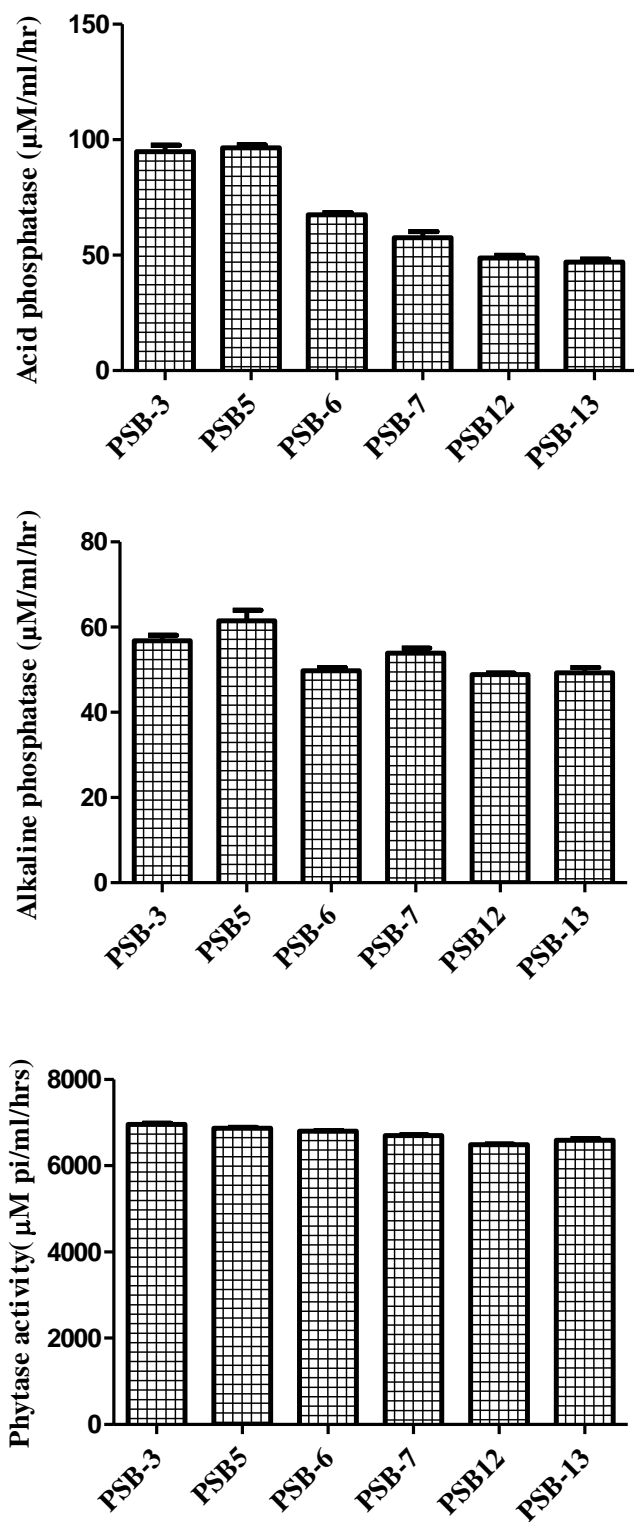


Fig. 4.1 Enzyme activities by selected bacterial isolates in Pikovskaya's broth supplemented with tri-calcium phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Table 4.5 Organic acid production by bacterial isolates in Pikovskaya's broth amended with tri-calcium phosphate (equivalent to 100 mg P₂O₅100 ml⁻¹).

Isolates	Oxalic acid (n mol/ml)	Citric acid (n mol/ml)	Malic acid (n mol/ml)	Succinic acid (n mol/ml)	Formic acid (n mol/ml)	Acetic acid (n mol/ml)	Gluconic acid (n mol/ml)
PSB-3	113 ± 14b	0	0	0	0	4986 ± 78b	4968 ± 26c
PSB-5	60 ± 16c	703 ± 10b	0	100 ± 3	0	82 ± 1c	13780 ± 483a
PSB-6	159 ± 16a	77 ± 1c	0	0	0	18667 ± 242a	0
PSB-7	106 ± 15b	0	0	0	303 ± 5	18072 ± 986a	0
PSB-12	57 ± 15c	52 ± 3cd	0	0	0	0	9282 ± 241b
PSB-13	105 ± 15b	3076 ± 47a	0	0	0	485 ± 16c	0
LSD (<i>P</i> <0.05)	27	39				739	393

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

4.1.5 Solubilization of rock phosphate by bacterial isolates

The selected six bacterial isolates namely PSB-3, PSB-5, PSB-6, PSB-7, PSB-12 and PSB-13 were further tested for solubilization of rock phosphate supplemented in (equivalent to 100 mg P₂O₅ 100 ml⁻¹) in PKV broth. The maximum amount of soluble P was recorded on day five of incubation and later decreased (Table 4.6). It was observed that with increase in solubilization, pH of the medium decreased from its initial value 7.2 to 3.65. P solubilization in all the isolates was in range of 231 to 271 µg ml⁻¹. The reduction in pH was observed corresponds to the respective increase in soluble P concentration (Table 4.6). Maximum P solubilization was observed in PSB-3 isolate that solubilized 253 µg P ml⁻¹ and in PSB-5 that solubilize 271 µg P ml⁻¹. P solubilization was accompanied by a decrease in pH of the culture filtrate up to 3.65 in both cases from the initial pH of 7.2. All the isolates were able to produce significant amounts of acid phosphatase, alkaline phosphatase and phytase enzymes in the RP-amended PKV broth (Fig. 4.2). Results in Table 4.7 showed that all isolates were produced organic acid in RP amended PKV broth. The main organic acids exudated by these isolates in RP amended PKV broth were oxalic acid and gluconic acid. Exudation of gluconic acid was higher than that of other organic acids.

Table 4.6 Release of soluble P and pH reduction at different time intervals (days) by selected bacterial isolates in Pikovskaya's broth supplemented with rock phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Isolates	3 rd day		5 th day		7 th day		9 th day	
	pH	Soluble 'P' (µg ml ⁻¹)	pH	soluble 'P' (µg ml ⁻¹)	pH	Soluble 'P' (µg ml ⁻¹)	pH	Soluble 'P' (µg ml ⁻¹)
PSB-3	3.79 ± 0.03d	172 ± 10a	3.65 ± 0.19a	253 ± 7b	3.80 ± 0.08c	231 ± 3ab	4.15 ± 0.04c	199 ± 8ab
PSB-5	4.14 ± 0.06a	135 ± 7bc	3.70 ± 0.05a	271 ± 6a	3.79 ± 0.03c	240 ± 4a	4.04 ± 0.07c	209 ± 7a
PSB-6	3.90 ± 0.03cd	162 ± 14ab	3.83 ± 0.10a	235 ± 4cd	3.83 ± 0.07c	190 ± 6d	4.42 ± 0.04b	158 ± 3d
PSB-7	3.87 ± 0.04d	153 ± 6abc	3.69 ± 0.13a	231 ± 6d	4.27 ± 0.04a	198 ± 12cd	4.50 ± 0.08ab	151 ± 6d
PSB-12	3.99 ± 0.05bc	132 ± 4c	3.86 ± 0.07a	243 ± 4bcd	4.10 ± 0.06b	218 ± 9bc	4.54 ± 0.04ab	188 ± 7ab
PSB-13	4.08 ± 0.03ab	180 ± 15a	3.79 ± 0.06a	245 ± 4bc	4.15 ± 0.04ab	224 ± 7ab	4.61 ± 0.09a	180 ± 6c
LSD	0.074	18	0.195	9.38	0.097	13.07	0.111	11.17

(*P*<0.05)

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

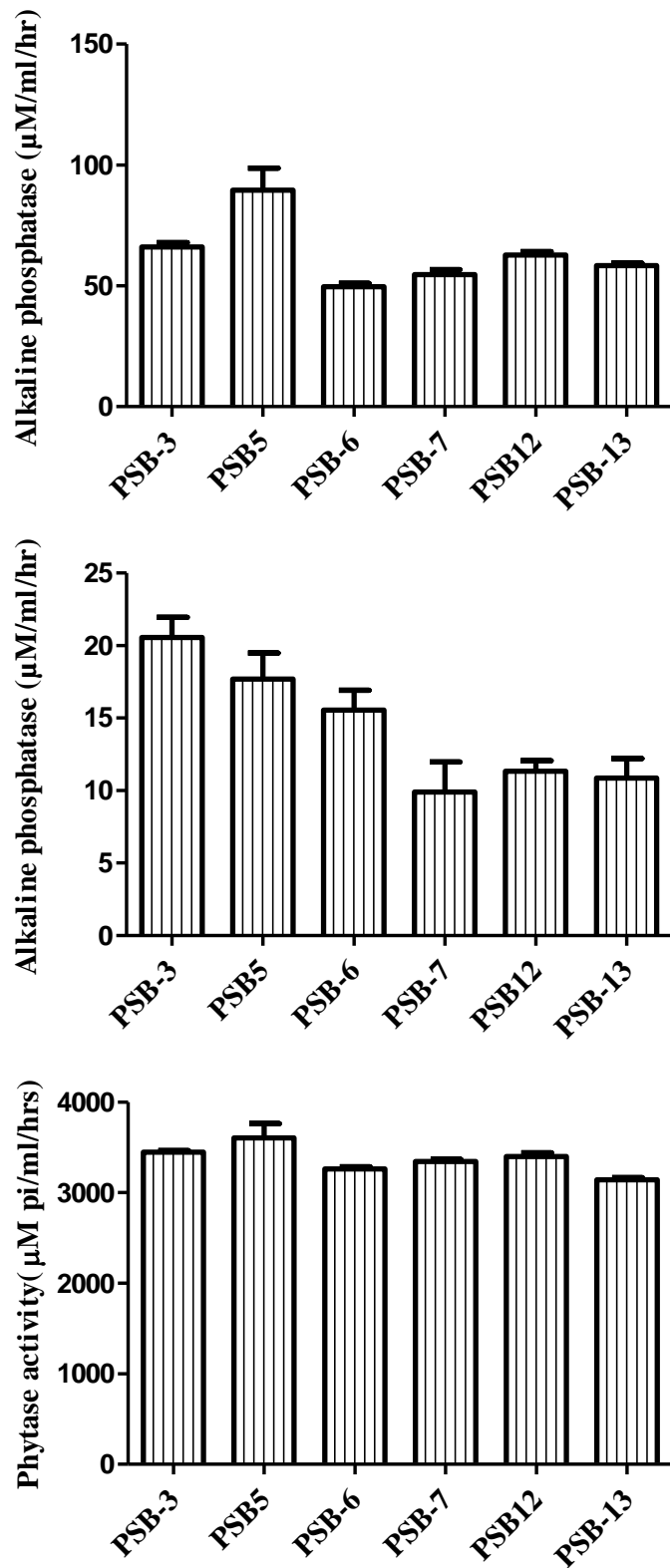


Fig. 4.2 Enzyme activities by selected bacterial isolates in Pikovskaya's broth supplemented with rock phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Table 4.7 Organic acid production by bacterial isolates in Pikovskaya's broth amended with rock phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Isolates	Oxalic acid (n mol/ml)	Citric acid (n mol/ml)	Malic acid (n mol/ml)	Succinic acid (n mol/ml)	Formic acid (n mol/ml)	Acetic acid (n mol/ml)	Gluconic acid (n mol/ml)
PSB-3	33 ± 0.9d	0	0	0	0	0	4222 ± 53a
PSB-5	84 ± 1.3b	0	0	61 ± 0.4	0	0	3816 ± 49b
PSB-6	4 ± 2.6e	0	169 ± 1	0	0	0	2746 ± 26c
PSB-7	38 ± 1.0c	0	0	0	0	0	536 ± 5d
PSB-12	100 ± 2.3a	13 ± 0.2	0	0	0	0	199 ± 7e
PSB-13	83 ± 1.0b	0	0	0	0	0	18 ± 1f
LSD (<i>P</i> <0.05)	2.81						56

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

4.1.6 Biochemical characterization of phosphate-solubilizing bacteria

The bacterial isolates namely PSB-3, PSB-5, PSB-6, PSB-7, PSB-12 and PSB-13 that showed maximum P solubilization in tri-calcium phosphate and rock phosphate supplemented PKV broth were further selected for biochemical characterization (Table 4.8). All the isolates were rod shaped gram negative bacteria and were motile in nature. All the isolates were found to be positive for catalase test, citrate test, nitrate reduction test, starch utilization test and for glucose fermentation test. Results in the Table 4.8 showed that all the bacterial isolates were showed negative results for indole production, methyl red test, urease test, cellulose utilization test and for lactose sugar fermentation and gas production.

Two bacterial isolates PSB-3 and PSB-6 were found to be negative and four bacterial isolates PSB-5, PSB-6, PSB-12 and PSB-13 were showed positive results for oxidase test. Two bacterial isolates PSB-3 and PSB-6 were positive and four bacterial isolates PSB-5, PSB-6, PSB-12 and PSB-13 were negative for Voges-Proskauer test, fructose and sucrose sugar fermentation and gas production and glucose suger gas production during fermentation.

Antibiotic profile of all the selected bacterial isolates is shown in Table 4.8. Result showed that all the bacterial isolates have positive antibiotic profile against Penicillin, Cephalothin, Clindamycin, Erythromycin, Amoxyclav, Vancomycin, Ofloxacin, Teicoplanin, Ceftazidime, Gentamicin and Cephoxithin. Bacterial isolate PSB-3 and PSB-6 were positive and PSB-5, PSB-6, PSB-12 and PSB-13 have negative antibiotic profile for Oxacillin.

Table 4.8 Biochemical characterization of bacterial isolates.

Biochemical reactions	PSB-3	PSB-5	PSB-6	PSB-7	PSB-12	PSB-13
Gram staining	–	–	–	–	–	–
Morphology	Rod	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	+
Oxidase	–	+	–	+	+	+
Catalase	+	+	+	+	+	+
IMViC test						
Indole production	–	–	–	–	–	–
Methyl red	–	–	–	–	–	–
Voges-Proskauer	+	–	+	–	–	–
Citrate (Simmons)	+	+	+	+	+	+
Urease	–	–	–	–	–	–
Nitrate reduction	+	+	+	+	+	+
Starch utilization	+	+	+	+	+	+
Cellulose utilization	–	–	–	–	–	–
Sugar fermentation						
Glucose	+	+	+	+	+	+
Fructose	+	–	+	–	–	–
Sucrose	+	–	+	–	–	–
Lactose	–	–	–	–	–	–
Gas production during sugar fermentation						
Glucose	+	–	+	–	–	–
Fructose	+	–	+	–	–	–
Sucrose	+	–	+	–	–	–
Lactose	–	–	–	–	–	–
Antibiotic profile						
Penicillin G (10 U) , Cephalothin (30 mcg), Clindamycin (2 mcg), Erythromycin (15 mcg), Amoxyclav (30 mcg), Vancomycin (30 mcg), Ofloxacin (5mcg), Teicoplanin (30 mcg), Ceftazidime (30 mcg), Gentamicin (10 mcg), Cephoxithin (30 mcg), Oxacillin (1 mcg)	+	+	+	+	+	+
	+	–	+	–	–	–

+: Positive; –: Negative

4.1.7 Identification of selected bacterial isolates

All the selected bacterial isolates were subjected to 16S rRNA amplification and about 1.5 kb amplicon was observed in all the isolates (Fig. 4.3). RFLP analysis was performed for 16S rRNA amplified product of all these isolates by using the restriction enzymes *MboI*, *AluI*, *TaqI* and *RsaI*. Results of RFLP with *MboI* (Fig. 4.4) showed that isolates PSB-3 (lane 3), PSB-5 (lane 4), PSB-6 (lane 5) and PSB-7 (lane 6) showed similar type of banding pattern and isolates PSB-12 (Lane7) and isolate PSB-13 (Lane 8) showed entirely different type of restriction pattern. Restriction digestion with *AluI* (Fig. 4.5) showed that isolates PSB-3 (lane 3 and 4), PSB-5 (lane 5), PSB-6 (lane 6) and PSB-7 (lane 7) showed similar type of banding pattern and isolates PSB-12 (Lane 8) and isolate PSB-13 (Lane 9) showed entirely different type of restriction pattern. Similarly restriction digestion with *TaqI* (Fig. 4.6) and *RsaI* (Fig 4.7) also showed that isolates PSB-3 (lane 4), PSB-5 (lane 5), PSB-6 (lane 6) and PSB-7 (lane 7) showed similar type of banding pattern and isolates PSB-12 (Lane 8) and isolate PSB-13 (Lane 9) showed entirely different type of restriction pattern.

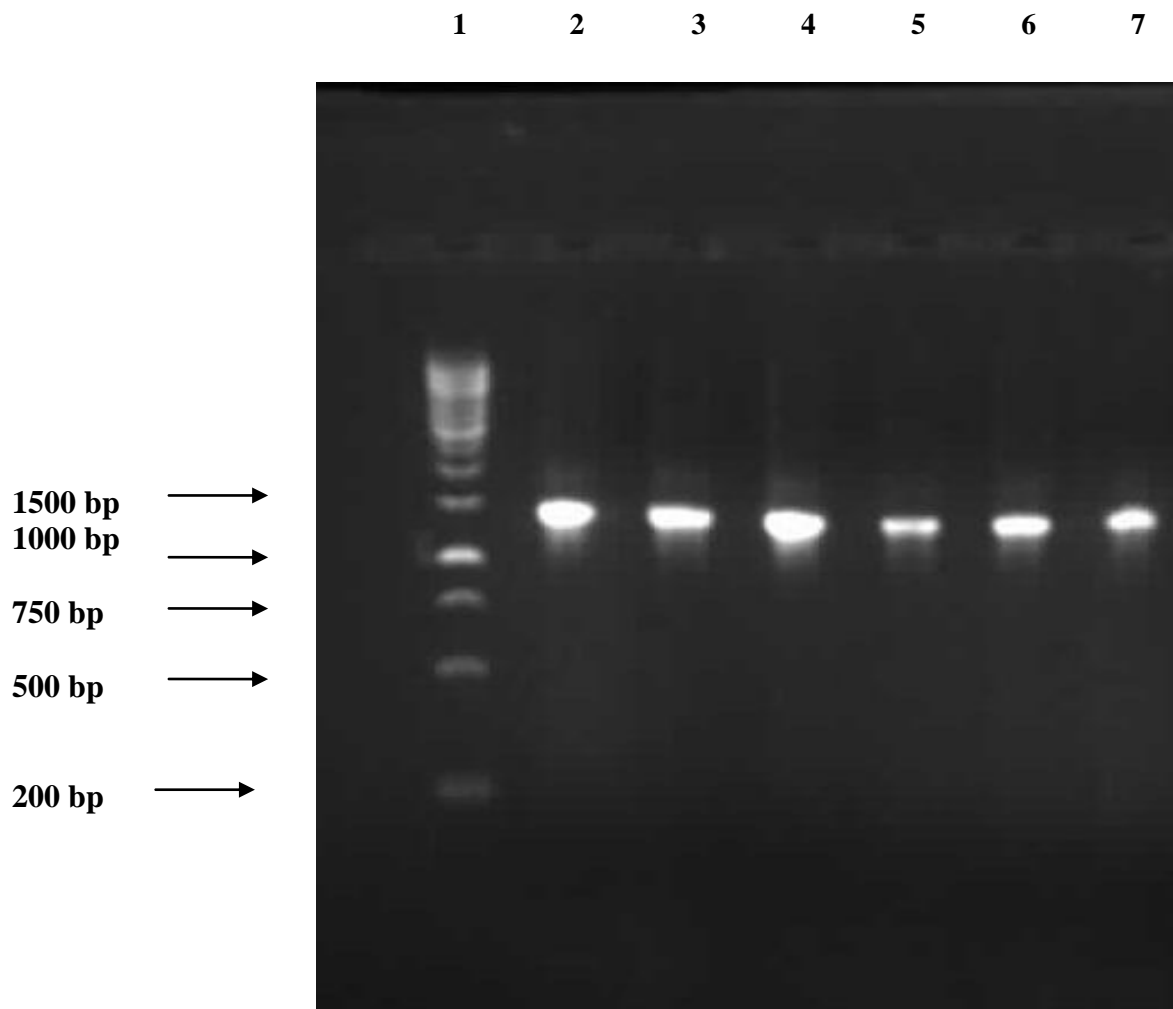


Fig. 4.3 16S rDNA amplified PCR products of isolates: Lane 1: 1 Kb ladder, Lane 2: PSB-3, Lane 3: PSB-5, Lane 4: PSB-6, Lane 5: PSB-7, Lane 6: PSB-12, Lane 7: PSB-13.

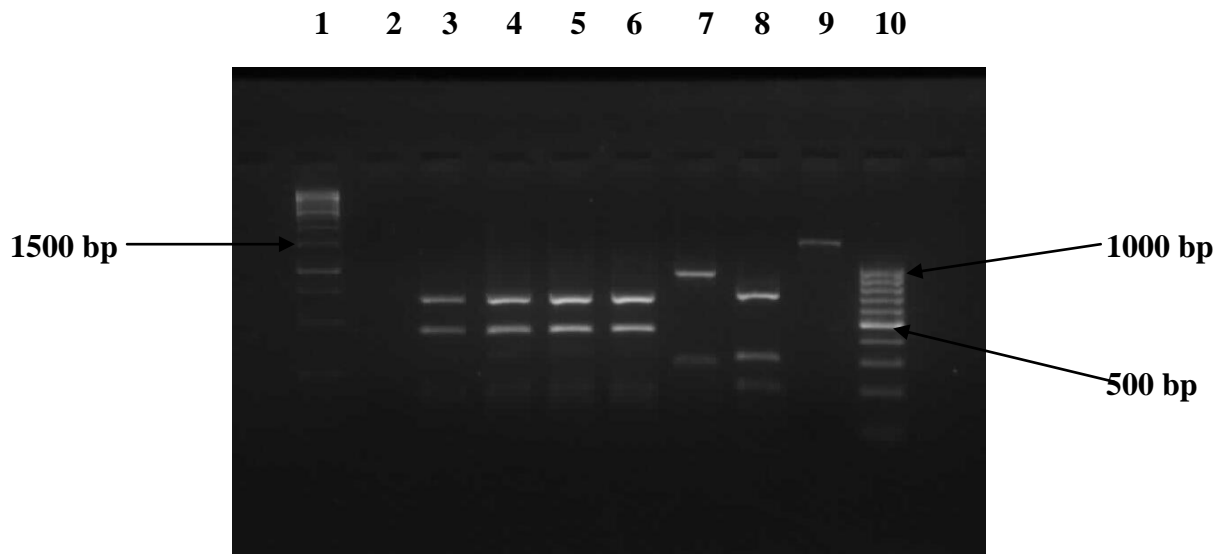


Fig. 4.4 Restriction digestion of 16S rRNA amplified PCR product with *MboI*. Lane 1: 1 Kb ladder, Lane 3: PSB-1, Lane 4: PSB-5, Lane 5: PSB-6, Lane 6: PSB-7, Lane 7: PSB-12, Lane 8: PSB-13, Lane 9: control (16S rRNA amplified PCR product without digestion), Lane 10: 100 bp ladder.

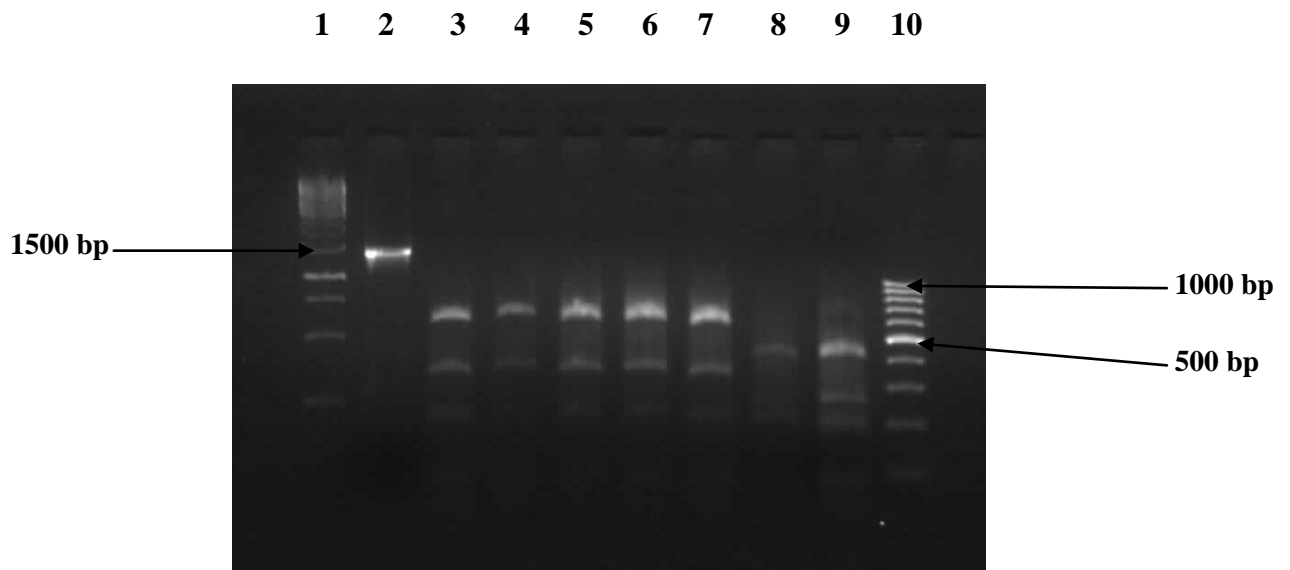


Fig. 4.5 Restriction digestion of 16S rRNA amplified PCR product with *AluI*. Lane 1: 1 Kb ladder, Lane 2: control (16S rRNA amplified PCR product without digestion), Lane 3-4: PSB-1, Lane 5: PSB-5, Lane 6: PSB-6, Lane 7: PSB-7, Lane 8: PSB-12, Lane 9: PSB-13, Lane 10: 100 bp ladder.

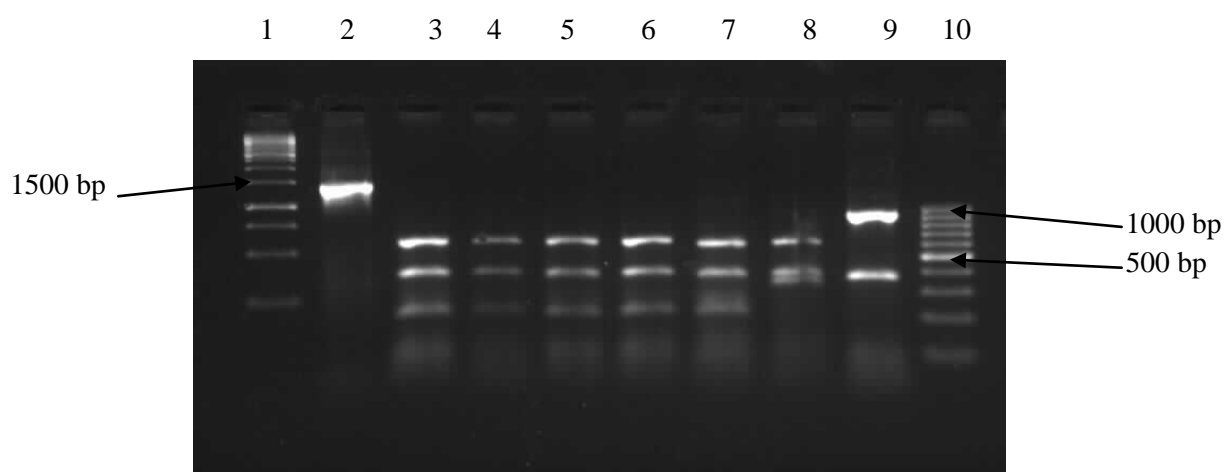


Fig. 4.6 Restriction digestion of 16S rRNA amplified PCR product with *TaqI*. Lane 1: 1 Kb ladder, Lane 2: control (16S rRNA amplified PCR product without digestion), Lane 3-4: PSB-1, Lane 5: PSB-5, Lane 6: PSB-6, Lane 7: PSB-7, Lane 8: PSB-12, Lane 9: PSB-13, Lane 10: 100 bp ladder.

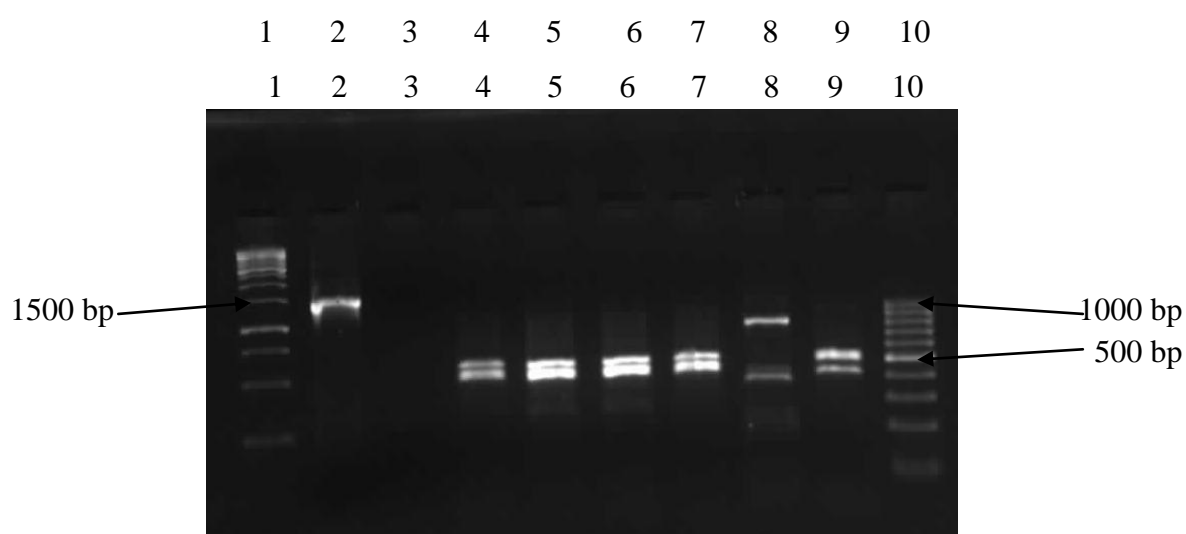


Fig. 4.7 Restriction digestion of 16S rRNA amplified PCR product with *RsaI*. Lane 1: 1 Kb ladder, Lane 2: control (16S rRNA amplified PCR product without digestion), Lane 4: PSB-1, Lane 5: PSB-5, Lane 6: PSB-6, Lane 7: PSB-7, Lane 8: PSB-12, Lane 9: PSB-13, Lane 10: 100 bp ladder.

Prior to sequencing, PCR products were purified and subcloned using Ins T/A clone PCR cloning kit (Fermentas, USA) according to the manufacturer's instructions and incorporated into transformed *E. coli* DH5 α cells. Transformants were screened for the 16S rRNA inserts and partially sequenced (DNA sequencing facility, University of Delhi, Delhi, India. Nucleotide sequence comparisons were performed using Ez Txzon-e database. Sequence analysis from Ez Txzon-e database showed that two isolates PSB-3 and PSB-6 have maximum similarity (98.45 %) with *Pantoea cyripedii* and four isolates PSB-5, PSB-7, PSB-12 and PSB-13 have maximum similarity (99 %) with *Pseudomonas plecoglossicida*. Out of these six isolates, two isolates PSB-3 and PSB-5 were selected for 16S rRNA full length sequence analysis. 16S rRNA full length sequence analysis using the Ez Txzon-e database revealed that the most closely related type strain for PSB-3 is *Pantoea cyripedii*, which showed 98.6% similarity (Fig. 4.8). For PSB-5, the most closely related sequence is *Pseudomonas plecoglossicida*, with 99.8% similarity (Fig. 4.8). Phylogenetic analysis also grouped PSB-3 with *P. cyripedii* and PSB-5 with *P. plecoglossicida* (Fig 4.9). 16S rRNA gene sequences determined in this study were deposited in GenBank of the NCBI under the accession numbers JX556216 and JX556217 for PSB-3 and PSB-5, respectively.

The screenshot displays the EzTaxon web interface. The top navigation bar includes the EzTaxon logo and links for 'How to cite', 'Hierarchy', 'Taxonomic Group', 'Cart', and 'Q&A'. The user 'gurdeep kaur' is logged in. The main content area is titled 'Analysis Results' and shows a table of search results. The table has columns for 'asks', 'Job Type', 'DB', 'Query (Click to view details)', 'Length (bp)', 'Closest match', and 'Similarity (%)'. Two results are visible:

asks	Job Type	DB	Query (Click to view details)	Length (bp)	Closest match	Similarity (%)
	Identify	EzTaxon	PSB-5, 16S rRNA	1394	<i>Pseudomonas plecoglossicida</i> FPC951(T)	99.78
	Identify	EzTaxon	PSB-3, 16S rRNA	1399	<i>Pantoea cyripedii</i> ATCC 29267(T)	98.67

Fig. 4.8 16S rRNA sequence analysis of PSB-3 and PSB-5 with Ez Txzon-e database.

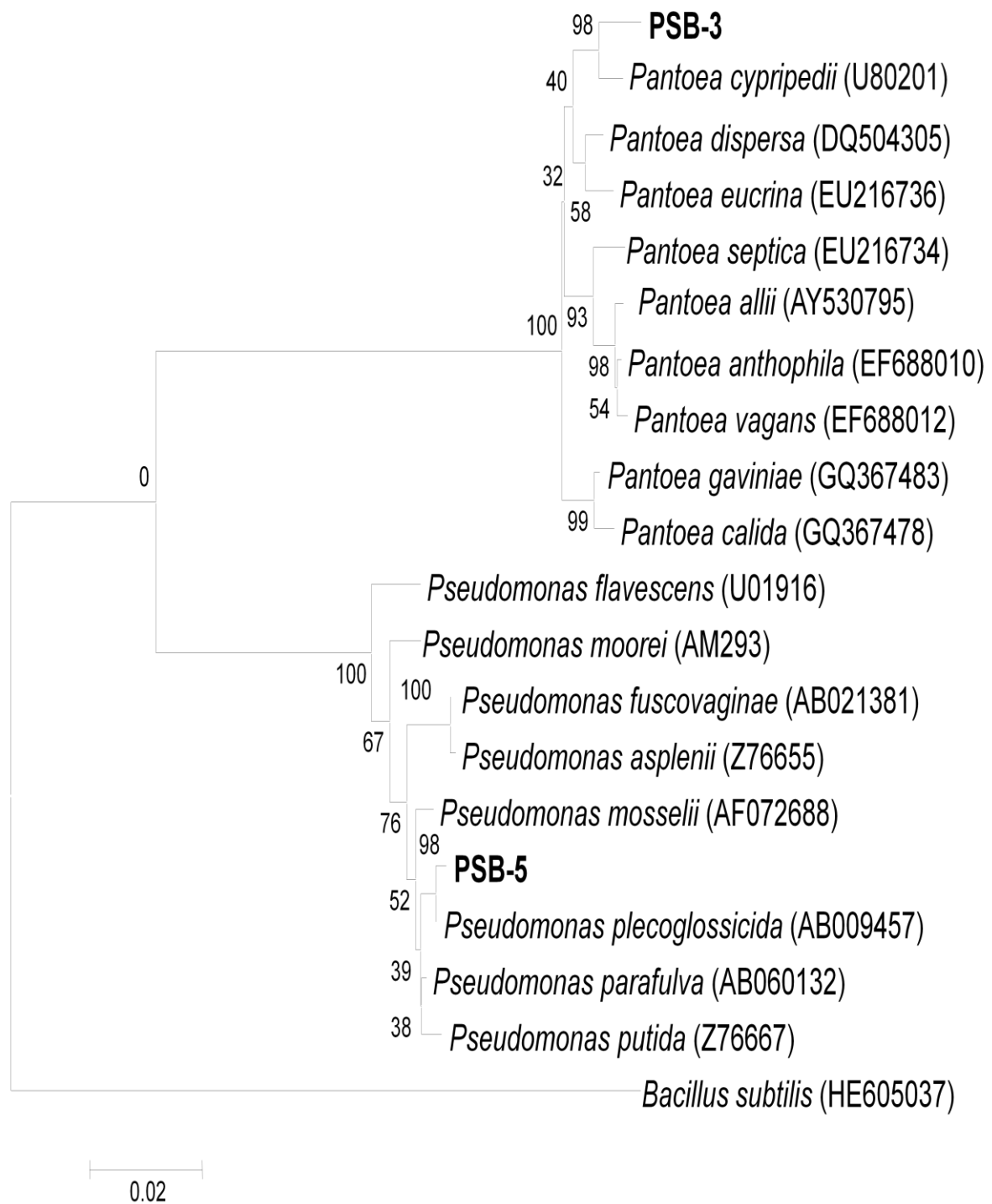


Fig. 4.9 Phylogenetic (Neighbor-Joining) tree based on 16S rRNA gene sequences, showing the relationship between present study isolates and representatives of other related taxa with validly published names. *Bacillus subtilis* (HE605037) was used as out group.

4.2 Isolation and identification of phosphate-solubilizing fungi

4.2.1 Isolation of phosphate-solubilizing fungi

From the selected soil niches a total of 69 fungi were isolated on Pikovskaya's agar plates by serial dilution method at 10^{-5} dilution (Table 4.9). Out of these 69 fungal isolates, only 9 fungal isolates formed a halo (zone) surrounding the colonies which were 13 % of total fungal population isolated (Table 4.9). Out of these 13 % phosphate-solubilizing fungal population, maximum phosphate-solubilizing fungal population was came from rhizosphere soil of *Stevia rebaudiana* grown in organic farm (45 %) followed by mature maize (*Zea mays*) rhizosphere (20 %), surface soil of potato (*Solanum tuberosum*) field (14 %), mustard (*Brassica campestris*) rhizosphere soil (11 %), rhizosphere soil of bamboo (*Bambusa vulgaris*) (8 %), rhizosphere soil of sunflower (*Helianthus annuus*), small maize (*Zea mays*) rhizosphere soil, surface soil of barseem (*Trifolium alexandrinum*) crop field, and surface soil of CORE field does not contain any phosphate solubilizing-fungal population (Table 4.9). Phosphate solubilization activity of fungal isolates is shown in Plate 4.3.

4.2.2 Test of purity and stability

To test the purity and stability, fungal isolates were point inoculated on BCG PKV agar plates. After three days of incubation, yellow colored zone formation was observed surrounding the colonies. Change in color, surrounding the colonies clearly indicated the drop in pH at this place, and acidification of the medium seems to be directly associated to the process of phosphate solubilization. All the tested fungal isolates were able to show halo zone on plates (Plate 4.4).

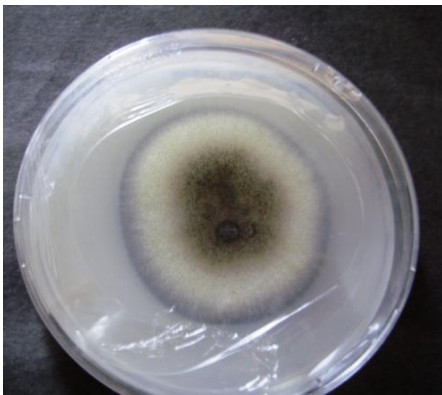
Table 4.9 Population density (P.D.) of phosphate-solubilizing fungi in different crop fields.

Serial No.	Soil samples of different crop fields	pH of soil	P.D. of fungi × 10 ⁵ /g. of soil	P.D. of PSF × 10 ⁵ /g. of soil	% age of PSB
1	Rhizospheric soil of bamboo (<i>Bambusa vulgaris</i>)	7.71 ± 0.02	12	1	8 %
2	Surface soil of potato (<i>Solanum tuberosum</i>) field	7.93 ± 0.04	7	1	14 %
3	Surface soil of CORE field	7.81 ± 0.03	5	0	0 %
4	Mustard (<i>Brassica campestris</i>) rhizospheric soil	7.82 ± 0.03	9	1	11 %
5	Mature maize (<i>Zea mays</i>) rhizosphere	8.09 ± 0.03	5	1	20 %
6	Small maize (<i>Zea mays</i>) rhizosphere	8.32 ± 0.03	4	0	0 %
7	Surface soil of berseem (<i>Trifolium alexandrinum</i>) field	8.18 ± 0.03	3	0	0 %
8	Sunflower (<i>Helianthus annuus</i>) rhizosphere	8.29 ± 0.02	13	0	8 %
9	Rhizosphere soil of <i>Stevia rebaudiana</i> grown in organic farm	8.37 ± 0.02	11	5	45 %

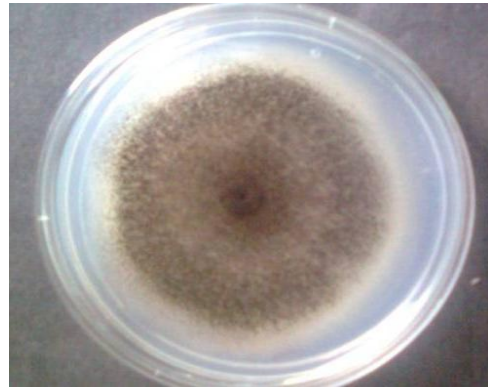
Pronounced results are represented in bold.



a)



b)

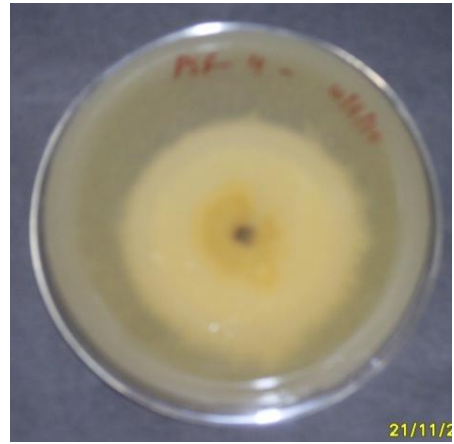


c)

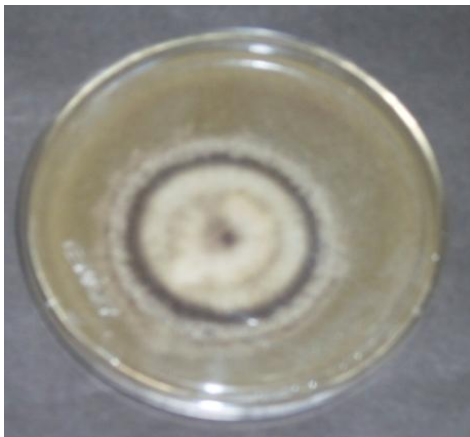
Plate 4.3 a) Control (Pikovskaya's agar plate). b) - c) phosphate-solubilizing fungi showing zone of solubilization on Pikovskaya's agar plates.



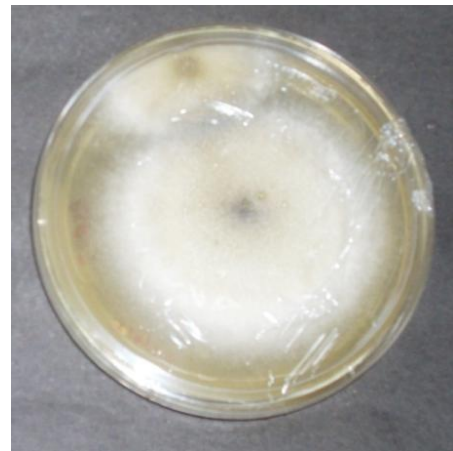
a)



b)



c)



d)

Plate 4.4 a) Control (Pikovskaya's agar plate with Bromo Cresol Green). b)-d) point inoculation of isolated P-solubilizing fungi to check the stability on PKV medium containing BCG as an indicator.

4.2.3 Quantitative estimation of tri-calcium phosphate solubilization by fungal isolates.

All the nine fungal isolates were tested for P solubilization in different time intervals (days) in PKV broth supplemented with tri-calcium phosphate (equivalent to 100 mg P_2O_5 100 ml⁻¹). It was observed that all fungal isolates showed maximum P solubilization at day three of incubation with decrease in pH from 7.2 to 2.12 (Table 4.10). At day five of incubation, soluble P was decreased and it was continued to decrease as the incubation time was increased. Results in the Table 4.10 showed that soluble P was increased as the pH was decreased. Soluble P was decreased and pH was increased with increased in incubation period. Fig. 4.10 showed that all the fungal isolates were produced acid phosphatase, alkaline phosphatase and phytase enzyme in PKV broth. Production of acid phosphates enzyme was higher than that of alkaline phosphatase enzyme. Results in Fig 4.10 showed that production of phytase enzyme was higher than that of acid phosphatase and alkaline phosphatase enzyme. Fungal dry biomass was also measured for all the isolates and results in Table 4.10 showed that fungal dry biomass was ranged in between 0.350 to 0.515 g 100 ml⁻¹. All the isolates were exudates organic acids in PKV broth supplemented with TCP (equivalent to 100 mg P_2O_5 100 ml⁻¹). Main organic acids exudated by these isolates in culture filtrate were oxalic acid, citric acid, malic acid, succinic acid acetic acid and gluconic acid (Table 4.11).

Table 4.10 Release of soluble P and pH reduction at different time intervals (days) by selected phosphate solubilizing fungi at different time intervals (days) in Pikovskaya's broth supplemented with tri-calcium phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Isolates	3 rd day		5 th day		7 th day		Dry wt. of fungi (g/100 ml)
	pH	Soluble 'P' (µg ml ⁻¹)	pH	Soluble 'P' (µg ml ⁻¹)	pH	Soluble 'P' (µg ml ⁻¹)	
PSF-1	2.36 ± 0.13ab	356 ± 8c	2.75 ± 0.30a	343 ± 6def	2.92 ± 0.02b	289 ± 5b	0.350 ± 0.03a
PSF-2	2.86 ± 0.38ab	416 ± 5b	2.87 ± 0.24a	332 ± 4ef	3.14 ± 0.30b	262 ± 4c	0.393 ± 0.13a
PSF-3	2.59 ± 0.79ab	346 ± 7c	3.09 ± 0.79a	329 ± 4f	3.53 ± 0.39b	235 ± 9d	0.411 ± 0.12a
PSF-4	2.12 ± 0.03b	459 ± 8a	2.60 ± 0.23a	394 ± 8a	2.90 ± 0.02b	291 ± 11b	0.514 ± 0.03a
PSF-5	2.41 ± 0.26ab	443 ± 7a	2.63 ± 0.26a	347 ± 7cde	3.02 ± 0.13b	250 ± 8cd	0.488 ± 0.002a
PSF-6	2.32 ± 0.10ab	444 ± 2a	2.45 ± 0.82a	361 ± 7bc	3.33 ± 0.70b	312 ± 4a	0.515 ± 0.02a
PSF-7	2.19 ± 0.06b	440 ± 7a	2.34 ± 0.12a	364 ± 6b	3.47 ± 0.10b	320 ± 7a	0.474 ± 0.01a
PSF-8	2.30 ± 0.52ab	416 ± 10b	3.01 ± 0.60a	358 ± 7bcd	3.70 ± 0.17ab	238 ± 3d	0.460 ± 0.01a
PSF-9	3.48 ± 0.68a	346 ± 10c	3.67 ± 0.35a	336 ± 3ef	4.40 ± 0.16a	249 ± 6cd	0.447 ± 0.04a
LSD (<i>P</i> <0.05)	0.72	13	0.82	10	0.52	11.3	0.108

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

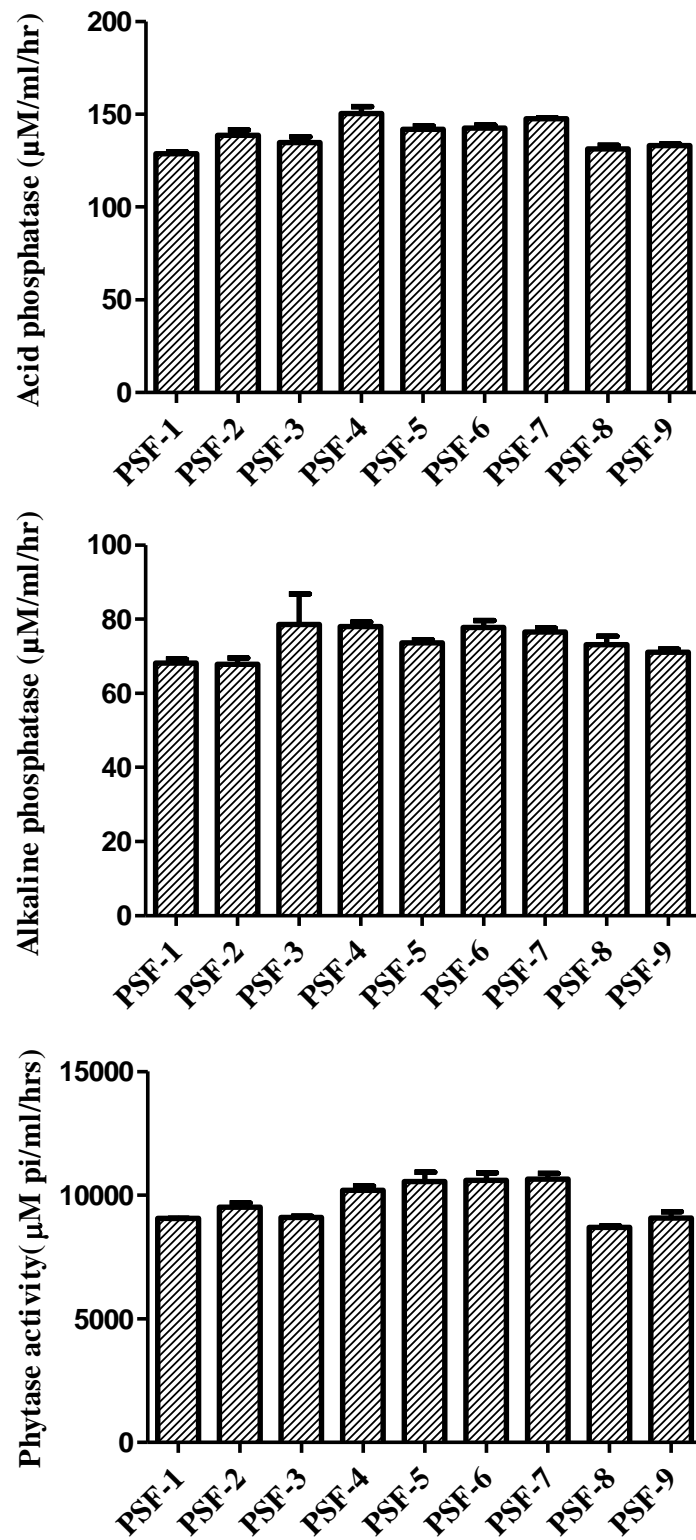


Fig. 4.10 Enzyme activities by selected fungal isolates in Pikovskaya's broth supplemented with tri-calcium phosphate (equivalent to 100 mg P_2O_5 100 ml⁻¹).

Table 4.11 Organic acid production by fungal isolates in Pikovskaya's broth amended with tri-calcium phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Isolates	Oxalic acid (n mol/ml)	Citric acid (n mol/ml)	Malic acid (n mol/ml)	Succinic acid (n mol/ml)	Formic acid (n mol/ml)	Acetic acid (n mol/ml)	Gluconic acid (n mol/ml)
PSF-1	4 ± 0.07e	0	12 ± 0.30fg	0	0	0	16 ± 0.3d
PSF-2	10 ± 0.10cd	1237 ± 4b	21 ± 0.37f	0	0	0	0
PSF-3	50 ± 1.0a	1077 ± 13c	290 ± 0.35d	0	0	0	0
PSF-4	9 ± 0.66d	7 ± 0.12e	571 ± 14b	2010 ± 61a	0	0	8063 ± 40a
PSF-5	4 ± 1.92e	2692 ± 73a	0	0	0	0	0
PSF-6	23 ± 0.09b	0	361 ± 6c	0	0	58 ± 0.9c	3988 ± 44b
PSF-7	50 ± 0.85a	719 ± 7d	662 ± 0.01a	101 ± 5b	0	82 ± 0.6a	8105 ± 43a
PSF-8	12 ± 0.28c	0	0	0	0	0	0
PSF-9	24 ± 0.12b	0	81 ± 0.78e	0	0	78 ± 0.9b	248 ± 4c
LSD (<i>P</i> <0.05)	1.48	43	8.9	35		0.873	42

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

4.2.4 Quantitative estimation of rock phosphate solubilization by fungal isolates

Fungal isolates were further tested for rock phosphate solubilization (equivalent to 100 mg P₂O₅ 100 ml⁻¹) supplemented in PKV broth. All isolates were showed highest P solubilization on day five of incubation (Table 4.12) ranging between 289 µg ml⁻¹ and 392 µg ml⁻¹. Soluble P released by fungal isolates in rock phosphate solubilization was less than tri-calcium phosphate. A decrease in pH up to 2.5 from initial value of 7.2 was observed as the soluble P in culture medium was increased. As the time of incubation increased, pH of culture filtrate was increased and soluble P was also started decreased. Fungal dry biomass of all the isolates was ranged between 0.370 to 0.464 g 100 ml⁻¹.

Culture filtrates of all the isolates were tested for acid phosphatase, alkaline phosphatase and phytase enzyme production and also for organic acid exudation. Results in Fig. 4.11 showed that all the isolates produced acid phosphatase, alkaline phosphatase and phytase enzyme. Acid phosphatase enzyme production was higher than that of alkaline phosphatase enzyme and production of phytase enzyme was higher than both acid phosphatase and alkaline phosphatase enzymes. Main organic acids exudated by all fungal isolates in PKV broth supplemented with rock phosphate were oxalic acid, citric acid, succinic acid and gluconic acid (Table 4.13).

Table 4.12 Release of soluble P and pH reduction at different time intervals (days) by selected phosphate solubilizing fungi at different time intervals (days) in Pikovskaya's broth supplemented with rock phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Isolates	3 rd day		5 th day		7 th day		Dry wt. of fungi (g/100 ml)
	pH	Soluble 'P' (µg ml ⁻¹)	pH	Soluble 'P' (µg ml ⁻¹)	pH	Soluble 'P' (µg ml ⁻¹)	
PSF-1	3.33 ± 0.04a	224 ± 5c	2.83 ± 0.12ab	337 ± 6de	3.80 ± 0.18a	282 ± 7b	0.405 ± 0.06a
PSF-2	3.13 ± 0.03ab	187 ± 2e	2.80 ± 0.01ab	323 ± 5e	3.75 ± 0.12a	249 ± 4c	0.387 ± 0.02a
PSF-3	3.29 ± 0.18a	213 ± 5cd	2.84 ± 0.08ab	289 ± 7ef	3.59 ± 0.08a	224 ± 4de	0.370 ± 0.09a
PSF-4	3.14 ± 0.07ab	251 ± 3a	2.77 ± 0.02ab	358 ± 10d	3.24 ± 0.05b	330 ± 4a	0.451 ± 0.02a
PSF-5	2.85 ± 0.14bc	245 ± 1ab	2.66 ± 0.10bc	364 ± 3c	3.12 ± 0.13b	298 ± 13b	0.453 ± 0.03a
PSF-6	2.73 ± 0.09bc	236 ± 4b	2.68 ± 0.05bc	366 ± 16b	3.19 ± 0.02b	255 ± 2c	0.464 ± 0.03a
PSF-7	2.61 ± 0.08c	242 ± 1ab	2.53 ± 0.06c	392 ± 7a	2.68 ± 0.01c	335 ± 4a	0.415 ± 0.02a
PSF-8	3.36 ± 0.26a	218 ± 8cd	2.95 ± 0.15a	230 ± 8f	3.07 ± 0.022b	218 ± 5e	0.373 ± 0.02a
PSF-9	3.30 ± 0.27a	212 ± 3d	2.61 ± 0.04bc	323 ± 6e	3.73 ± 0.04a	241 ± 8cd	0.381 ± 0.06a
LSD (<i>P</i> <0.05)	0.26	6.88	0.14	14	0.199	11	0.079

Values are Mean ± SD (*n* = 3). Means sharing a common letter within the column are not significantly different at *P* < 0.05. Pronounced results are represented in bold.

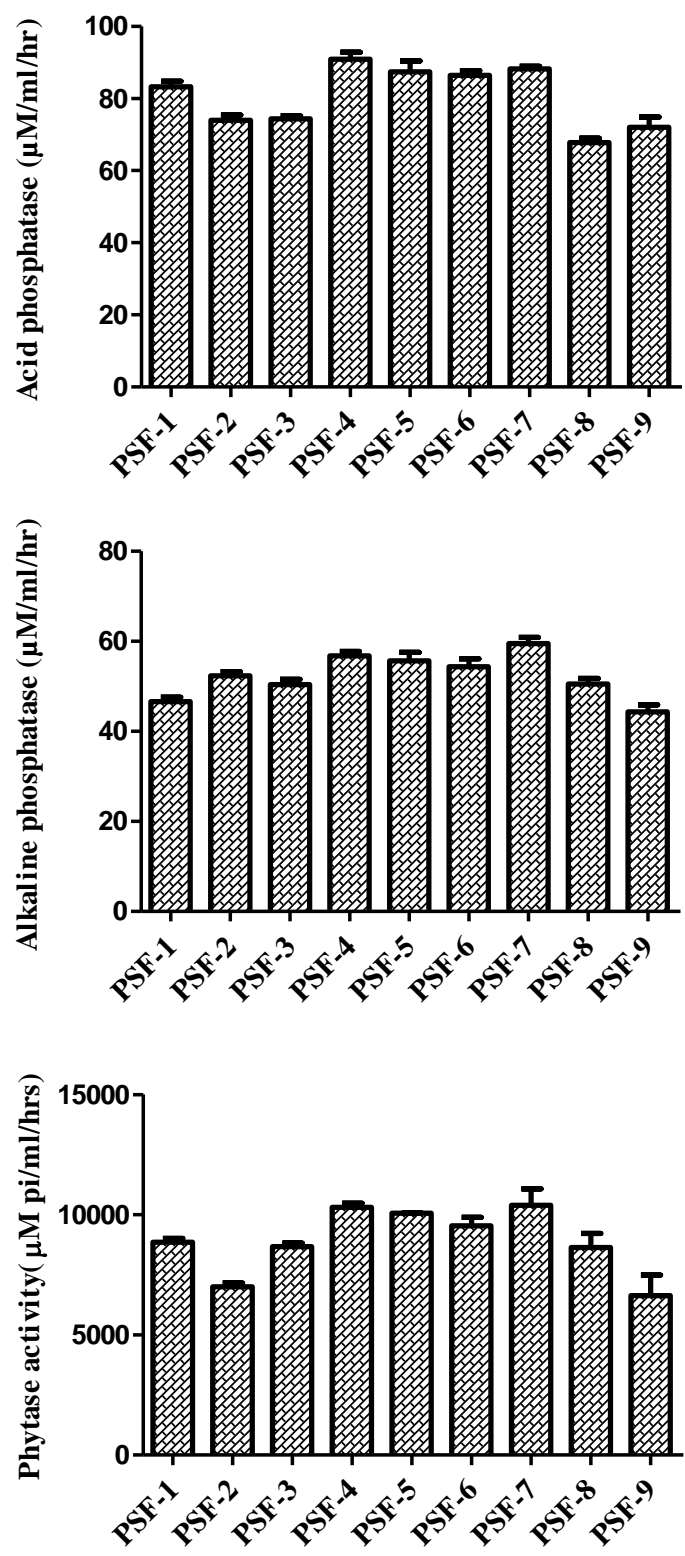


Fig. 4.11 Enzyme activities by selected fungal isolates in Pikovskaya's broth supplemented with rock phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Table 4.13 Organic acid production by fungal isolates in Pikovskaya's broth amended with rock phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Isolates	Oxalic acid (n mol/ml)	Citric acid (n mol/ml)	Malic acid (n mol/ml)	Succinic acid (n mol/ml)	Formic acid (n mol/ml)	Acetic acid (n mol/ml)	Gluconic acid (n mol/ml)
PSF-1	44 ± 0.56d	355 ± 18c	0	42 ± 1cd	0	0	5619 ± 42b
PSF-2	5 ± 0.72h	0	0	0	0	0	2838 ± 48e
PSF-3	20 ± 0.85g	0	0	0	0	0	1886 ± 32g
PSF-4	150 ± 0.78a	0	0	5218 ± 53a	0	0	655 ± 4h
PSF-5	50 ± 1.89c	515 ± 4a	0	0	0	0	6737 ± 26a
PSF-6	30 ± 1.17f	320 ± 5d	0	61 ± 1c	0	0	4315 ± 36c
PSF-7	75 ± 3.50b	375 ± 4b	3198 ± 45	1959 ± 35b	0	0	0
PSF-8	36 ± 1.32e	0	0	0	0	0	3871 ± 36d
PSF-9	27 ± 0.92f	0	0	38 ± 1cd	0	0	1977 ± 21f
LSD (<i>P</i> <0.05)	2.82	11.11		36			54

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

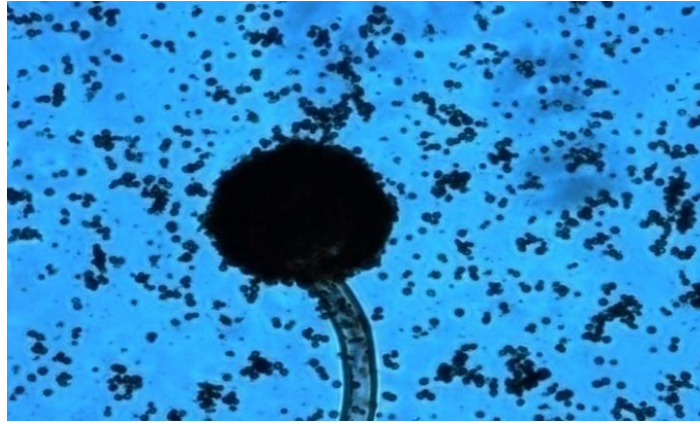
4.2.5 Characterization of fungi.

The nine fungal isolates were screened for solubilization efficiency. Morphological studies showed that all nine fungal isolates (PSF-1, PSF-2, PSF-3, PSF-4, PSF-5, PSF-6, PSF-7, PSF-8, PSF-9) belonged to black mold i.e. genus *Aspergillus*. Out of these 9 *Aspergilli*, some showed characteristic morphology of *A. tubingensis*, while others showed morphological similarities with *A. niger*.

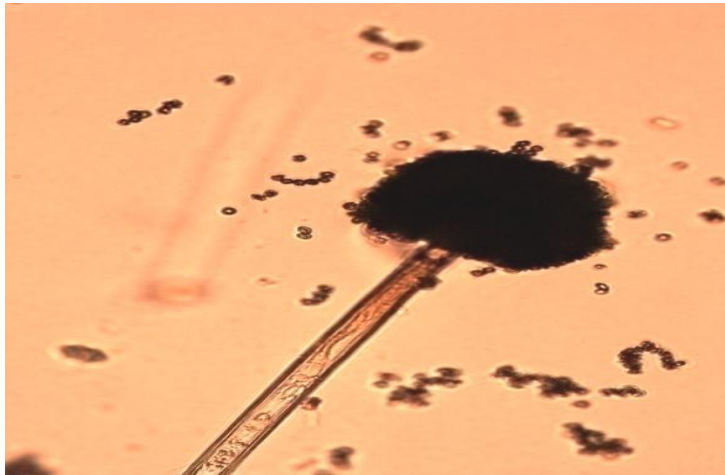
***Aspergillus tubingensis*:** The purified colonies had radial villus and white mycelia at the edge of each colony in early culture stages (3 to 5 days). After five days, the colony became black at the head of mycelium and white in the reverse side in PKV agar plate, respectively. The vesicle of *A. tubingensis* was round and radial. Diaphragms in hypha were visible under microscope. Sporangia consisted of many spores (Fig. 4.12).

***Aspergillus niger*:** *A. niger* on PKV agar, after 5 days at 28 °C, the initial growth was white, which later on turned to black due to formation of black spores and reverse turning pale yellow. Microscopic morphology showed septate hyphae with smooth-walled, simple conidiophores measuring up to 1 mm in length. Conidiophores end in vesicle, which was globose and entirely covered (radiating) with two series of sterigmata (biseriate). Conidia produced from these sterigmata ranged between brown to black in color, round, rough walled.

Aspergillus niger was differentiated from *Aspergillus tubingensis* by its microscopically long conidiophores, larger conidial diameter, yellow at reverse in agar plate, whereas *A. tubingensis* had small conidiophores, two times smaller conidial diameter, white at reverse in agar plate, shaggy hairy/villus look.



a)



b)

Fig. 4.12 Microscopic view of a) *A. tubingensis* and b) *A. niger* under $40\times$ magnification.

4.2.6 Identification of fungal isolates.

For molecular identification, genomic DNA of all the nine fungal isolates was isolated. ITS region of the rRNA from genomic DNA was amplified by the PCR using ITS1 and ITS4 primers. Resultant PCR products were viewed after electrophoresis in agarose gel. The results showed that all isolates produced a single band of approximately 600 bp (Fig. 4.13).

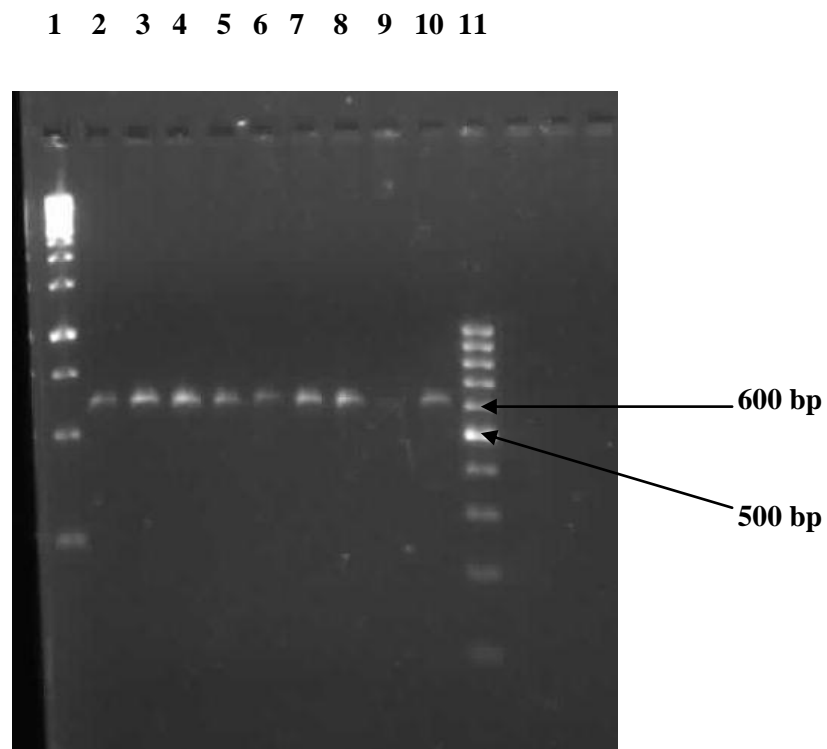


Fig. 4.13 ITS-PCR products of nine different fungal isolates. Lane 1: 1 Kb ladder, Lane 2: PSF-1, Lane 3: PSF-2, Lane 4: PSF-3, Lane 5: PSF-4, Lane 6: PSF-5, Lane 7: PSF-6, Lane 8: PSF-7, Lane 9: PSF-8, Lane 10: PSF-9, Lane 11: 100 bp ladder.

The PCR products of nine fungal isolates were digested with *RsaI* enzyme to check the variation between *Aspergillus tubingensis* and *Aspergillus niger*. RFLP results (Fig. 4.14) revealed that isolates PSF-1(lane2), PSF-2 (lane 3), PSF-3 (lane 4), PSF-5 (lane 6), PSF-6 (lane 7), PSF-7 (lane 8), PSF-8 (lane 9), PSF-9 (lane 10) show same banding pattern and showed positive for *RsaI* that can be grouped together as *Aspergillus niger*. Isolate PSF-4 (lane 5) showed different banding pattern compared to other isolates and was negative for *RsaI* so that can be grouped as *Aspergillus tubingensis*. Isolates PSF-4, PSF-5, PSF-6 and PSF-7 on the basis of their P solubilization efficiency were selected for sequencing.

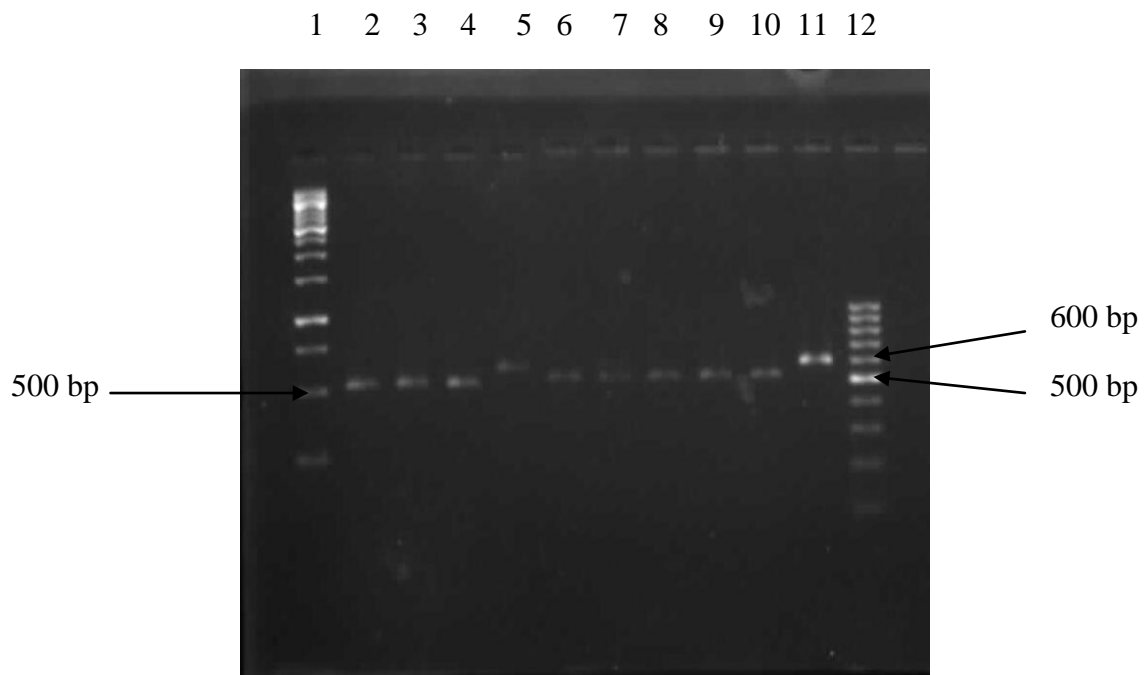


Fig. 4.14 Restriction digestion of ITS amplified PCR product with *RsaI*. Lane 1: 1 Kb ladder, Lane 2: PSF-1, Lane 3: PSF-2, Lane 4: PSF-3, Lane 5: PSF-4, Lane 6: PSF-5, Lane 7: PSF-6, Lane 8: PSF-7, Lane 9: PSF-8, Lane 10: PSF-9, Lane 11: control (ITS amplified PCR product without digestion), Lane 12: 100 bp ladder.

The ITS-PCR products were cloned into pTZ57R/T Vector. Prior to sequencing, PCR products were purified and subcloned using Ins T/A clone PCR cloning kit (Fermentas, USA) according to the manufacturer's instructions and incorporated into transformed *E. coli* DH5 α cells. Transformants were screened for the ITS/5.8S rRNA inserts and partially sequenced (DNA sequencing facility, University of Delhi, Delhi, India). Nucleotide sequence comparisons were performed using the BLAST database (Altschul et al 1997). Sequence data from BLAST results showed that out of four, three fungal isolates (PSF-5-, PSF-6, PSF-7) showed maximum similarity (99 %) with *Aspergillus niger* and one isolate PSF-4 showed maximum similarity (99 %) with *Aspergillus tubingensis* (Fig. 4.14 and 4.16) . Phylogenetic analysis also grouped PSF-4, PSF-5, PSF-6 and PSF-7 with *Aspergillus* (Fig 4.17). ITS/5.8S rRNA gene sequences determined in this study for fungal isolates were deposited in GenBank of the NCBI under the accession numbers KJ410674, KJ410675, KJ410676 and KJ410677 for PSF-4, PSF-5, PSF-6 and PSF-7, respectively.

Sequences producing significant alignments:

Select: [All](#) [None](#) Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
<input type="checkbox"/> Aspergillus tubingensis isolate A2S2_D32 18S ribosomal RNA gene, partial sequence: internal tr	1092	1092	100%	0.0	99%	JX501380.1
<input type="checkbox"/> Aspergillus tubingensis isolate A2S5_3 18S ribosomal RNA gene, partial sequence: internal trans	1092	1092	100%	0.0	99%	JX501390.1
<input type="checkbox"/> Aspergillus niger strain MUM05.13 18S ribosomal RNA gene, partial sequence: internal transcribe	1092	1092	100%	0.0	99%	JF838357.1
<input type="checkbox"/> Aspergillus niger strain WM10.74 18S ribosomal RNA gene, partial sequence: internal transcribec	1092	1092	100%	0.0	99%	HQ014696.1
<input type="checkbox"/> Aspergillus niger strain WM10.68 18S ribosomal RNA gene, partial sequence: internal transcribec	1092	1092	100%	0.0	99%	HQ014690.1
<input type="checkbox"/> Aspergillus sp. 06 SMR-2010 18S ribosomal RNA gene, partial sequence: internal transcribed sp	1090	1090	99%	0.0	99%	HM801881.1
<input type="checkbox"/> Aspergillus awamori 18S ribosomal RNA gene, partial sequence: internal transcribed spacer 1, 5:	1090	1090	99%	0.0	99%	DQ235784.1
<input type="checkbox"/> Aspergillus niger genes for small subunit rRNA, ITS1, 5.8S rRNA, ITS2 and large subunit rRNA, p	1090	1090	99%	0.0	99%	AB369898.1
<input type="checkbox"/> Aspergillus niger strain EIM-6 18S ribosomal RNA gene, partial sequence: internal transcribed sp	1090	1090	99%	0.0	99%	FJ040211.1
<input type="checkbox"/> Aspergillus niger strain 91718 18S ribosomal RNA gene, partial sequence: internal transcribed sp	1088	1088	99%	0.0	99%	JN565296.1

Fig. 4.15 BLAST results of ITS /18S RNA Sequence of PSF-4 isolate.

Descriptions

Sequences producing significant alignments:

Select: [All](#) [None](#) Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
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<input type="checkbox"/> Aspergillus niger strain KAML02 18S ribosomal RNA gene, partial sequence: internal transcribed	1103	1103	100%	0.0	99%	KC119204.1
<input type="checkbox"/> Aspergillus niger isolate F6-01 18S ribosomal RNA gene, partial sequence: internal transcribed sp	1103	1103	100%	0.0	99%	JN561269.1
<input type="checkbox"/> Aspergillus niger strain WM10.70 18S ribosomal RNA gene, partial sequence: internal transcribec	1103	1103	100%	0.0	99%	HQ014692.1
<input type="checkbox"/> Aspergillus niger strain WM10.69 18S ribosomal RNA gene, partial sequence: internal transcribec	1103	1103	100%	0.0	99%	HQ014691.1
<input type="checkbox"/> Uncultured fungus clone f2HSc82 18S ribosomal RNA gene, partial sequence: internal transcribe	1103	1103	100%	0.0	99%	GU721579.1
<input type="checkbox"/> Aspergillus niger isolate UOA/HCPF 4289 18S ribosomal RNA gene, partial sequence: internal tr	1103	1103	100%	0.0	99%	FJ878650.1
<input type="checkbox"/> Aspergillus niger isolate UOA/HCPF 3875 18S ribosomal RNA gene, partial sequence: internal tr	1103	1103	100%	0.0	99%	FJ878649.1

Fig. 4.16 BLAST results of ITS /18S RNA Sequence of PSF-5, PSF-6 and PSF-7 isolates.

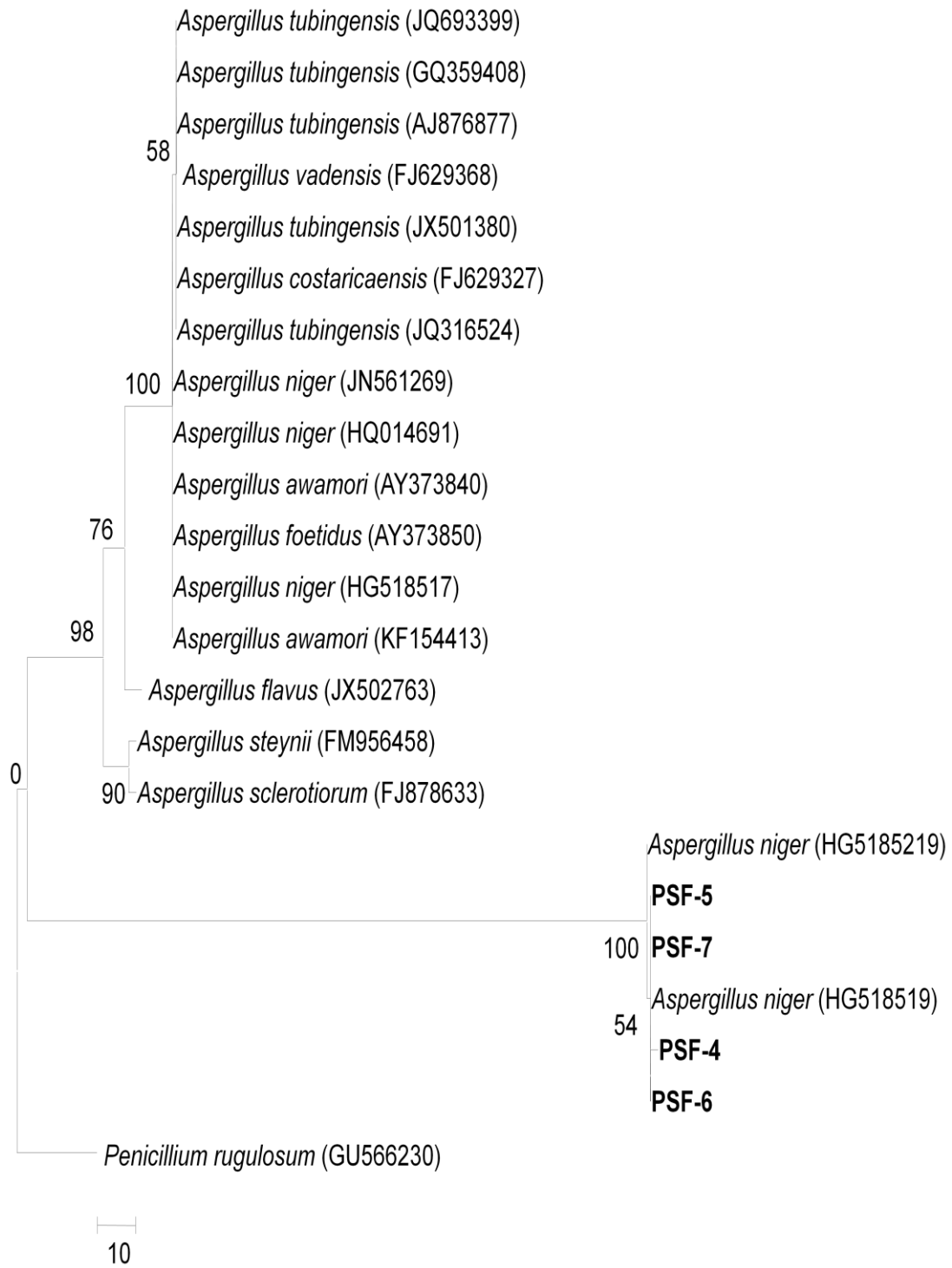


Fig. 4.17 Phylogenetic tree based on ITS/18S rRNA gene sequences of fungi, generated from Maximum Parsimony analysis of ITS sequence rooted with *Penicillium* as out group, showing the relationship between present study isolates and representatives of other related taxa with validly published names.

Chapter 5

Physiological characterization and elucidation of mechanism of phosphate solubilization

5.1 Effect of different carbon and nitrogen sources on phosphate solubilization by bacterial isolates

For biological assessment of phosphate solubilization, effect of different carbon and nitrogen sources must be considered. For this purpose, Pikovskaya's broth containing different carbon (fructose, arabinose, galactose, mannitol, maltose, lactose, sucrose, xylose and glucose) and nitrogen sources (NaNO_3 , NH_4NO_3 , NaNO_2 , KNO_3 , tryptophan, NH_4Cl , urea and $(\text{NH}_4)_2\text{SO}_4$) with tri-calcium phosphate ($100 \text{ mg P}_2\text{O}_5 \text{ 100 ml}^{-1}$) were prepared and inoculated with selected bacterial isolates PSB-3 (*Pantoea cyripedii*) and PSB-5 (*Pseudomonas plecoglossicida*). Production of acid phosphatase, alkaline phosphatase, phytase enzyme and organic acids in the presence of different carbon and nitrogen sources were also recorded to check their effect on P solubilization.

5.1.1 Carbon sources

P. cyripedii showed different pattern of P solubilization in presence of different carbon sources. Maximum P solubilization was observed in the presence of glucose at day five of incubation. Reduction of pH was observed with increase in soluble P in culture supernatant. The pattern of P solubilization by *P. cyripedii* in different carbon sources was glucose > xylose > galactose > fructose > arabinose > lactose = sucrose > mannitol > maltose (Fig. 5.1). *P. plecoglossicida* also showed maximum P solubilization and pH reduction in presence of glucose. Pattern of P solubilization by *P. plecoglossicida* in the presence of different carbon sources was glucose > xylose > galactose > sucrose > maltose > lactose > fructose > mannitol > arabinose (Fig. 5.2).

In both the isolates, enzyme activities (acid phosphatase, alkaline phosphatase and phytase) were higher in presence of glucose compared to other carbon sources. Results in the Table 5.1 and 5.2 showed that pattern of enzyme production in the presence of different carbon

sources was similar to P solubilization pattern by these isolates in different carbon sources, it suggested that nature of different carbon source also effect the enzyme production in both the isolates.

Results of organic acids production by both the isolates in presence of different carbon sources were given in (Table 5.3 and 5.4). It was observed that organic acid production was higher when glucose was used as a carbon source compared to other carbon sources. Main organic acids exudated by *P. cyripedii* in different carbon sources were oxalic acid, acetic acid and gluconic acid. *P. plecoglossicida* produced oxalic acid, citric acid and gluconic acid in the presence of different carbon sources.

5.1.2 Nitrogen sources

By *Pantoea cyripedii* pattern of P solubilization with different nitrogen sources was $(\text{NH}_4)_2\text{SO}_4 > \text{KNO}_3 > \text{NH}_4\text{NO}_3 > \text{NaNO}_2 > \text{tryptophan} > \text{NaNO}_3 > \text{NH}_4\text{Cl} > \text{urea}$ (Fig. 5.3). In case of *Pseudomonas plecoglossicida* pattern was $(\text{NH}_4)_2\text{SO}_4 > \text{urea} > \text{tryptophan} > \text{KNO}_3 > \text{NH}_4\text{Cl} > \text{NaNO}_2 > \text{NaNO}_3 > \text{NH}_4\text{NO}_3$ (Fig. 5.4). Maximum P solubilization was observed in $(\text{NH}_4)_2\text{SO}_4$ as compared to other nitrogen source with both isolates. Both the isolates were able to produce acid phosphatase, alkaline phosphatase and phytase enzymes in presence of all these different nitrogen sources (Table 5.5 and 5.6). Production of enzymes was maximum in $(\text{NH}_4)_2\text{SO}_4$ and it is comparable with the P solubilization with decreased in pH in culture supernatant compared to other nitrogen sources. Organic acid production by these isolates in different nitrogen sources is presented in Table 5.7 and 5.8. *P. cyripedii* produced oxalic acid and gluconic acid as main organic acid in all the nitrogen sources but in presence of $(\text{NH}_4)_2\text{SO}_4$ it also produce acetic acid. The organic acids produced by *P. plecoglossicida* in different nitrogen sources were oxalic acid, citric acid, malic acid and gluconic acid,

maximum acid production was observed in presence of $(\text{NH}_4)_2\text{SO}_4$ as a nitrogen source (Table 5.8).

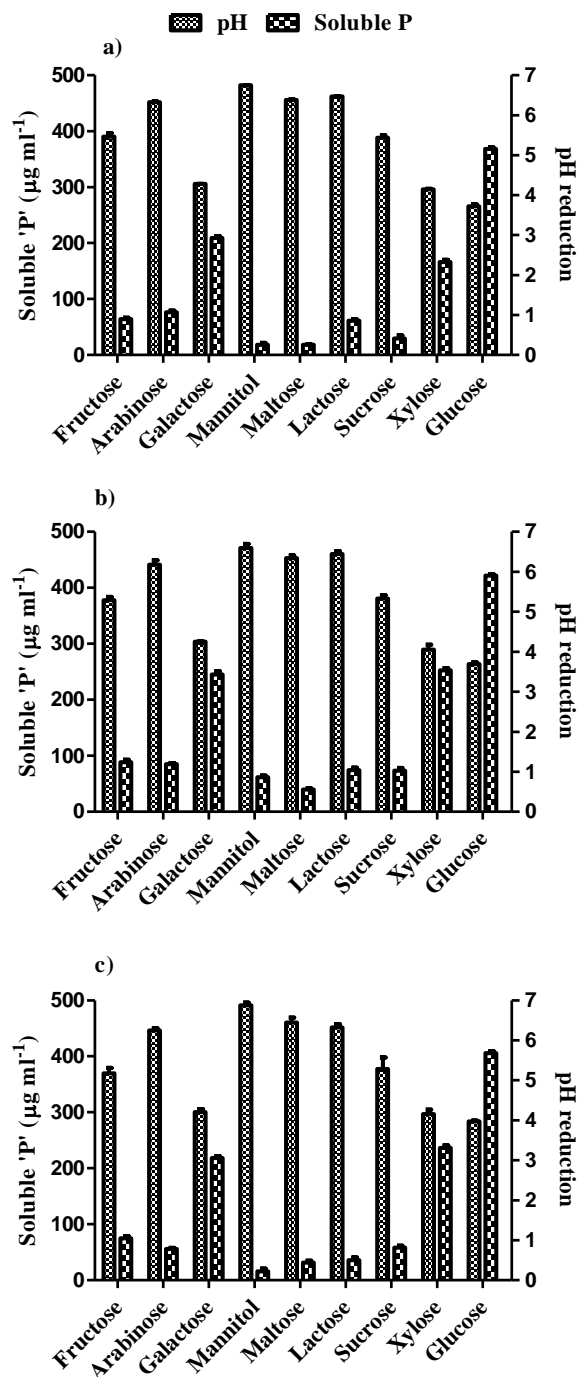


Fig. 5.1 Influence of carbon sources on pH reduction and on soluble P by *Pantoea cypripedii* in presence of different C sources in PKV media having TCP (100 mg P₂O₅ 100 ml⁻¹) on different time intervals, a) 3rd day, b) 5th day and c) 7th day.

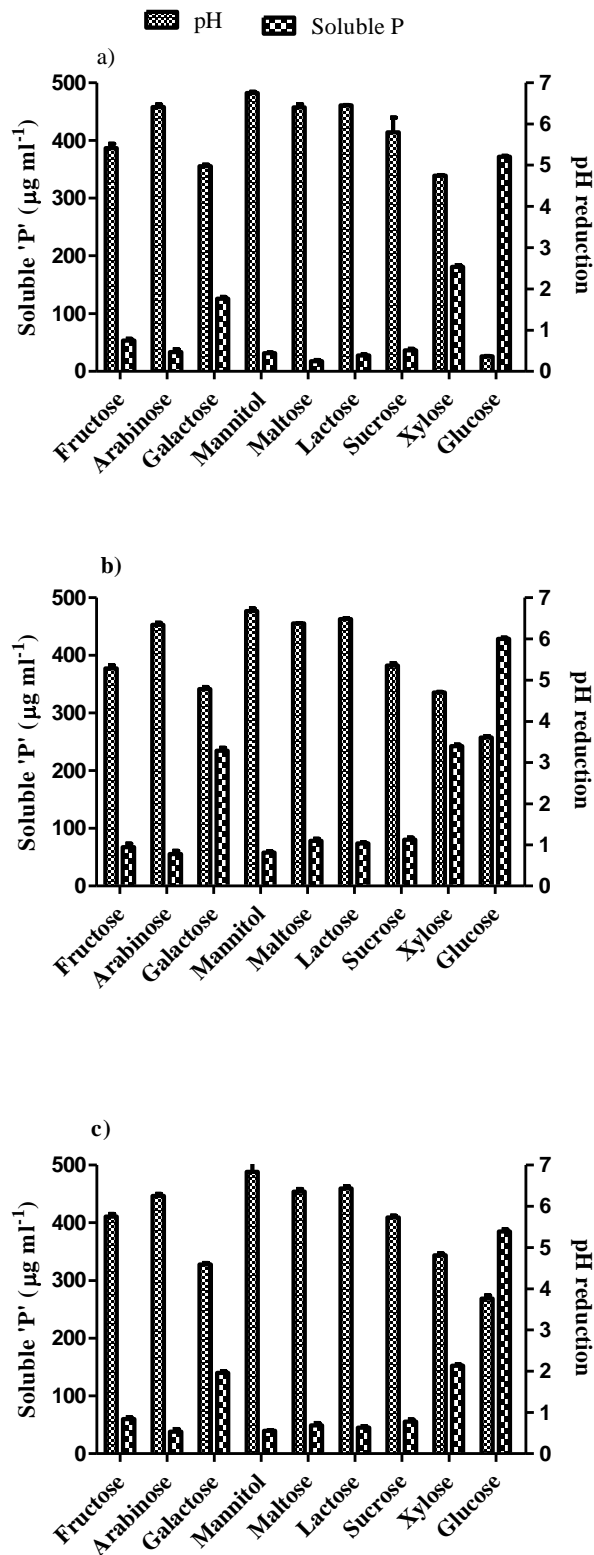


Fig. 5.2 Influence of carbon sources on pH reduction and on soluble P by *Pseudomonas plecoglossicida* in presence of different C sources in PKV media having TCP (100 mg P₂O₅ 100 ml⁻¹) on different time intervals, a) 3rd day, b) 5th day and c) 7th day of incubation.

Table 5.1 Acid phosphatase, alkaline phosphatase and phytase enzyme activities by *Pantoea cypripedii* in presence of different C sources in PKV broth supplemented with tri-calcium phosphate (100 mg P₂O₅ 100 ml⁻¹).

Carbon Sources	Acid phosphatase ($\mu\text{M ml}^{-1} \text{ h}^{-1}$)	Alkaline phosphatase ($\mu\text{M ml}^{-1} \text{ h}^{-1}$)	Phytase ($\mu\text{M ml}^{-1} \text{ h}^{-1}$)
Fructose	52 ± 5b	25 ± 7c	3162 ± 39d
Arabinose	19 ± 2d	20 ± 3c	2631 ± 57e
Galactose	88 ± 12a	62 ± 8ab	6033 ± 8b
Mannitol	22 ± 9cd	22 ± 4c	2396 ± 61f
Maltose	50 ± 11bc	23 ± 4c	2290 ± 73f
Lactose	44 ± 7bcd	25 ± 4c	2698 ± 77e
Sucrose	47 ± 6bcd	48 ± 7b	2431 ± 72f
Xylose	89 ± 18a	60 ± 8ab	4927 ± 37c
Glucose	104 ± 13a	68 ± 9a	6659 ± 25a
LSD ($P < 0.05$)	17	11	93

Values are Mean ± SD ($n = 3$). Means sharing a common letter within the column are not significantly different at $P < 0.05$. Pronounced results are represented in bold.

Table 5.2 Acid phosphatase, alkaline phosphatase and phytase enzyme activities by *Pseudomonas plecoglossicida* in presence of different C sources in PKV broth supplemented with tri-calcium phosphate (100 mg P₂O₅ 100 ml⁻¹).

Carbon Sources	Acid phosphatase (μM ml ⁻¹ h ⁻¹)	Alkaline phosphatase (μM ml ⁻¹ h ⁻¹)	Phytase (μM ml ⁻¹ h ⁻¹)
Fructose	54 ± 9ab	27 ± 3cd	3026 ± 87d
Arabinose	40 ± 9b	22 ± 3d	1207 ± 43f
Galactose	80 ± 11ab	62 ± 9a	6241 ± 129b
Mannitol	67 ± 27ab	22 ± 3d	2593 ± 132e
Maltose	63 ± 34ab	22 ± 3d	2775 ± 266de
Lactose	46 ± 8ab	20 ± 3d	2596 ± 25e
Sucrose	39 ± 3b	43 ± 8bc	3636 ± 93c
Xylose	85 ± 13ab	51 ± 7ab	6171 ± 65b
Glucose	93 ± 13a	65 ± 9a	6884 ± 36a
LSD (<i>P</i> <0.05)	29	10	205

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 5.3 Effect of different carbon sources on organic acid production by *Pantoea cypripedii* in Pikovskaya's broth amended with tri-calcium phosphate (equivalent to 100 mg P₂O₅100 ml⁻¹).

Carbon sources	Oxalic acid (n mol/ml)	Citric acid (n mol/ml)	Malic acid (n mol/ml)	Succinic acid (n mol/ml)	Formic acid (n mol/ml)	Acetic acid (n mol/ml)	Gluconic acid (n mol/ml)
Fructose	43 ± 0.03d	0	0	0	0	23 ± 0.48de	7 ± 0.07e
Arabinose	17 ± 0.09e	0	0	0	0	65 ± 0.36d	9 ± 0.19e
Galactose	89 ± 0.18b	0	0	0	0	4685 ± 19b	3384 ± 8b
Mannitol	14 ± 0.09f	0	0	0	0	0	3 ± 0.86e
Maltose	3 ± 0.01h	0	0	0	0	0	0
Lactose	7 ± 0.13g	0	0	0	0	0	24 ± 0.18d
Sucrose	17 ± 0.10e	2 ± 0.2	9 ± 0.23	0	0	0	0
Xylose	78 ± 0.08c	0	0	0	0	3640 ± 31c	2311 ± 7c
Glucose	121 ± 0.02a	0	0	0	0	5384 ± 28a	4551 ± 0a
LSD (<i>P</i> <0.05)	0.33					26	6

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 5.4 Effect of different carbon sources on organic acid production by *Pseudomonas plecoglossicida* in Pikovskaya's broth amended with tri-calcium phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Carbon sources	Oxalic acid (n mol/ml)	Citric acid (n mol/ml)	Malic acid (n mol/ml)	Succinic acid (n mol/ml)	Formic acid (n mol/ml)	Acetic acid (n mol/ml)	Gluconic acid (n mol/ml)
Fructose	3 ± 0.02h	0	0	0	0	0	17 ± 0.3d
Arabinose	5 ± 0.10g	0	0	0	0	0	12 ± 0.3d
Galactose	12 ± 0.19f	2 ± 0.20c	0	0	0	0	10559 ± 5b
Mannitol	21 ± 0.20d	0	0	0	0	0	0
Maltose	16 ± 0.19e	0	0	0	0	0	23 ± 0.03d
Lactose	0.56 ± 0.01i	7 ± 0.1b	0	0	0	0	25 ± 2d
Sucrose	40 ± 0.02b	0	0	0	0	0	7 ± 0.12d
Xylose	35 ± 0.02c	0	8 ± 0.56	0	0	70 ± 0.32a	9985 ± 57c
Glucose	76 ± 0.7a	513 ± 0.6a	0	85 ± 0.10	0	57 ± 3b	11127 ± 176a
LSD (<i>P</i> <0.05)	0.47	0.33				1.78	105

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

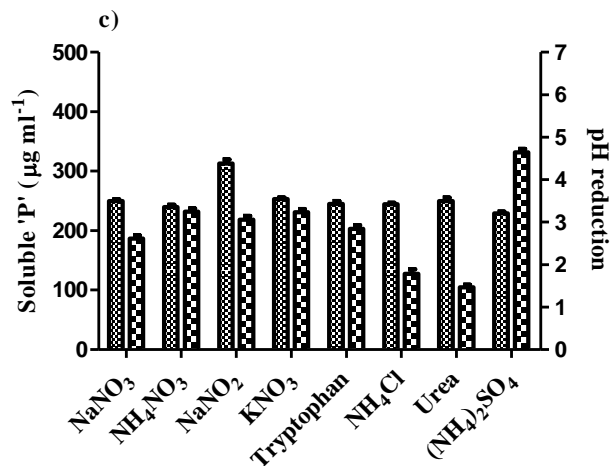
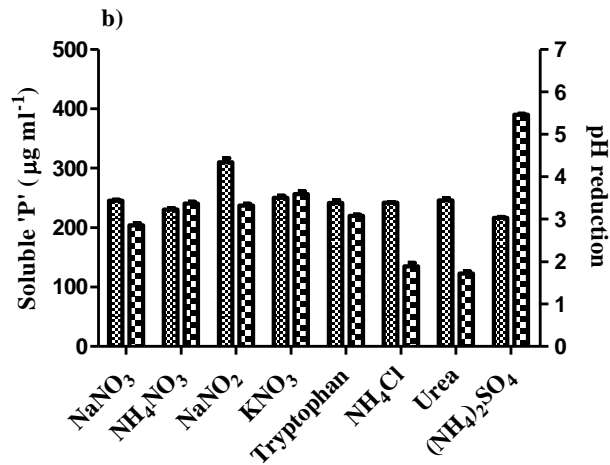
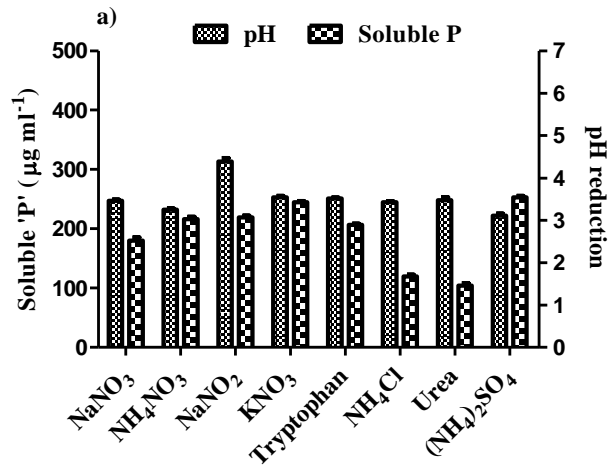


Fig. 5.3 pH reduction and soluble P by *Pantoea cyripedii* in presence of different N sources in PKV media supplemented with TCP ($100 \text{ mg P}_2\text{O}_5 \text{ 100 ml}^{-1}$) on different time intervals, a) 3rd day, b) 5th day and c) 7th day of incubation.

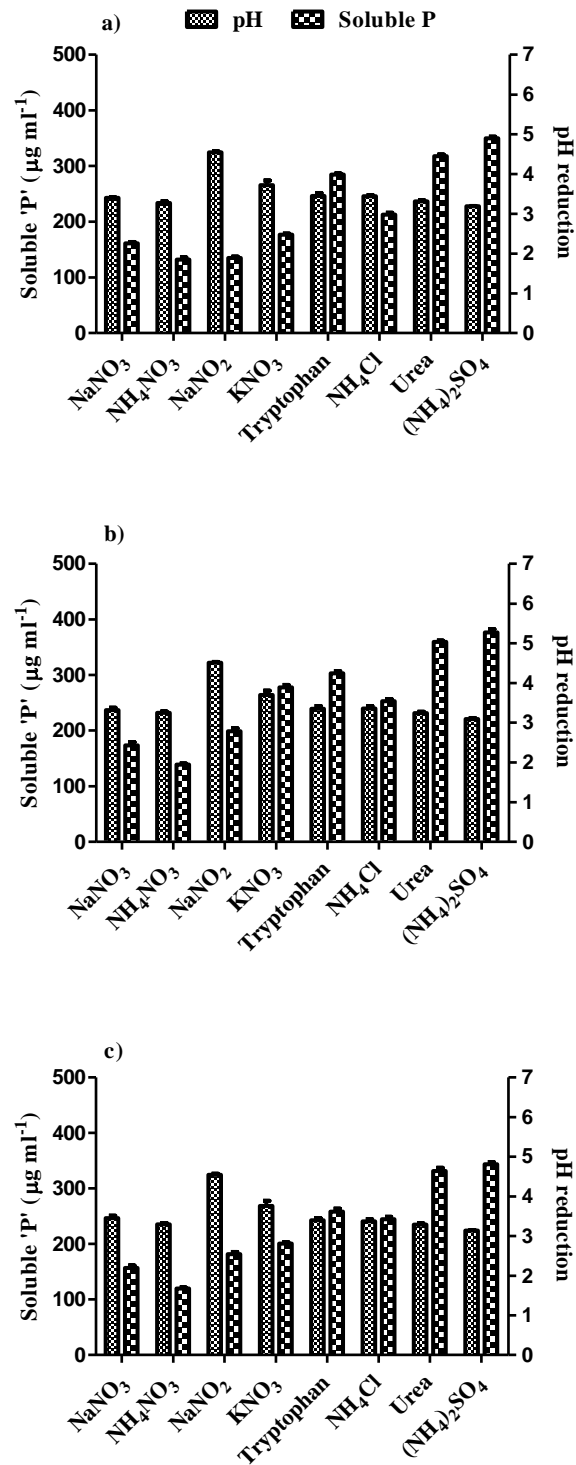


Fig. 5.4 Influence of N sources on pH reduction and soluble P by *Pseudomonas plecoglossicida* in presence of different N sources in PKV media supplemented with TCP (100 mg P₂O₅ 100 ml⁻¹) on different time intervals, a) 3rd day, b) 5th day and c) 7th day of incubation.

Table 5.5 Acid phosphatase, alkaline phosphatase and phytase enzyme activities by *Pantoea cypripedii* in presence of different N sources in PKV broth supplemented with tri-calcium phosphate (100 mg P₂O₅ 100 ml⁻¹).

Nitrogen sources	Acid phosphatase ($\mu\text{M ml}^{-1} \text{ h}^{-1}$)	Alkaline phosphatase ($\mu\text{M ml}^{-1} \text{ h}^{-1}$)	Phytase ($\mu\text{M ml}^{-1} \text{ h}^{-1}$)
NaNO ₃	66 ± 3de	39 ± 3cd	5667 ± 28b
NH ₄ NO ₃	73 ± 4cd	44 ± 1.5bc	4644 ± 88c
NaNO ₂	61 ± 5e	19 ± 5e	1264 ± 72f
KNO ₃	76 ± 4cd	22 ± 1.8e	3633 ± 44d
Tryptophan	81 ± 3bc	29 ± 3de	3552 ± 30d
NH ₄ Cl	76 ± 4cd	21 ± 3e	2517 ± 31e
Urea	89 ± 3ab	54 ± 11ab	4747 ± 102c
(NH ₄) ₂ SO ₄	95 ± 3a	62 ± 4a	6998 ± 104a
LSD (<i>P</i> <0.05)	6.31	8.78	120

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 5.6 Acid phosphatase, alkaline phosphatase and phytase enzyme activities by *Pseudomonas plecoglossicida* in presence of different N sources in PKV broth supplemented with tri-calcium phosphate (100 mg P₂O₅ 100 ml⁻¹).

Nitrogen sources	Acid phosphatase ($\mu\text{M ml}^{-1} \text{ h}^{-1}$)	Alkaline phosphatase ($\mu\text{M ml}^{-1} \text{ h}^{-1}$)	Phytase ($\mu\text{M ml}^{-1} \text{ h}^{-1}$)
NaNO ₃	58 ± 8cd	27 ± 6d	5866 ± 82b
NH ₄ NO ₃	71 ± 7bc	51 ± 7bc	2865 ± 554d
NaNO ₂	78 ± 5ab	45 ± 6c	1845 ± 102e
KNO ₃	43 ± 4de	13 ± 5d	1894 ± 68e
Tryptophan	47 ± 3de	43 ± 4c	2698 ± 34d
NH ₄ Cl	40 ± 7e	59 ± 1.3ab	1849 ± 37e
Urea	87 ± 3ab	60 ± 1.8ab	4857 ± 17c
(NH ₄) ₂ SO ₄	93 ± 7a	68 ± 5a	6825 ± 76a
LSD (<i>P</i> <0.05)	10	8.65	356

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 5.7 Effect of different nitrogen sources on organic acid production by *Pantoea cypripedii* in Pikovskaya's broth amended with tri-calcium phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Nitrogen sources	Oxalic acid (n mol/ml)	Citric acid (n mol/ml)	Malic acid (n mol/ml)	Succinic acid (n mol/ml)	Formic acid (n mol/ml)	Acetic acid (n mol/ml)	Gluconic acid (n mol/ml)
NaNO ₃	2602±17a	0	0	0	0	0	0
NH ₄ NO ₃	171 ± 019e	0	0	0	0	0	4153 ± 1.3d
NaNO ₂	219 ± 2d	0	0	0	0	0	4296 ± 6c
KNO ₃	253 ± 0.8c4	0	0	0	0	0	4371 ± 25b
Tryptophan	12 ± 0.02g	0	0	0	0	0	3966 ± 44e
NH ₄ Cl	290 ± 8b	0	0	0	0	0	1921 ± 28f
Urea	15 ± 0.22g	0	0	0	0	0	1566 ± 5g
(NH ₄) ₂ SO ₄	126 ± 0.65f	0	0	0	0	3484 ± 2	5514 ± 29a
LSD (<i>P</i> <0.05)	11.26						39.63

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 5.8 Effect of different nitrogen sources on organic acid production by *Pseudomonas plecoglossicida* in Pikovskaya's broth amended with tri-calcium phosphate (equivalent to 100 mg P₂O₅/100 ml).

Isolates	Oxalic acid (n mol/ml)	Citric acid (n mol/ml)	Malic acid (n mol/ml)	Succinic acid (n mol/ml)	Formic acid (n mol/ml)	Acetic acid (n mol/ml)	Gluconic acid (n mol/ml)
NaNO ₃	932 ± 7b	0	0	0	0	0	1573 ± 14e
NH ₄ NO ₃	1168 ± 18a	0	131 ± 2b	0	0	0	0
NaNO ₂	77 ± 2d	5 ± 0.18b	4417 ± 5a	0	0	0	0
KNO ₃	912 ± 14b	0	0	0	0	0	7876 ± 90d
Tryptophan	56 ± 0.02d	0	0	0	0	0	8928 ± 167c
NH ₄ Cl	137 ± 2c	0	0	0	0	0	7737 ± 78d
Urea	121 ± 0.13c	0	0	0	0	0	11360 ± 14b
(NH ₄) ₂ SO ₄	57 ± 0.09d	618 ± 0.32a	0	74 ± 1.17	0	56 ± 3	12335 ± 5a
LSD (<i>P</i> <0.05)	14.56	0.24	3.49				126

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

5.2 Effect of different carbon and nitrogen sources on phosphate solubilization by fungal isolates

To study the effect of different carbon and nitrogen sources on P solubilization by fungal isolates (*Aspergillus tubingensis* and *Aspergillus niger*), fructose, arabinose, galactose, mannitol, maltose, lactose, sucrose, xylose and glucose were used as carbon sources and NaNO_3 , NH_4NO_3 , NaNO_2 , KNO_3 , tryptophan, NH_4Cl , urea and $(\text{NH}_4)_2\text{SO}_4$ were used as nitrogen sources in Pikovskaya's broth supplemented with tri-calcium phosphate (100 mg P_2O_5 100 ml⁻¹).

5.2.1 Carbon sources

A. tubingensis was grown in different carbon sources for different time interval and it showed maximum P solubilization on day three of incubation in all the carbon sources (Fig. 5.5). Maximum P solubilization was observed when glucose was used as a carbon source. Maximum pH reduction was observed with increased in P solubilization. *A. tubingensis* in presence of different carbon sources showed pattern of P solubilization as glucose > mannitol > sucrose > xylose > maltose > fructose > galactose > arabinose > lactose (Fig 5.5). *A. tubingensis* was able to produce acid phosphatase, alkaline phosphatase and phytase enzyme in the presence of different carbon sources but maximum enzyme production was observed in medium having glucose as a carbon source (Table 5.9). Results in Table 5.9 indicated that nature of carbon sources affect the enzyme actives in culture broth. Main organic acids produced by *A. tubingensis* in presence of different carbon sources were oxalic acid, succinic acid and gluconic acid. Maximum organic acid production was found in presence of glucose as a carbon source

In case of *A. niger* (Fig. 5.6) the pattern of P solubilization in presence of different carbon sources was glucose > mannitol > maltose > xylose > sucrose > fructose > galactose >

arabinose > lactose. Maximum P solubilization was observed in presence of glucose. Table 5.11 showed that maximum acid phosphatase, alkaline phosphatase and phytase enzyme production was observed in medium contained glucose. Main organic acids produced by *A. niger* were oxalic acid, citric acid, malic acid and gluconic acid and maximum acid production was observed in presence of glucose compared to other carbon sources (Table 5.12). Table 5.9 and 5.11 showed that fungal dry biomass was also varied with different carbon sources present in growth medium. Maximum fungal growth was observed in glucose compared to other carbon sources.

5.2.2 Nitrogen sources

Both fungal isolates were able to solubilize tri-calcium phosphate in presence of different nitrogen sources. Isolates were tested for acid phosphatase, alkaline phosphatase and phytase enzyme production and for organic acid production. P solubilization, enzyme production and organic acid production in both the fungal isolates were varied with different nitrogen sources.

P solubilization in presence of different nitrogen source in *A. tubingensis* was as follows: $(\text{NH}_4)_2\text{SO}_4 > \text{KNO}_3 > \text{NH}_4\text{NO}_3 > \text{NH}_4\text{Cl} > \text{urea} > \text{tryptophan} > \text{NaNO}_3 > \text{NaNO}_2$ (Fig. 5.7). Maximum pH reduction was observed in ammonium sulphate amended PKV broth. Acid phosphatase, alkaline phosphatase and phytase enzyme production was also higher in presence of ammonium sulphate (Table 5.13). Main organic acids produced by *A. tubingensis* were oxalic acid, citric acid, malic acid, succinic acid and gluconic acid.

In case of *A. niger*, P solubilization in the presence of different nitrogen sources was, $(\text{NH}_4)_2\text{SO}_4 > \text{KNO}_3 > \text{NaNO}_2 > \text{NaNO}_3 > \text{tryptophan} > \text{NH}_4\text{Cl} > \text{urea} > \text{NH}_4\text{NO}_3$ (Fig. 5.8). Maximum P solubilization was observed in medium having ammonium sulphate compared to other nitrogen sources. Acid phosphatase, alkaline phosphatase and phytase enzyme production was higher in presence of ammonium sulphate compared to other nitrogen

sources. Main organic acids produced by *A. niger* were oxalic acid, malic acid and gluconic acid (Table 5.16). Results in Table 5.13 and 5.15 showed that fungal dry biomass was also varied in presence of different nitrogen sources. Maximum fungal growth was observed in ammonium sulphate compared to other nitrogen sources.

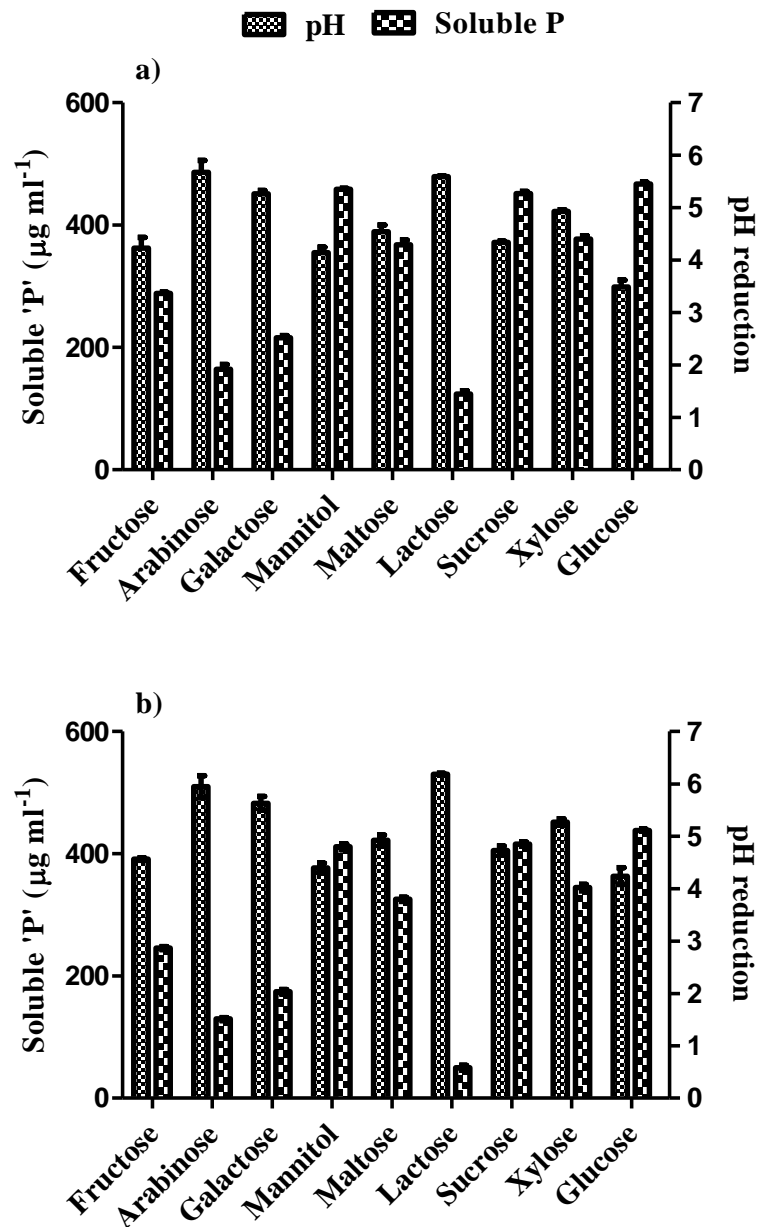


Fig. 5.5 Influence of different carbon sources on pH reduction and P solubilization by *Aspergillus tubingensis* in PKV media supplemented with tri-calcium phosphate (100 mg P₂O₅ 100 ml⁻¹) at different time intervals, a) 3rd day and b) 5th day of incubation.

Table 5.9 Acid phosphatase, alkaline phosphatase and phytase enzyme activity by *Aspergillus tubingensis* in presence of different C sources in PKV broth supplemented with tri-calcium phosphate (100 mg P₂O₅ 100 ml⁻¹).

Carbon sources	Acid phosphatase (μM ml ⁻¹ h ⁻¹)	Alkaline phosphatase (μM ml ⁻¹ h ⁻¹)	Phytase (μM ml ⁻¹ h ⁻¹)	Dry weight (g/100 ml)
Fructose	91 ± 0.7c	48 ± 2.2c	7854 ± 14d	0.428 ± 0.04a
Arabinose	69 ± 1.8d	44 ± 2.5c	6685 ± 33e	0.200 ± 0.12b
Galactose	81 ± 13cd	45 ± 2.0c	7520 ± 25d	0.314 ± 0.04ab
Mannitol	141 ± 1.8a	87 ± 2.2a	9661 ± 19b	0.429 ± 0.02a
Maltose	117 ± 2.2b	83 ± 1.1a	8788 ± 611c	0.442 ± 0.07a
Lactose	38 ± 0.4e	25 ± 1.3d	2729 ± 28f	0.215 ± 0.01b
Sucrose	118 ± 3.1b	83 ± 2.2a	9400 ± 28b	0.370 ± 0.06ab
Xylose	116 ± 1.8b	73 ± 1.7b	9174 ± 25bc	0.406 ± 0.02ab
Glucose	148 ± 2.2a	87 ± 3.1a	10252 ± 36a	0.511 ± 0.15a
LSD (P<0.05)	7.9	3.86	352	0.13

Values are Mean ± SD (n =3). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

Table 5.10 Effect of different carbon sources on organic acid production by *Aspergillus tubingensis* in Pikovskaya's broth amended with tri-calcium phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Isolates	Oxalalic acid (n mol/ml)	Citric acid (n mol/ml)	Malic acid (n mol/ml)	Succinic acid (n mol/ml)	Formic acid (n mol/ml)	Aceticacid (n mol/ml)	Gluconic acid (n mol/ml)
Fructose	21 ± 0.09de	0	0	1524 ± 27b	0	0	781 ± 1.2h
Arabinose	14 ± 0.05g	0	0	327 ± 2f	0	0	976 ± 1.67g
Galactose	17 ± 0.08f	0	0	0	0	0	3166 ± 3.4d
Mannitol	21 ± 0.09e	0	0	1120 ± 9d	0	0	5557 ± 4.3b
Maltose	67 ± 0.68a	0	0	0	0	0	2658 ± 15e
Lactose	11 ± 0.02h	110 ± 0.38b	0	0	0	0	690 ± 0.9i
Sucrose	30 ± 0.02b	129 ± 0.38a	0	706 ± 17e	0	0	3402 ± 14c
Xylose	22 ± 0.19d	0	540 ± 9	1218 ± 16c	0	0	1173 ± 39f
Glucose	25 ± 0.65c	0	0	2348 ± 3a	0	0	6167 ± 15a
LSD (<i>P</i> <0.05)	0.66	0.33		21.07			27

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

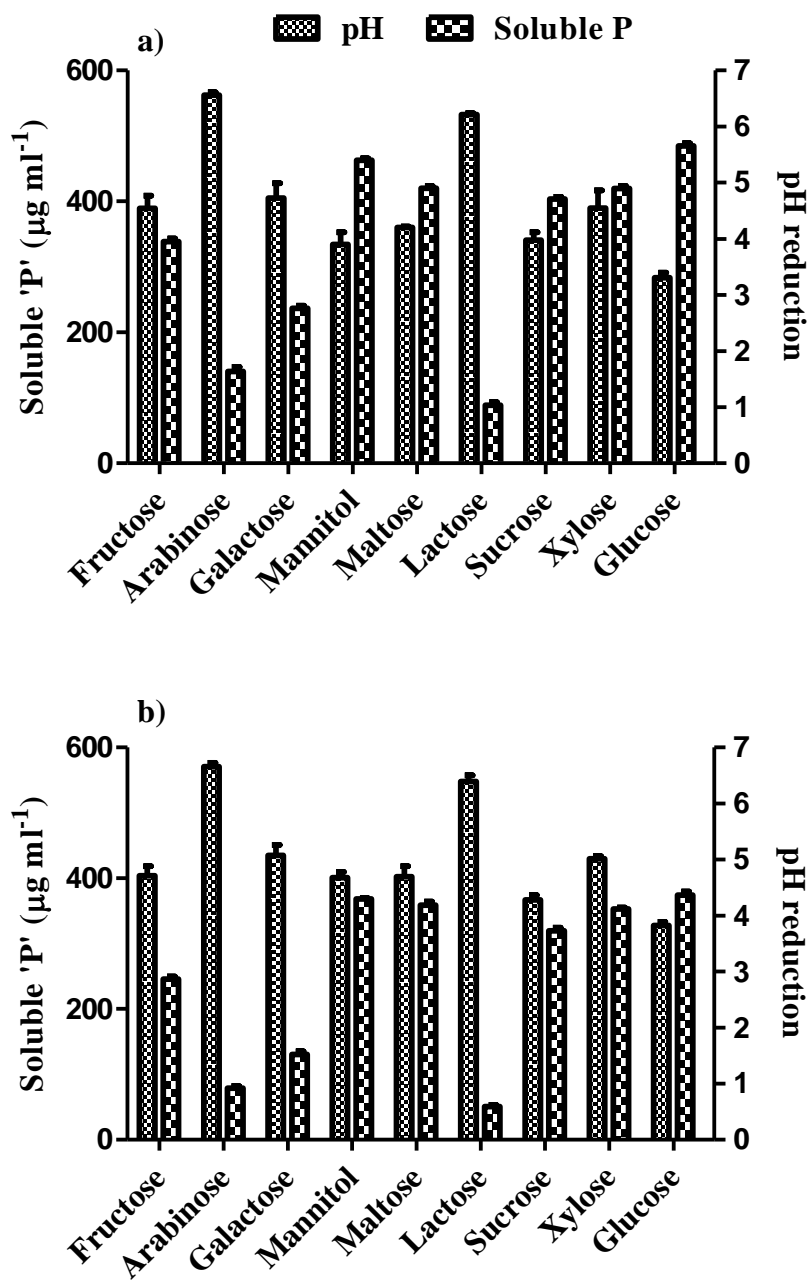


Fig. 5.6 Influence of carbon sources on pH reduction and P solubilization by *Aspergillus niger* in PKV media supplemented with tri-calcium phosphate (100 mg P₂O₅ 100 ml⁻¹), a) 3rd day and b) 5th day of incubation.

Table 5.11 Acid phosphatase, alkaline phosphatase and phytase enzyme activities by *Aspergillus niger* in presence of different C sources in PKV broth supplemented with tri-calcium phosphate (100 mg P₂O₅ 100 ml⁻¹).

Carbon sources	Acid phosphatase (μM ml ⁻¹ h ⁻¹)	Alkaline phosphatase (μM ml ⁻¹ h ⁻¹)	Phytase (μM ml ⁻¹ h ⁻¹)	Dry weight (g/100 ml)
Fructose	114 ± 2.4cd	51 ± 8.6c	7251 ± 17d	0.425 ± 0.06a
Arabinose	51 ± 2.9f	17 ± 3.8d	6687 ± 268e	0.174 ± 0.11bc
Galactose	76 ± 1.2e	62 ± 3.4b	7237 ± 22d	0.184 ± 0.03bc
Mannitol	109 ± 1.9d	77 ± 0.7a	10221 ± 33a	0.423 ± 0.06a
Maltose	132 ± 1.3b	80 ± 1.8a	9062 ± 26c	0.448 ± 0.03a
Lactose	35 ± 0.9g	13 ± 1.4d	4144 ± 25f	0.137 ± 0.06c
Sucrose	118 ± 1.8c	80 ± 1.8a	9300 ± 33bc	0.341 ± 0.02ab
Xylose	118 ± 3.2c	83 ± 0.9a	9532 ± 22b	0.398 ± 0.08a
Glucose	141 ± 2.0a	86 ± 0.9a	10390 ± 19a	0.476 ± 0.06a
LSD (<i>P</i> <0.05)	3.55	5.96	159	0.107

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 5.12 Effect of different carbon sources on organic acid production by *Aspergillus niger* in Pikovskaya's broth amended with tri-calcium phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Isolates	Oxalic acid (n mol/ml)	Citric acid (n mol/ml)	Malic acid (n mol/ml)	Succinic acid (n mol/ml)	Formic acid (n mol/ml)	Acetic acid (n mol/ml)	Gluconic acid (n mol/ml)
Fructose	42 ± 1bc	33 ± 0.12d	0	0	0	0	4456 ± 14f
Arabinose	0	0	5 ± 0.17d	0	0	0	2598 ± 3h
Galactose	0	214 ± 9c	0	0	0	0	3882 ± 30g
Mannitol	41 ± 1.1c	722 ± 0.4a	333 ± 0.9b	0	0	72 ± 0.57b	4871 ± 23e
Maltose	10 ± 0.11d	0	0	0	0	0	6075 ± 29b
Lactose	6 ± 0.06e	0	0	0	0	0	1123 ± 8i
Sucrose	0	210 ± 0.6c	0	0	51 ± 0.6	0	5666 ± 30d
Xylose	43 ± 0.06b	0	68 ± 0.95c	0	0	0	5826 ± 6c
Glucose	57 ± 0.7a	661 ± 4b	648 ± 2a	135 ± 2	0	131 ± 4a	7882 ± 21a
LSD (<i>P</i> <0.05)	1.14	5.93	1.78			2.33	36

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

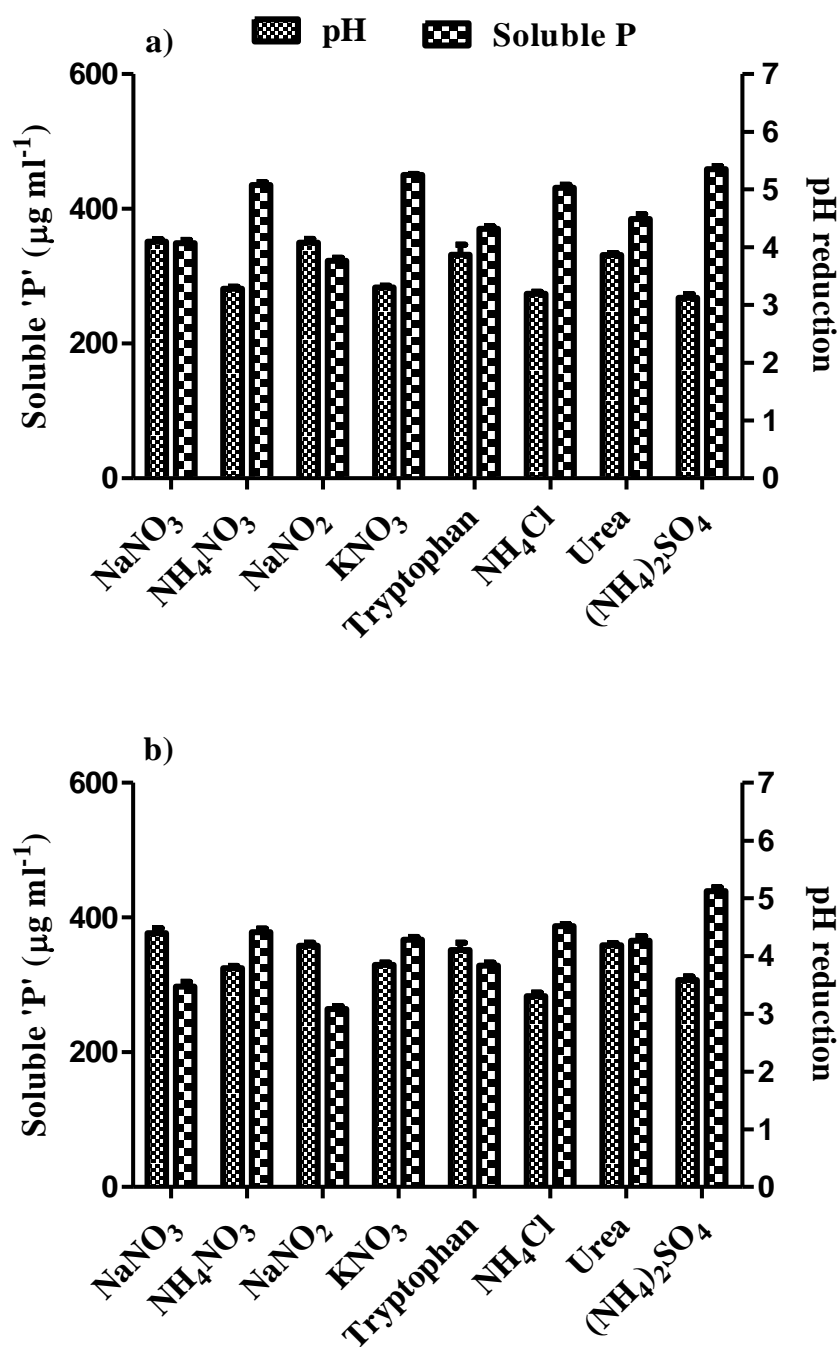


Fig. 5.7 pH reductions and phosphate solubilization by *Aspergillus tubingensis* in presence of different N sources in PKV media supplemented with TCP (100 mg P₂O₅ 100 ml⁻¹) on different days of incubation, a) 3rd day and b) 5th day.

Table 5.13 Acid phosphatase, alkaline phosphatase and phytase enzyme activity by *Aspergillus tubingensis* in presence of different N sources in PKV broth supplemented with tri-calcium phosphate (100 mg P₂O₅ 100 ml⁻¹).

Nitrogen sources	Acid phosphatase ($\mu\text{M ml}^{-1} \text{h}^{-1}$)	Alkaline phosphatase ($\mu\text{M ml}^{-1} \text{h}^{-1}$)	Phytase ($\mu\text{M ml}^{-1} \text{h}^{-1}$)	Dry weight (g/100 ml)
NaNO ₃	128 ± 0.9cde	73 ± 2.8bcd	8416 ± 50c	0.413 ± 0.01b
NH ₄ NO ₃	125 ± 1.8e	58 ± 3.0f	9150 ± 52b	0.395 ± 0.03b
NaNO ₂	128 ± 1.8de	62 ± 4.3ef	7670 ± 30e	0.368 ± 0.02b
KNO ₃	139 ± 3.6b	79 ± 2.4ab	8357 ± 30c	0.297 ± 0.10b
Tryptophan	134 ± 2.4bcd	66 ± 1.4de	6739 ± 22f	0.418 ± 0.02b
NH ₄ Cl	134 ± 3.4bc	76 ± 1.3bc	9203 ± 80b	0.413 ± 0.04b
Urea	1.31 ± 2.2cde	69 ± 2.0cd	7900 ± 22d	0.407 ± 0.02b
(NH ₄) ₂ SO ₄	147 ± 1.4a	83 ± 1.4a	10462 ± 14a	0.557 ± 0.05a
LSD (<i>P</i> <0.05)	4.09	4.31	74	0.077

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 5.14 Effect of different nitrogen sources on organic acid production by *Aspergillus tubingensis* in Pikovskaya's broth amended with tri-calcium phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Isolates	Oxalic acid (n mol/ml)	Citric acid (n mol/ml)	Malic acid (n mol/ml)	Succinic acid (n mol/ml)	Formic acid (n mol/ml)	Acetic acid (n mol/ml)	Gluconic acid (n mol/ml)
NaNO ₃	51 ± 0.9d	0	52 ± 1.5d	0	0	0	4437 ± 31f
NH ₄ NO ₃	1175 ± 0.19b	0	6 ± 0.24f	0	0	0	6562 ± 4d
NaNO ₂	0	5112 ± 35a	189 ± 6c	0	0	0	0
KNO ₃	1449 ± 14a	0	31 ± 0.24e	759 ± 46b	0	0	6733 ± 8b
Tryptophan	27 ± 0.5e	0	726 ± 7a	0	0	0	3452 ± 42g
NH ₄ Cl	100 ± 0.05c	0	28 ± 0.28e	0	0	0	6659 ± 7c
Urea	0	2004 ± 128b	0f	372 ± 7c	0	0	4513 ± 42e
(NH ₄) ₂ SO ₄	11 ± 0.03f	11 ± 0.29c	610 ± 2b	1897 ± 16a	0	0	7189 ± 0.8a
LSD (<i>P</i> <0.05)	8.35	82	5.89	30			42

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

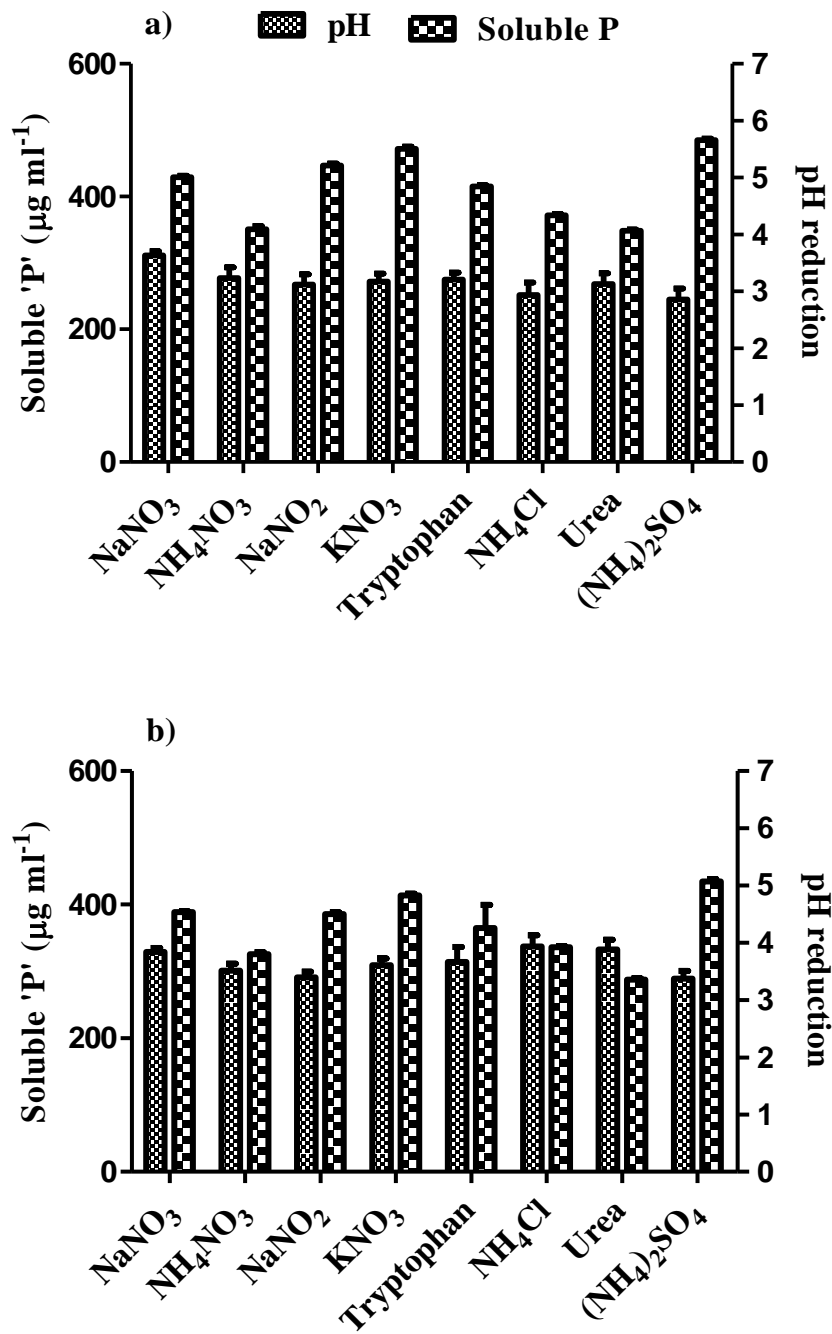


Fig. 5.8 Influence of N sources on pH reduction by *Aspergillus niger* in presence of different N sources in PKV media having TCP (100 mg P₂O₅ 100 ml⁻¹) on different intervals, a) 3rd day, b) 5th day of incubation.

Table 5.15 Acid phosphatase, alkaline phosphatase and phytase enzyme activity by *Aspergillus niger* in presence of different N sources in PKV broth supplemented with tri-calcium phosphate (100 mg P₂O₅ 100 ml⁻¹).

Nitrogen Sources	Acid phosphatase (μM ml ⁻¹ h ⁻¹)	Alkaline phosphatase (μM ml ⁻¹ h ⁻¹)	Phytase (μM ml ⁻¹ h ⁻¹)	Dry weight (g/100 ml)
NaNO ₃	126 ± 1.6de	63 ± 2.6bc	8593 ± 28c	0.407 ± 0.02a
NH ₄ NO ₃	118 ± 1.4f	57 ± 1.5c	7933 ± 22g	0.425 ± 0.01a
NaNO ₂	134 ± 2.7c	72 ± 2.7ab	8481 ± 39d	0.403 ± 0.02a
KNO ₃	145 ± 2.9b	67 ± 8.0ab	7022 ± 48h	0.385 ± 0.02a
Tryptophan	131 ± 4.2cd	69 ± 3.0ab	9295 ± 28b	0.436 ± 0.02a
NH ₄ Cl	129 ± 2.2cde	72 ± 1.0ab	8365 ± 22e	0.401 ± 0.03a
Urea	121 ± 2.0ef	64 ± 1.5bc	8187 ± 33f	0.397 ± 0.02a
(NH ₄) ₂ So ₄	159 ± 3.2a	77 ± 2.0a	10050 ± 17a	0.467 ± 0.07a
LSD (<i>P</i> <0.05)	4.54	6.10	54	0.055

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 5.16 Effect of different nitrogen sources on organic acid production by *Aspergillus niger* in Pikovskaya's broth amended with tri-calcium phosphate (equivalent to 100 mg P₂O₅ 100 ml⁻¹).

Isolates	Oxalic acid (n mol/ml)	Citric acid (n mol/ml)	Malic acid (n mol/ml)	Succinic acid (n mol/ml)	Formic acid (n mol/ml)	Acetic acid (n mol/ml)	Gluconic acid (n mol/ml)
NaNO ₃	72 ± 0.22f	36 ± 0.38b	0	0	0	0	7046 ± 33c
NH ₄ NO ₃	394 ± 9c	0	0	15 ± 5b	0	0	3639 ± 29h
NaNO ₂	278 ± 9d	0	9 ± 0.6e	14 ± 6b	0	0	6971 ± 2d
KNO ₃	544 ± 8b	0	102 ± 2d	0	0	0	7281 ± 3b
Tryptophan	1300 ± 0.8a	0	782 ± 15a	0	0	0	6425 ± 11f
NH ₄ Cl	188 ± 0.9e	0	427 ± 12b	0	0	0	3737 ± 0.85g
Urea	83 ± 1.23f	0	328 ± 3c	0	0	0	6493 ± 15e
(NH ₄) ₂ SO ₄	39 ± 0.55g	547 ± 7a	778 ± 18a	134 ± 3a	0	65 ± 0.09	7628 ± 9a
LSD (<i>P</i> <0.05)	9.18	4.24	15	4.9			30

Values are Mean ± SD (*n* =3). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

5.3 Plant growth promotion activities of bacteria isolates

Bacterial isolates *P. cyripedii* and *P. plecoglossicida* were tested for their ability to produce indole acetic acid (IAA), hydrogen cyanide (HCN), siderophore production and nitrogen fixation ability.

5.3.1 Indole acetic acid production

Both the bacterial isolates were tested for IAA production with and without addition of L-tryptophan in growth medium. Indole acetic acid (IAA) production was detected in both isolates with and without addition of L-tryptophan, but the rate of IAA production was higher in presence of L-tryptophan. Maximum production was observed at day five of incubation in nutrient broth supplemented with 0.1 % L-tryptophan. IAA production in presence of L-tryptophan was found to be more in case of *P. cyripedii* (92.9 $\mu\text{g ml}^{-1}$) compared to *P. plecoglossicida* (26.9 $\mu\text{g ml}^{-1}$) (Table 5.17).

Table 5.17 Indole acetic acid production by selected phosphate-solubilizing bacteria with and without tryptophan.

Isolates	Without tryptophan ($\mu\text{g ml}^{-1}$)	With tryptophan ($\mu\text{g ml}^{-1}$)
<i>Pantoea cyripedii</i>	7.6 \pm 0.42a	92.9 \pm 0.44a
<i>Pseudomonas plecoglossicida</i>	6.8 \pm 0.19b	26.9 \pm 0.04b
LSD ($P < 0.05$)	0.73	0.93

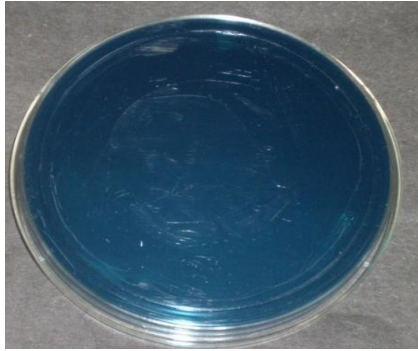
Values are Mean \pm SD ($n = 3$). Means sharing a common letter within the column are not significantly different at $P < 0.05$.

5.3.2 Siderophore production by bacterial isolates

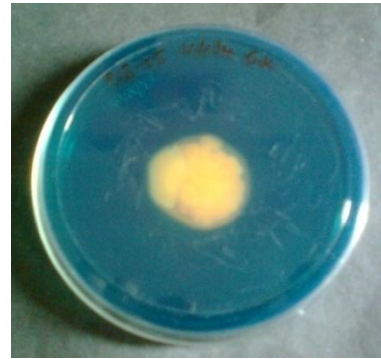
Pantoea cyripedii and *Pseudomonas plecoglossicida* were found to be positive for the siderophore production. Siderophore production was confirmed by the development of an orange halo zone around the bacterial colonies on CAS agar plate (Plate 5.1). Siderophore production activity of both the isolates was almost similar (1.5 and 1.6) as calculated by Z/C ratio where Z is the colony diameter plus orange halo zone diameter and C is the diameter of colony. Plate 5.2 showed the siderophore production by both the isolates in CAS broth in which after three days of incubation, color of broth was changed from blue to red-orange confirming the production of siderophore. The supernatant was further tested for the detection of catechol and hydroxamate type of siderophores, respectively, as CAS assay does not indicate the type of siderophores being produced. When 3 ml of freshly prepared 2 % of aqueous FeCl_3 solution was added to 1 ml of culture supernatant of both the isolates, and the absorbance between 200-600 nm was recorded where maximum absorbance was obtained at 425 nm (Fig.5.9). This indicates the hydroxamate nature of siderophore produced by both isolates.

5.3.3 Hydrogen cyanide (HCN) production by bacterial isolates

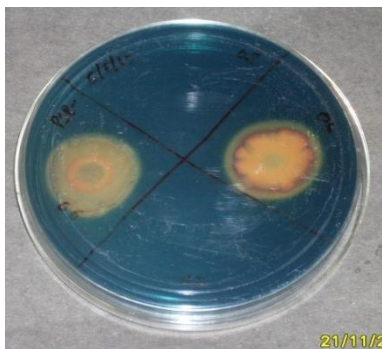
Both the isolates were tested for HCN production. Results in Plate 5.3 showed that, there was no change in color of filter paper from yellow to reddish brown (in nutrient agar plates supplemented with glycine (4.4 g l^{-1}), a whatman no. 1 filter paper soaked in 2 % sodium carbonate in 0.5 % picric acid solution was placed inside the lid of Petri plates) after three days of incubation that confirmed the negative results for HCN production. Hence both the isolates *P. cyripedii* and *P. plecoglossicida* were negative for HCN production.



a)



b)



c)



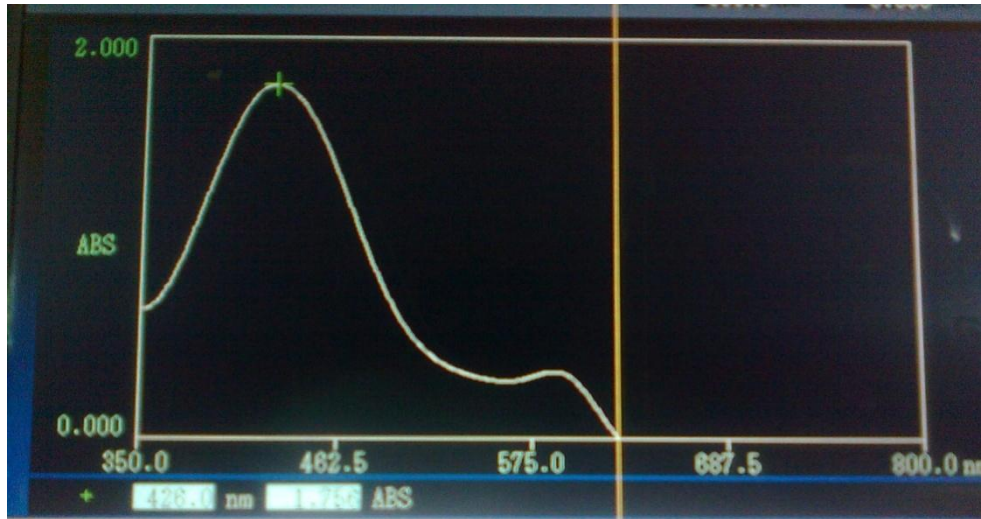
d)

Plate 5.1 a) Control (Chromo Azurol 'S' agar plate). b), c) and d) point inoculation of P-solubilizing bacteria forming pink colored zone surrounding the colony (Siderophore production).

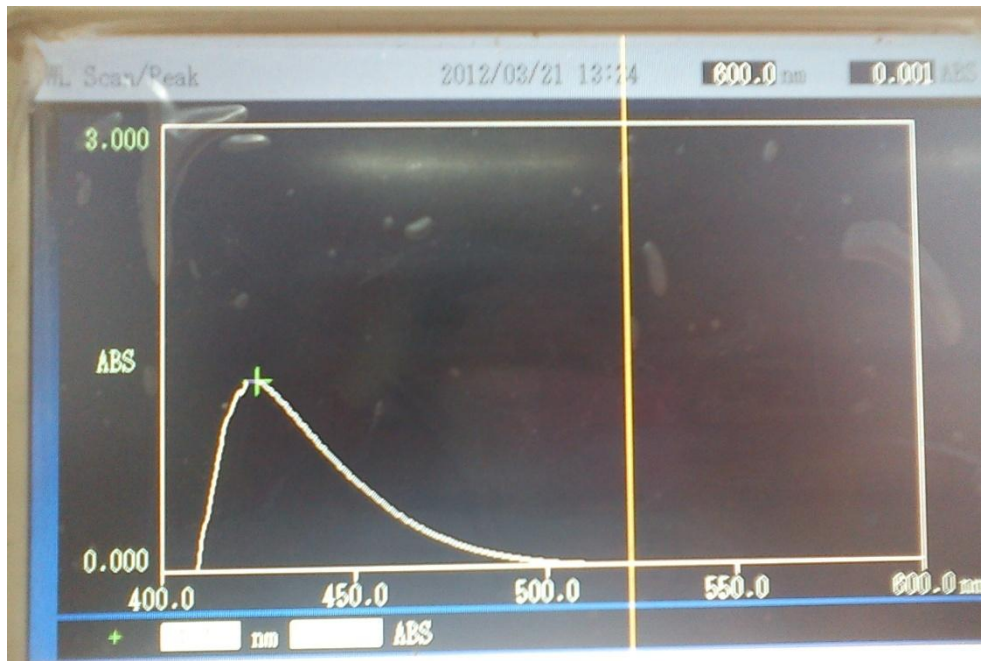


a) Control b) PSB-3 c) PSB-5

Plate 5.2 a) Control (un-inoculated CAS broth of blue color). b) and c) in case of bacterial isolates color of CAS broth was converted from blue to red-orange indicated that these isolates are siderophore producers.

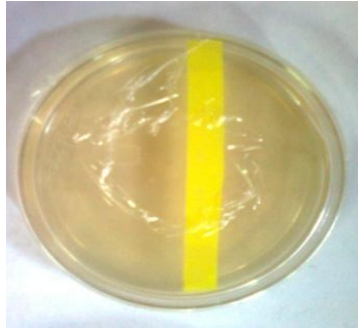


a)

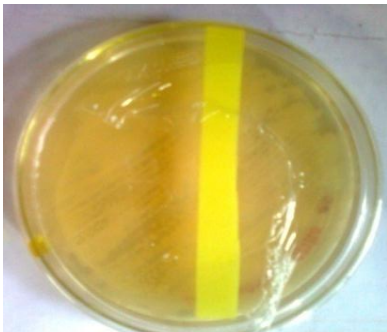


b)

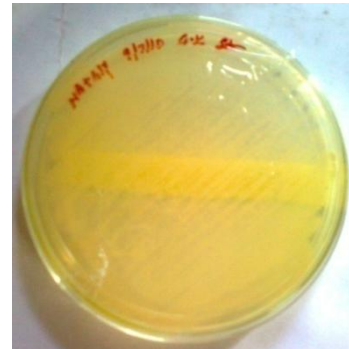
Fig. 5.9 Absorption spectrum of siderophore by a) *Pantoea cyripedii* b) *Pseudomonas plecoglossicida*. Absorption between 420-450 nm indicates hydroxamate type of siderophore.



a)



b)



c)

Plate 5.3 a) Un-inoculated control (nutrient agar plate amended with glycine, yellow color strip of filter paper with picric acid solution). b) - c) selected bacterial inoculation; no change in color of filter paper from yellow to reddish brown indicates that there is no HCN production.

5.3.4 Nitrogen fixation ability of bacterial isolates

The ability of *Pantoea cyripedii* and *Pseudomonas plecoglossicida* to fix di-nitrogen was measured based on acetylene reduction activity. The bacterial culture (100 µl of OD₆₀₀ = 1.0) was inoculated in 10 ml of Jensen broth (Jensen 1942) in test tubes. After three days of incubation at 30 °C, the cotton plugs were replaced with a rubber stopper, and 1 ml of air was removed from the tube with a syringe and 1 ml of acetylene (0.1 atm) was injected into the tubes which were then incubated for 24 hours. One ml of the gas was sampled and the ethylene concentration was measured by gas chromatography. No acetylene to ethylene reduction was detected in gas chromatography indicating both the isolates were not able to fix nitrogen.

5.4 Plant growth promotion activities of fungal isolates

5.4.1 Indole acetic acid production

Both fungal isolates *Aspergillus tubingensis* and *Aspergillus niger* were tested for indole acetic acid production with and without addition of L-tryptophan in growth medium. IAA production was detected in both isolates with and without addition of L-tryptophan, but the rate of IAA production was higher in presence of L- tryptophan. Maximum production was observed after five days of incubation in Czapek's Dox broth supplemented with 0.1% L-tryptophan. IAA production in presence of L-tryptophan in case of *Aspergillus tubingensis* was 50 µg ml⁻¹ and in case of *Aspergillus niger* it was 57 µg ml⁻¹ (Table 5.18).

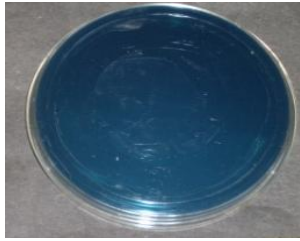
Table 5.18 Indole acetic acid production by selected phosphate-solubilizing fungi with and without tryptophan.

Isolates	Without tryptophan ($\mu\text{g ml}^{-1}$)	With tryptophan ($\mu\text{g ml}^{-1}$)
<i>Aspergillus tubingensis</i>	18 \pm 0.14 b	50 \pm 0.23 b
<i>Aspergillus niger</i>	20 \pm 0.12 a	57 \pm 0.17a
LSD ($P < 0.05$)	0.29	0.47

Values are Mean \pm SD ($n = 3$). Means sharing a common letter within the column are not significantly different at $P < 0.05$.

5.4.2 Siderophore production

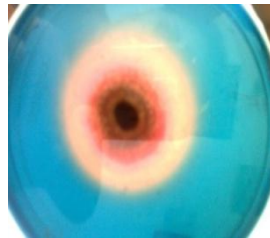
Both fungal isolates *A. tubingensis* and *A. niger* were positive for the siderophore production. Siderophore production was confirmed by the development of an orange halo zone around the fungal colonies on CAS agar plate (Plate 5.4). Plate 5.5 showed the siderophore production, by both the isolates in CAS broth in which after three days of incubation, the color of broth was changed from blue to red-orange confirming the production of siderophore by both isolates. The supernatant was further tested for the detection of type of siderophore, and the results indicated the hydroxamate nature of siderophore production by both fungal isolates (Fig.5.10).



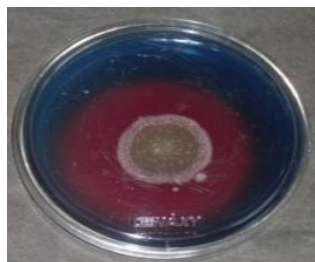
a)



b)



c)

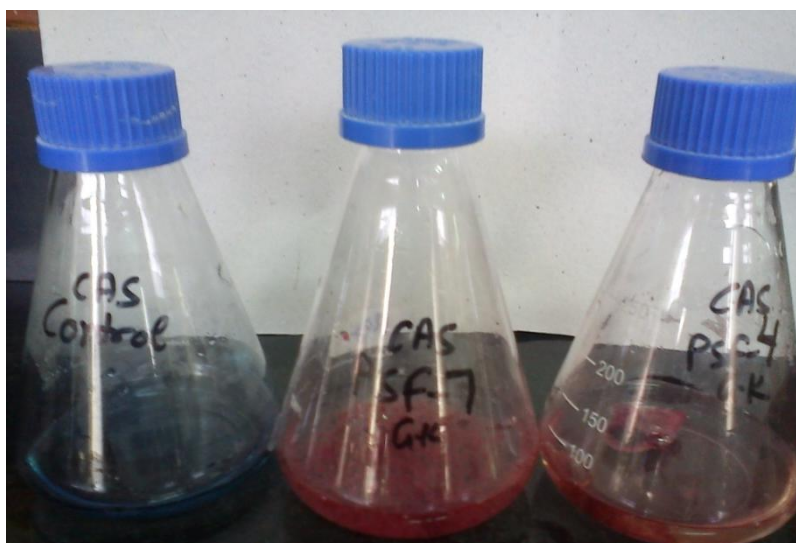


d)



e)

Plate 5.4 a) Control (Chromo Azurol 'S' agar plate). b) - e) point inoculation of P-solubilizing fungi forming pink colored zone surrounding the colony (Siderophore production).



a) Control

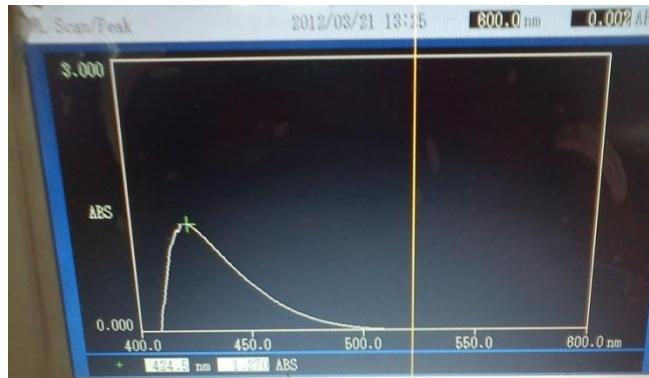
b) PSF-7

c) PSF-4

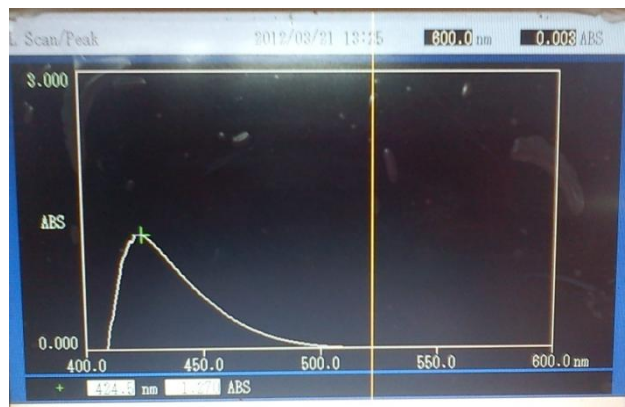
Plate 5.5 a) Control (un-inoculated CAS broth of blue color). b) and c) in case of selected fungal isolates color of CAS broth was converted from blue to red-orange indicated that these isolates are siderophore producers.

5.4.3 Hydrogen cyanide (HCN) production by fungal isolates

Both *A. tubingensis* and *A. niger* were tested for HCN production. Results in Plate 5.6 showed that there was no change in color of filter paper from yellow to reddish brown (in Czapek's Dox agar plates supplemented with glycine (4.4 g l^{-1}), a whatman no. 1 filter paper soaked in 2 % sodium carbonate in 0.5 % picric acid solution was placed inside the lid of Petri plates) after three days of incubation. Hence both the isolates *A. tubingensis* and *A. niger* were negative for HCN production.

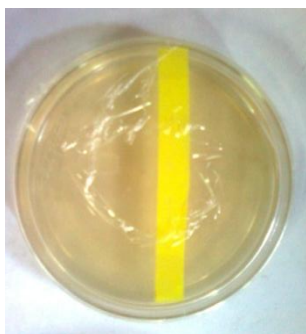


a)

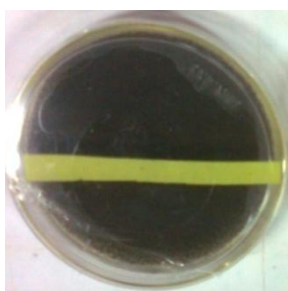


b)

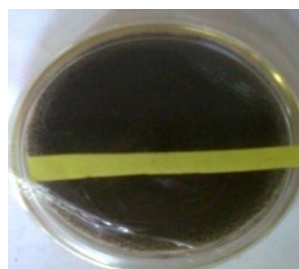
Fig. 5.10 Absorption spectrum of siderophore by a) *Aspergillus tubingensis* b) *Aspergillus niger*. Absorption between 420-450 nm indicates hydroxamate type of siderophore.



a)



b)



c)

Plate 5.6 a) Control (Czapek's Dox agar plate amended with glycine, yellow color strip of filter paper with picric acid solution. b) and c) fungal inoculation, no change in color of filter paper from yellow to reddish brown indicates that there is no HCN production.

5.5 Effect of buffering of media on P solubilization by phosphate-solubilizing bacteria

Phosphate solubilization efficiency of *P. cypripedii* (PSB-3) and *P. plecoglossicida* (PSB-5) was tested in buffered medium. For this, isolates were inoculated into Pikovskaya's broth having TCP and the pH of the growth medium was maintained at 7.0, 8.0 and 9.0 with 0.1 M Tris-HCl buffer. Phosphate solubilization by bacterial isolates was compared with un-buffered medium of pH 7.0, 8.0 and 9.0. Un-buffered PKV broth pH was adjusted to 7.0, 8.0, and 9.0 with 0.1 M NaOH. Normal PKV broth having TCP inoculated with *P. cypripedii* and *P. plecoglossicida* was kept as control. Acid phosphatase, alkaline phosphates and phytase enzyme production in buffered, un-buffered and control broth were also recorded. Results in Table 5.19 showed that, there was no effect of buffering of media on P solubilization when compared with un-buffered medium of different pH ranges and with control medium. P-concentration was increased with reduction in pH of the buffered and un-buffered medium. Results in Table 5.20 showed that both the isolates were able produced acid phosphatase, alkaline phosphatase and phytase enzyme in buffered and un-buffered conditions and this enzyme production was comparable with normal broth (control). The amount of enzyme production is decreased in growth medium with Tris-HCl buffer of pH 8.0 and 9.0 in comparison to respective un-buffered medium and control broth (Table 5.20).

Table 5.19 Quantitative screening of selected phosphate-solubilizing bacteria at different time intervals (days) in PKV broth amended with tri-calcium phosphate (TCP) (equivalent to 100 mg P₂O₅ 100 ml⁻¹) at different pH maintained with 0.1 M Tris-HCl buffer and with 0.1 M NaOH.

Isolates	5 th day		7 th day	
	pH	Soluble P (µg ml ⁻¹)	pH	Soluble P (µg ml ⁻¹)
Control (without buffer)				
PSB-3	3.65 ± 0.12	469 ± 5	3.97 ± 0.09	428 ± 6
PSB-5	3.68 ± 0.01	457 ± 6	4.05 ± 0.09	419 ± 3
Buffer pH 7				
PSB-3	3.75 ± 0.04	437 ± 2	4.10 ± 0.08	382 ± 2
PSB-5	3.71 ± 0.03	451 ± 8	4.12 ± 0.04	386 ± 4
Buffer pH 8				
PSB-3	4.08 ± 0.02	430 ± 6	4.13 ± 0.01	374 ± 10
PSB-5	3.99 ± 0.03	437 ± 3	4.17 ± 0.09	409 ± 3
Buffer pH 9				
PSB-3	4.20 ± 0.03	446 ± 5	4.59 ± 0.28	434 ± 11
PSB-5	4.31 ± 0.03	424 ± 4	4.36 ± 0.04	392 ± 6
0.1 M NaOH pH 7				
PSB-3	3.70 ± 0.06	446 ± 11	4.1 ± 0.06	380 ± 5
PSB-5	3.77 ± 0.15	478 ± 13	3.96 ± 0.04	392 ± 4
0.1 M NaOH pH 8				
PSB-3	3.70 ± 0.04	466 ± 9	4.09 ± 0.11	412 ± 5
PSB-5	3.87 ± 0.21	475 ± 10	4.20 ± 0.10	425 ± 5
0.1 M NaOH pH 9				
PSB-3	3.75 ± 0.06	474 ± 12	3.92 ± 0.08	429 ± 7
PSB-5	3.67 ± 0.04	481 ± 5	4.17 ± 0.09	420 ± 7

Values are Mean ± SD (*n* =3).

Table 5.20 Enzyme activity of selected phosphate-solubilizing bacteria in PKV broth amended with tri-calcium phosphate (TCP) (equivalent to 100 mg P₂O₅ 100 ml⁻¹) at different pH maintained with Tris buffer and with 0.1 M NaOH.

Isolates	Acid phosphatase ($\mu\text{M ml}^{-1} \text{h}^{-1}$)	Alkaline phosphatase ($\mu\text{M ml}^{-1} \text{h}^{-1}$)	Phytase ($\mu\text{M ml}^{-1} \text{h}^{-1}$)
Control (without buffer)			
PSB-3	98 \pm 5	56 \pm 2	6786 \pm 24
PSB-5	86 \pm 9	49 \pm 3	6457 \pm 31
Buffer pH 7			
PSB-3	82 \pm 4	54 \pm 3	6687 \pm 85
PSB-5	92 \pm 2	75 \pm 1	6614 \pm 31
Buffer pH 8			
PSB-3	64 \pm 3	51 \pm 1	6904 \pm 155
PSB-5	82 \pm 4	59 \pm 1	6768 \pm 100
Buffer pH 9			
PSB-3	95 \pm 2	52 \pm 4	5967 \pm 61
PSB-5	77 \pm 2	75 \pm 5	6702 \pm 51
0.1 M NaOH pH 7			
PSB-3	115 \pm 9	55 \pm 5	6895 \pm 74
PSB-5	97 \pm 2	60 \pm 3	7012 \pm 73
0.1 M NaOH pH 8			
PSB-3	89 \pm 18	58 \pm 6	6825 \pm 70
PSB-5	102 \pm 22	61 \pm 3	6805 \pm 187
0.1 M NaOH pH 9			
PSB-3	108 \pm 3	62 \pm 5	7108 \pm 129
PSB-5	98 \pm 3	71 \pm 1	7034 \pm 108

Values are Mean \pm SD ($n = 3$).

5.6 Effect of buffering of media on P solubilization of phosphate-solubilizing fungi

Aspergillus tubingensis (PSF-4) and *Aspergillus niger* (PSF-7) were inoculated in Pikovskaya's broth having TCP and pH of growth medium was maintained 7.0, 8.0 and 9.0 with 0.1 M Tris-HCl buffer. Similarly isolates were grown in un-buffered medium of pH 7.0, 8.0 and 9.0 adjusted with 0.1 M NaOH. Normal PKV broth having TCP (equivalent to 100 mg P₂O₅ 100 ml⁻¹) inoculated with *A. tubingensis* and *A. niger* was kept as control. P solubilization by isolates in buffered, un-buffered and control medium were recorded and it was observed that buffering of media does not affect the P solubilization when compared with control medium and un-buffered medium of different pH ranges (Table 5.21). pH reduction was observed with increased in soluble P concentration in buffered and un-buffered broth medium that is comparable with normal broth inoculated with isolates. Results in Table 5.21 showed that dry biomass of fungal culture in buffered, un-buffered and control broth was in same range. Both fungal isolates were able produced acid phosphatase, alkaline phosphatase and phytase enzymes in buffered and un-buffered conditions and this enzymes production was comparable with normal broth (control). Results presented in Table 5.22 showed that enzyme production in PKV growth medium was not affected with Tris-HCl buffer of pH 7.0, 8.0 and 9.0 when compared with enzyme activities of un-buffered growth medium of different pH and control broth.

Table 5.21 Quantitative screening of selected phosphate-solubilizing fungus at different time intervals (days) in PKV broth amended with tri-calcium phosphate (TCP) (equivalent to 100 mg P₂O₅ 100 ml⁻¹) at different pH maintained with 0.1 M Tris-HCl buffer and 0.1 M NaOH.

Isolates	5 th day		7 th day		Dry weight (g/100 ml)
	pH	Soluble P (µg ml ⁻¹)	pH	Soluble P (µg ml ⁻¹)	
Control (without buffer)					
PSF-4	2.57 ± 0.04	446 ± 3	3.81 ± 0.47	376±12	0.441±0.03
PSF-7	2.87 ± 0.63	492 ± 4	3.51 ± 0.06	422±6	0.414±0.02
Buffer pH 7					
PSF-4	2.63 ± 0.02	486 ± 4	3.82 ± 0.07	327 ± 11	0.432 ± 0.02
PSF-7	3.51 ± 0.02	448 ± 6	4.21 ± 0.33	380 ± 8	0.460 ± 0.08
Buffer pH 8					
PSF-4	3.75 ± 0.03	463 ± 5	4.40 ± 0.04	413 ± 5	0.458 ± 0.04
PSF-7	3.56 ± 0.07	447 ± 6	4.17 ± 0.06	418 ± 7	0.434 ± 0.06
Buffer pH 9					
PSF-4	3.68 ± 0.08	475 ± 4	4.59 ± 0.03	363 ± 5	0.452 ± 0.10
PSF-7	3.79 ± 0.03	452 ± 7	4.81 ± 0.02	339 ± 7	0.422 ± 0.06
0.1 M NaOH pH 7					
PSF-4	3.77 ± 0.11	465 ± 10	3.81 ± 0.13	358 ± 3	0.440 ± 0.05
PSF-7	3.66 ± 0.06	480 ± 2	3.95 ± 0.02	369 ± 2	0.449 ± 0.04
0.1 M NaOH pH 8					
PSF-4	3.63 ± 0.05	480 ± 17	3.75 ± 0.11	417 ± 7	0.431 ± 0.04
PSF-7	3.61 ± 0.07	486 ± 7	3.72 ± 0.11	386 ± 10	0.419 ± 0.06
0.1 M NaOH pH 9					
PSF-4	3.69 ± 0.07	445 ± 6	3.75 ± 0.08	393 ± 6	0.458 ± 0.05
PSF-7	3.56 ± 0.09	475 ± 3	3.74 ± 0.18	419 ± 5	0.442 ± 0.03

Values are Mean ± SD (*n* =3).

Table 5.22 Enzyme activity of selected phosphate solubilizing fungus in PKV broth amended with tri-calcium phosphate (TCP) (equivalent to 100 mg P₂O₅ 100 ml⁻¹) at different pH maintained with 0.1 M Tris-HCl buffer and 0.1 M NaOH.

Isolates	Acid phosphatase ($\mu\text{M ml}^{-1} \text{h}^{-1}$)	Alkaline phosphatase ($\mu\text{M ml}^{-1} \text{h}^{-1}$)	Phytase ($\mu\text{M ml}^{-1} \text{h}^{-1}$)
Control (without buffer)			
PSF-4	161 \pm 3	79 \pm 7	10894 \pm 33
PSF-7	140 \pm 3	90 \pm 1	10982 \pm 58
Buffer pH 7			
PSF-4	163 \pm 3	76 \pm 2	10973 \pm 28
PSF-7	149 \pm 2	91 \pm 6	11079 \pm 16
Buffer pH 8			
PSF-4	149 \pm 5	81 \pm 1	10532 \pm 61
PSF-7	153 \pm 3	89 \pm 5	10640 \pm 75
Buffer pH 9			
PSF-4	136 \pm 4	83 \pm 5	10449 \pm 77
PSF-7	146 \pm 1	71 \pm 4	10776 \pm 37
0.1 M NaOH pH 7			
PSF-4	147 \pm 7	81 \pm 2	10465 \pm 42
PSF-7	156 \pm 3	76 \pm 3	10510 \pm 33
0.1 M NaOH 8			
PSF-4	134 \pm 1	77 \pm 7	10662 \pm 44
PSF-7	145 \pm 3	84 \pm 2	10723 \pm 105
0.1 M NaOH 9			
PSF-4	128 \pm 2	86 \pm 3	10350 \pm 96
PSF-7	125 \pm 2	78 \pm 1	10601 \pm 99

Values are Mean \pm SD ($n = 3$).

Chapter 6

Field application of phosphate solubilizing microorganisms in organic farming

6.1 Role of phosphate-solubilizing bacteria in organic farming

Organic farming is an eco-friendly system of farming which helps to maintain health of soil in terms of soil biological fertility and productivity. Crop production in organic farming mainly depends on nutrients released as a result of mineralization processes in soils. An active soil microflora and a considerable pool of accessible nutrients are, therefore, important priorities in organic farming. Organic farming avoids the input of synthetic chemicals and their consequences. The build-up of a large and active soil microbial biomass is, therefore, critically important for sustaining the productivity of soils in organic farming system. A field experiment was conducted in which *Pantoea cypripedii* and *Pseudomonas plecoglossicida* isolates were inoculated alone or along with rock phosphate and compared with chemical fertilizer treatments. Maize and wheat was selected as test crops for field experiments. The site is situated at 31.65° N latitude and 76.26° E longitude and falls under sub-mountain undulating agroclimatic region of Punjab state. The region possesses dry sub humid type of climate. Soil was loamy sand (Typic Ustorthents), PH - 8.37, organic carbon - 0.42 %, organic matter - 0.7 mg, available P - 4.3 mg kg⁻¹, total P - 245 mg kg⁻¹ and total nitrogen - 0.035 %.

6.1.1 Field study with Maize crop

A field experiment of maize was conducted during first year of field study in an organic farm (Plate 6.1). Inoculation of *P. cypripedii* and *P. plecoglossicida* in maize crop with and without rock phosphate fertilization were tested for the improvement in crop yield and soil fertility. The shoot height, shoot and root dry biomass and grain yield was significantly increased due to inoculation and inoculation along with RP fertilization compared to un-inoculation control soil. Total P in plant tissues (grains, shoots and roots) was increased significantly in inoculated and RP fertilized treatments compared to control soil (Table 6.2). It was observed that plant growth parameters, grain yield and total P uptake was significantly increased in all the inoculated and

RP fertilized soil treatments, but the results were more pronounced when inoculation was done along with RP fertilization than individual inoculation and control. Grain yield was increased to 17.9 % with inoculation, 15.8 % with RP fertilization and 20.7 % when inoculation was done along with RP fertilization. On the other hand di-ammonium phosphate (DAP) chemical P fertilizer treatment increased grain yield up to 13.0 % compared to control. Similarly, total P uptake in grains, shoots and roots was increased due to inoculation and RP fertilization treatments but the results were more pronounced and significant when RP fertilization was done along with seed inoculation.

Effect of inoculation alone or along with RP fertilization and in chemical fertilizer treatments was tested on soil physiochemical properties, which played an important role in improvement of soil fertility such as organic carbon, organic matter, total P, available P, total nitrogen and soil enzyme activities. Soil pH decreased slightly from its initial value in all the treatments compared to control soil treatments (Table 6.3). Organic carbon of soil was significantly improved in inoculation (44 %) and RP fertilization treatments along with inoculation (47 %) compared to RP fertilization alone (17 %) and chemical fertilizer treatment (20 %) compared to control soil (Table 6.4). There was a significant improvement in total P of soil with RP fertilization. Available P was increased up to 42 % with bacterial inoculation and it rose up to 113 % when RP fertilization was done along with bio-inoculation compared to control soil. A significant increase in total P was observed in soil with DAP treatment but increase in available P (23 %) due to DAP fertilization was not comparable with increase in available P in inoculation and RP fertilization treatments (113 %). Total nitrogen was not increased with DAP, bio-inoculation and RP fertilization treatments (Table 6.4). Soil enzyme activities such as acid phosphatase, alkaline phosphatase, phytase and dehydrogenase enzyme activities were significantly improved in all the treatments, but the results were more pronounced with inoculation treatments along with RP fertilization (Table 6.5). Phytase enzyme activities in soil

were significantly higher than that of acid and alkaline phosphatase. Phosphate-solubilizing bacterial population density was significantly improved due to inoculation, RP fertilization and RP fertilization along with bacterial inoculation compared to DAP and control treatments. Population density of phosphate solubilizing-bacteria was tested in rhizospheric soil of each plot after harvesting of the crop and it was 1.8×10^6 cfu g^{-1} in control treatment, $1.0-1.1 \times 10^8$ cfu g^{-1} in inoculation treatments, 3.5×10^6 cfu g^{-1} in RP fertilization alone, $2.3-2.5 \times 10^8$ cfu g^{-1} in inoculation along with RP fertilization and 2.1×10^6 cfu g^{-1} in DAP treatments. There was a significant increase in population density of phosphate-solubilizing bacteria in inoculation treatments along with RP fertilization compared to other treatments. Field study of maize crop in organic farming showed a significant improvement in crop yield, total P uptake and soil fertility in context to organic carbon, total P, available P, soil enzyme activities and population density of P-solubilizing bacteria in bio-inoculation treatments along with RP fertilization compared to other treatments.



a)



b)



c)



d)

Plate 6.1 Maize field experiment with different growth stages a) tillering b) stem extension c) heading d) ripening.

Table 6.1 Effect of *Pantoea cypripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on the growth parameters of maize plants grown in an organic field.

Treatments	Shoot height (cm)	Shoot dry weight (g)	Root dry weight (g)	Grain yield (ton ha ⁻¹)
Soil	225 ± 3.3b	36 ± 1.9d	9.70 ± 1.0b	5.66 ± 0.14c
Soil + Pc	236 ± 4.7b	47 ± 1.3cd	11.3 ± 0.3b	6.61 ± 0.05ab
Soil + Pp	238 ± 3.1b	47 ± 10cd	11.3 ± 0.3b	6.66 ± 0.03ab
Soil + BC	239 ± 3.5b	51 ± 2.6bc	11.6 ± 0.9b	6.68 ± 0.05ab
Soil + RP	237 ± 3.0b	51 ± 5.1bc	10.0 ± 0.5b	6.55 ± 0.04ab
Soil + RP + Pc	257 ± 4.7a	63 ± 6.6ab	13.7 ± 1.0a	6.74 ± 0.05ab
Soil + RP + Pp	265 ± 6.1a	69 ± 2.1a	14.2 ± 0.7a	6.79 ± 0.02ab
Soil + RP + BC	260 ± 4.7a	70 ± 2.5a	15.0 ± 0.81a	6.83 ± 0.41a
DAP	239 ± 6.3b	49 ± 1.5cd	10.6 ± 0.8b	6.40 ± 0.04b
LSD (<i>P</i> <0.05)	9.74	8.12	1.26	0.26

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.2 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on P uptake of maize plants grown in an organic field.

Treatments	Total P (mg kg ⁻¹)		
	Grains	Shoot	Root
Soil	143 ± 10d	136 ± 9e	127 ± 10d
Soil + Pc	177 ± 9bcd	162 ± 4cd	165 ± 10bc
Soil + Pp	180 ± 17bc	165 ± 10bcd	180 ± 13b
Soil + BC	183 ± 17bc	166 ± 4bcd	186 ± 9b
Soil + RP	179 ± 13bcd	170 ± 11abcd	148 ± 6cd
Soil + RP + Pc	198 ± 13ab	186 ± 6ab	220 ± 7a
Soil + RP + Pp	223 ± 13a	183 ± 4abc	226 ± 10a
Soil + RP + BC	227 ± 6a	188 ± 2a	230 ± 6a
DAP	155 ± 13cd	150 ± 11de	138 ± 6d
LSD (<i>P</i> <0.05)	22	13	15

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.3 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of maize plants grown in an organic field.

Treatments	pH	EC (mScm ⁻¹)	TDS (ppm)
Soil	8.33 ± 0.03a	0.18 ± 0.02c	0.12 ± 0.02c
Soil + Pc	7.84 ± 0.02cd	0.26 ± 0.03ab	0.17 ± 0.02ab
Soil + Pp	7.85 ± 0.01c	0.24 ± 0.03abc	0.16 ± 0.02abc
Soil + BC	7.81 ± 0.06cde	0.27 ± 0.02ab	0.17 ± 0.01ab
Soil + RP	7.94 ± 0.02b	0.19 ± 0.02bc	0.13 ± 0.01bc
Soil + RP + Pc	7.78 ± 0.02cde	0.27 ± 0.03a	0.18 ± 0.02a
Soil + RP + Pp	7.76 ± 0.01de	0.26 ± 0.03ab	0.17 ± 0.02ab
Soil + RP + BC	7.75 ± 0.03e	0.26 ± 0.01ab	0.17 ± 0.01ab
DAP	8.01 ± 0.04b	0.25 ± 0.02abc	0.17 ± 0.01abc
LSD (<i>P</i> <0.05)	0.047	0.04	0.028

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.4 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of maize plants grown in an organic field.

Treatments	Organic carbon (%)	Organic matter (%)	Total P (mg kg ⁻¹)	Available P (mg kg ⁻¹)	Total nitrogen (%)
Soil	0.39 ± 0.05d	0.66 ± 0.09d	237 ± 8b	4.01 ± 0.17c	0.034 ± 0.002a
Soil + Pc	0.52 ± 0.02abc	0.89 ± 0.04abc	241 ± 4b	5.62 ± 0.16b	0.039 ± 0.004a
Soil + Pp	0.55 ± 0.05ab	0.94 ± 0.08ab	256 ± 2b	5.63 ± 0.05b	0.040 ± 0.001a
Soil + BC	0.56 ± 0.02a	0.96 ± 0.04a	261 ± 6b	5.66 ± 0.10b	0.039 ± 0.013a
Soil + RP	0.46 ± 0.01cd	0.78 ± 0.02cd	465 ± 9a	5.29 ± 0.07b	0.042 ± 0.017a
Soil + RP + Pc	0.54 ± 0.01abc	0.92 ± 0.01abc	480 ± 35a	8.07 ± 0.22a	0.039 ± 0.009a
Soil + RP + Pp	0.55 ± 0.02ab	0.94 ± 0.03ab	504 ± 17a	8.48 ± 0.33a	0.048 ± 0.010a
Soil + RP + BC	0.57 ± 0.03a	0.97 ± 0.05a	497 ± 6a	8.53 ± 0.10a	0.041 ± 0.002a
DAP	0.47 ± 0.03bcd	0.79 ± 0.05bcd	487 ± 8a	4.94 ± 0.09b	0.039 ± 0.005a
LSD (<i>P</i> <0.05)	0.05	0.088	24	0.47	0.016

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.5 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on enzyme activities of rhizosphere soil of maize plants grown in an organic field.

Treatments	Acid phosphatase ($\mu\text{M g}^{-1} \text{hr}^{-1}$)	Alkaline phosphatase ($\mu\text{M g}^{-1} \text{hr}^{-1}$)	Phytase activity ($\mu\text{M g}^{-1} \text{hr}^{-1}$)	Dehydrogenase activity (ppm)
Soil	370 \pm 1.2g	530 \pm 1.8d	6237 \pm 500c	8.63 \pm 0.92b
Soil + Pc	471 \pm 2.8d	712 \pm 13ab	11904 \pm 1704a	10.41 \pm 0.12ab
Soil + Pp	479 \pm 3.6cd	699 \pm 62b	11665 \pm 1663a	9.82 \pm 0.10ab
Soil + BC	481 \pm 1.1c	710 \pm 0.9ab	11913 \pm 762a	10.14 \pm 0.03ab
Soil + RP	425 \pm 2.7e	703 \pm 2.9ab	8735 \pm 126b	9.94 \pm 2.8ab
Soil + RP + Pc	490 \pm 3.8b	746 \pm 4.0ab	12446 \pm 183a	12.14 \pm 0.09a
Soil + RP + Pp	523 \pm 1.5a	762 \pm 1.3a	12960 \pm 287a	12.14 \pm 0.07a
Soil + RP + BC	521 \pm 2.8a	753 \pm 3.6ab	13052 \pm 250a	12.44 \pm 0.12a
DAP	411 \pm 5.1f	615 \pm 5.9c	8175 \pm 203bc	9.24 \pm 0.05b
LSD ($P<0.05$)	5.15	36	1483	1.71

Values are Mean \pm SD ($n=10$). Means sharing a common letter within the column are not significantly different at $P<0.05$. Pronounced results are represented in bold.

6.1.2 Field study with Wheat crop

During the second year of field study wheat crop was grown and it was observed that bacterial inoculation alone and along with RP fertilization have significant effects on plant shoot height, shoot and root dry weight, and grain yield (Table 6.6). Inoculation along with RP fertilization showed more pronounced effects compared to individual inoculation treatments. Inoculation increased the grain yield of wheat 14 %, RP fertilization 11 %, and inoculations along with RP fertilization 20 % and DAP treatments increased the yield up to 6 % compared to control treatments. Total P in seeds, shoots and roots was increased 29 %, 41 % and 36 % in inoculation treatments; in RP fertilization, it was 48 %, 81 % and 33 %; in inoculation treatments along with RP fertilization it was increased 64 %, 194 % and 94 % and in DAP treatments 4 %, 23 % and 11 % compared to uninoculated control (Table 6.7).

With inoculation and RP fertilization treatments a slight decrease in soil pH was observed (Table 6.8). EC and TDS of soil were not affected. Organic carbon of rhizospheric soil was increased to 26 % in bio-inoculation, 33 % in RP fertilization along with inoculation, 3 % in RP fertilization and 13 % in chemical fertilizer treatments (Table 6.9). There was significant improvement in total P of soil with RP fertilization in soil. Available P was increased up to 41 % with bio-inoculation and it rose up to 86 % when RP fertilization was done along with inoculation compared to control soil. With DAP fertilization and RP fertilization, total P of the respective plots was significantly increased, but the available P was improved significantly only in RP fertilization and bio-inoculation treatments (86 %) compared to DAP treatments (12 %). Total nitrogen was not affected with any of the treatment (Table 6.9). Soil enzyme activities such as acid phosphatase, alkaline phosphatase, phytase and dehydrogenase enzyme activities were significantly improved in all the treatments, but the results were more pronounced with inoculation treatments along with RP fertilization (Table 6.10). Phytase enzyme activities in soil were significantly higher than that of acid and alkaline phosphatase

(Table 6.10). After harvesting of the wheat crop phosphate-solubilizing bacterial population density was tested in rhizospheric soil of each plot and it was 2.0×10^6 cfu g^{-1} in control treatment, $2.1-2.2 \times 10^8$ cfu g^{-1} in inoculation treatments, 4.1×10^6 cfu g^{-1} in alone RP fertilization treatments, $3.6-4.1 \times 10^8$ cfu g^{-1} in inoculation along with RP fertilization and 2.1×10^6 cfu g^{-1} in DAP treatments. Inoculation alone or along with RP fertilization significantly increased the population density of phosphate-solubilizing bacteria in rhizospheric soil compared to chemical fertilizer (DAP) treatments. Second year of field experiment in organic farming showed that RP fertilization done only once in previous maize experiment is still effective in improvement of second year crop yield, P uptake and soil fertility, in comparison to the chemical fertilization done on regular basis during each crop.



a)



b)



c)



d)

Plate 6.2 Wheat field experiment with different growth stages a) tillering b) stem extension c) heading d) ripening.

Table 6.6 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on the growth parameters of wheat plants grown in an organic field.

Treatments	Shoot height (cm)	Shoot dry weight (g)	Root dry weight (g)	Grain yield (ton ha ⁻¹)
Soil	99 ± 8.1b	1.44 ± 0.10b	0.66 ± 0.07d	3.85 ± 0.18b
Soil + Pc	104 ± 5.8ab	1.82 ± 0.03b	0.79 ± 0.07cd	4.17 ± 0.18ab
Soil + Pp	104 ± 2.0ab	1.86 ± 0.01b	0.84 ± 0.08bcd	4.27 ± 0.36ab
Soil + BC	109 ± 3.8ab	1.87 ± 0.07b	0.82 ± 0.07bcd	4.36 ± 0.31ab
Soil + RP	109 ± 3.8ab	1.70 ± 0.05b	0.79 ± 0.16cd	4.27 ± 0.18ab
Soil + RP + Pc	119 ± 0.9ab	2.56 ± 0.16a	0.93 ± 0.05abc	4.17 ± 0.18ab
Soil + RP + Pp	120 ± 1.4a	2.54 ± 0.16a	1.03 ± 0.06ab	4.48 ± 0.18ab
Soil + RP + BC	122 ± 2.2a	2.55 ± 0.22a	1.12 ± 0.003a	4.63 ± 0.11a
DAP	100 ± 1.1ab	1.58 ± 0.46b	0.75 ± 0.04cd	4.06 ± 0.31ab
LSD ($P < 0.05$)	1071	0.33	0.131	0.408

Values are Mean ± SD ($n = 10$). Means sharing a common letter within the column are not significantly different at $P < 0.05$. Pronounced results are represented in bold.

Table 6.7 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on P uptake of wheat plants grown in an organic field.

Treatments	Total P (mg kg ⁻¹)		
	Grains	Shoot	Root
Soil	227 ± 10e	38 ± 10d	222 ± 6e
Soil + Pc	263 ± 10cde	44 ± 6d	265 ± 9cd
Soil + Pp	294 ± 5ab	52 ± 6cd	301 ± 6b
Soil + BC	273 ± 15cd	54 ± 4cd	302 ± 6b
Soil + RP	336 ± 23ab	69 ± 6c	295 ± 8bc
Soil + RP + Pc	372 ± 10a	109 ± 6ab	405 ± 10a
Soil + RP + Pp	350 ± 11a	93 ± 6b	429 ± 8a
Soil + RP + BC	366 ± 18a	112 ± 4a	430 ± 13a
DAP	236 ± 20de	47 ± 6d	245 ± 26de
LSD (<i>P</i> <0.05)	25	11	21

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.8 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of wheat plants grown in an organic field.

Treatments	pH	EC (mScm ⁻¹)	TDS (ppm)
Soil	8.30 ± 0.02a	0.14 ± 0.01c	0.09 ± 0.007c
Soil + Pc	7.67 ± 0.02d	0.14 ± 0.02c	0.09 ± 0.010c
Soil + Pp	7.61 ± 0.01ef	0.15 ± 0.01c	0.10 ± 0.007c
Soil + BC	7.65 ± 0.01de	0.22 ± 0.02a	0.15 ± 0.014a
Soil + RP	8.13 ± 0.02c	0.19 ± 0.01ab	0.13 ± 0.007ab
Soil + RP + Pc	7.56 ± 0.03fg	0.19 ± 0.02ab	0.13 ± 0.01ab
Soil + RP + Pp	7.55 ± 0.01g	0.16 ± 0.01bc	0.11 ± 0.004bc
Soil + RP + BC	7.58 ± 0.03fg	0.22 ± 0.01a	0.15 ± 0.007a
DAP	8.19 ± 0.03b	0.19 ± 0.02ab	0.13 ± 0.010ab
LSD (<i>P</i> <0.05)	0.032	0.023	0.015

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.9 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of wheat plants grown in an organic field.

Treatments	Organic carbon (%)	Organic matter (%)	Total P (mg kg ⁻¹)	Available P (mg kg ⁻¹)	Total nitrogen (%)
Soil	0.46 ± 0.04c	0.78 ± 0.06c	204 ± 7c	4.94 ± 0.18d	0.045 ± 0.005a
Soil + Pc	0.55 ± 0.02abc	0.94 ± 0.03abc	234 ± 25bc	6.72 ± 0.18b	0.050 ± 0.008a
Soil + Pp	0.57 ± 0.05abc	0.96 ± 0.08abc	245 ± 8bc	6.94 ± 0.18b	0.055 ± 0.011a
Soil + BC	0.58 ± 0.02abc	0.99 ± 0.08abc	250 ± 8b	6.88 ± 0.39b	0.051 ± 0.009a
Soil + RP	0.47 ± 0.08bc	0.80 ± 0.14bc	455 ± 6a	5.90 ± 0.18c	0.045 ± 0.010a
Soil + RP + Pc	0.57 ± 0.02abc	0.96 ± 0.03abc	445 ± 8a	9.11 ± 0.11a	0.061 ± 0.006a
Soil + RP + Pp	0.60 ± 0.06ab	1.01 ± 0.10ab	434 ± 28a	8.81 ± 0.11a	0.060 ± 0.016a
Soil + RP + BC	0.61 ± 0.06a	1.04 ± 0.10a	441 ± 8a	9.18 ± 0.29a	0.062 ± 0.028a
DAP	0.52 ± 0.02abc	0.88 ± 0.03abc	437 ± 19a	5.50 ± 0.11cd	0.046 ± 0.003a
LSD (<i>P</i> <0.05)	0.077	0.13	27	0.36	0.022

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.10 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on enzyme activities of rhizosphere soil of wheat plants grown in an organic field.

Treatments	Acid phosphatase ($\mu\text{M g}^{-1} \text{hr}^{-1}$)	Alkaline phosphatase ($\mu\text{M g}^{-1} \text{hr}^{-1}$)	Phytase activity ($\mu\text{M g}^{-1} \text{hr}^{-1}$)	Dehydro- genase activity (ppm)
Soil	439 \pm 1.4f	329 \pm 0.9g	6485 \pm 84e	12.3 \pm 0.16e
Soil + Pc	540 \pm 1.6c	467 \pm 0.4d	8056 \pm 97cd	14.0 \pm 0.06c
Soil + Pp	541 \pm 1.0c	466 \pm 1.1d	8212 \pm 99c	14.2 \pm 0.05c
Soil + BC	539 \pm 1.0c	469 \pm 0.7d	8184 \pm 55c	14.2 \pm 0.16c
Soil + RP	465 \pm 1.2e	403 \pm 0.4f	8092 \pm 89cd	13.9 \pm 0.11c
Soil + RP + Pc	575 \pm 1.4b	491 \pm 1.1c	9838 \pm 55b	14.1 \pm 0.06c
Soil + RP + Pp	574 \pm 1.0b	495 \pm 1.3b	9939 \pm 136b	15.6 \pm 0.07a
Soil + RP + BC	580 \pm 1.7a	499 \pm 0.7a	10664 \pm 397a	15.1 \pm 0.29b
DAP	482 \pm 1.2d	444 \pm 2.5e	7679 \pm 161d	13.4 \pm 0.08d
LSD ($P < 0.05$)	2.1	2.09	282	0.24

Values are Mean \pm SD ($n = 10$). Means sharing a common letter within the column are not significantly different at $P < 0.05$. Pronounced results are represented in bold.

6.2 Phosphate-solubilizing fungi as bio-inoculants in organic farming

6.2.1 Maize experiment

Similar to the field study with bacterial inoculants, fungal bio-inoculation of *Aspergillus tubingensis* and *Aspergillus niger* in maize crop with and without rock phosphate fertilization was tested for the improvement of crop yield and soil fertility. It was observed that both fungal isolates, have stimulatory effect on growth parameters (shoot height, shoot and root dry biomass and on grain yield) of maize crop but the effects were more pronounced when RP fertilization was done along with inoculation compared to other treatments (Table 6.11). P uptake in plant tissues (grains, shoots and roots) was significantly increased in inoculated and RP fertilized treatments compared to control treatment (Table 6.12). Increase in grain yield was 19 % with fungal inoculation, 15.8 % with RP fertilization alone and it was increased up to 25.4 % when inoculation was done along with RP fertilization. On the other hand, DAP chemical fertilization increase grain yield up to 13.0 % compared to control treatment.

Soil pH decreased slightly from its initial value in all the treatments compared to control soil (Table 6.13). Organic carbon of the soil was significantly improved due to inoculation and RP fertilization compared to chemical fertilization and control (Table 6.14). Available P in soil was increased up to 87 % with fungal inoculation, and it rose up to 146 % when RP fertilization was done along with fungal inoculation compared to control soil. A significant increase in total P was observed in soil with DAP treatment but increase in available P was significantly higher in fungal inoculation and RP fertilization treatments (146 %) compared to DAP treatments (23 %). All treatments have no significant effect on improvement of soil total nitrogen (Table 6.14). Soil enzyme activities, such as acid phosphates, alkaline phosphatase, phytase and dehydrogenase enzyme activities were significantly improved in all the treatments (Table 6.15). Phytase enzyme activities in soil were significantly higher than

that of acid and alkaline phosphatase enzyme activities. Population density of P-solubilizing fungi in rhizospheric soil, after harvesting was 2.0×10^6 cfu g⁻¹ in control treatment, $5.3-7.0 \times 10^6$ cfu g⁻¹ in inoculation treatments alone, 3.3×10^6 cfu g⁻¹ in RP fertilization alone, $3.0-4.7 \times 10^7$ cfu g⁻¹ in inoculation treatments along with RP fertilization and 2.3×10^6 cfu g⁻¹ in DAP treatments. Field study of maize crop in organic farming showed that there was a significant improvement in yield, P uptake and soil fertility in RP fertilization treatments along with fungal inoculation compared to individual inoculation and DAP treatments.

Aflatoxins in the maize grain samples were tested by HPLC analysis and the results revealed that aflatoxins such as B1, B2, G1, and G2 were absent in the grains.

Table 6.11 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on the growth parameters of maize plants grown in an organic field.

Treatments	Shoot length (cm)	Shoot dry weight (g)	Root dry weight (g)	Grain yield (ton ha ⁻¹)
Soil	225 ± 3.3d	36 ± 1.9g	9.70 ± 1.0b	5.66 ± 0.14d
Soil + At	258 ± 4.7ab	58 ± 1.3de	14.2 ± 0.9a	6.72 ± 0.06b
Soil + An	250 ± 3.0bc	64 ± 6.0cd	15.0 ± 0.9a	6.74 ± 0.08b
Soil + FC	252 ± 6.3bc	67 ± 1.0bc	14.8 ± 1.1a	6.73 ± 0.12b
Soil + RP	238 ± 3.0cd	51 ± 5.1ef	10.0 ± 0.5b	6.55 ± 0.04bc
Soil + RP + At	271 ± 3.0a	74 ± 0.7ab	15.3 ± 0.4a	7.03 ± 0.07a
Soil + RP + An	270 ± 1.8a	75 ± 1.2ab	15.8 ± 0.9a	7.06 ± 0.07a
Soil + RP + FC	272 ± 4.7a	78 ± 2.6a	15.3 ± 1.2a	7.10 ± 0.10a
DAP	239 ± 6.3cd	49 ± 1.5f	10.6 ± 0.8b	6.40 ± 0.04c
LSD (<i>P</i> <0.05)	9.44	5.19	1.56	0.149

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.12 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on P uptake of maize plants grown in an organic field.

Treatments	Total P (mg kg ⁻¹)		
	Grains	Shoot	Root
Soil	143 ± 10e	136 ± 9d	127 ± 10e
Soil + At	187 ± 11cd	172 ± 4bcd	176 ± 9cd
Soil + An	197 ± 17bc	177 ± 10abc	194 ± 24bc
Soil + FC	191 ± 4cd	176 ± 19abc	191 ± 4c
Soil + RP	179 ± 13cde	170 ± 11bcd	148 ± 6de
Soil + RP + At	230 ± 15ab	212 ± 17a	229 ± 18ab
Soil + RP + An	238 ± 15a	197 ± 15ab	236 ± 10a
Soil + RP + FC	248 ± 17a	201 ± 17ab	244 ± 15a
DAP	155 ± 13de	150 ± 11cd	139 ± 6e
LSD (<i>P</i> <0.05)	23	24	23

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.13 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of maize plants grown in an organic field.

Treatments	pH	EC (mScm ⁻¹)	TDS (ppm)
Soil	8.33 ± 0.03a	0.18 ± 0.02c	0.12 ± 0.01c
Soil + At	7.77 ± 0.01de	0.25 ± 0.02abc	0.17 ± 0.02abc
Soil + An	7.74 ± 0.01def	0.23 ± 0.03bc	0.16 ± 0.02bc
Soil + FC	7.81 ± 0.01d	0.23 ± 0.05bc	0.15 ± 0.03bc
Soil + RP	7.94 ± 0.02c	0.19 ± 0.02bc	0.13 ± 0.01bc
Soil + RP + At	7.67 ± 0.03g	0.32 ± 0.04a	0.22 ± 0.02a
Soil + RP + An	7.70 ± 0.02fg	0.27 ± 0.04ab	0.18 ± 0.02ab
Soil + RP + FC	7.73 ± 0.04efg	0.33 ± 0.4a	0.22 ± 0.02a
DAP	8.01 ± 0.04b	0.25 ± 0.02abc	0.17 ± 0.01abc
LSD (<i>P</i> <0.05)	0.041	0.05	0.034

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.14 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of maize plants grown in an organic field.

Treatments	Organic carbon (%)	Organic matter (%)	Total P (mg kg ⁻¹)	Available P (mg kg ⁻¹)	Total nitrogen (%)
Soil	0.39 ± 0.05c	0.66 ± 0.09c	237 ± 8d	4.02 ± 0.17e	0.035 ± 0.002a
Soil + At	0.58 ± 0.04a	0.98 ± 0.06a	248 ± 2d	7.51 ± 0.14b	0.045 ± 0.018a
Soil + An	0.56 ± 0.02a	0.96 ± 0.03a	250 ± 3d	7.23 ± 0.09b	0.042 ± 0.008a
Soil + FC	0.53 ± 0.02ab	0.91 ± 0.04ab	250 ± 4d	7.37 ± 0.07b	0.039 ± 0.005a
Soil + RP	0.46 ± 0.01bc	0.78 ± 0.02bc	465 ± 9c	5.29 ± 0.07c	0.042 ± 0.017a
Soil + RP + At	0.56 ± 0.02a	0.96 ± 0.04a	459 ± 9c	9.96 ± 0.12a	0.053 ± 0.010a
Soil + RP + An	0.56 ± 0.03a	0.95 ± 0.05a	508 ± 8a	9.86 ± 0.05a	0.050 ± 0.008a
Soil + RP + FC	0.57 ± 0.01a	0.96 ± 0.01a	469 ± 9bc	9.91 ± 0.15a	0.048 ± 0.013a
DAP	0.47 ± 0.03bc	0.79 ± 0.05bc	487 ± 8b	4.94 ± 0.10d	0.039 ± 0.005a
LSD (<i>P</i> <0.05)	0.049	0.084	12	0.20	0.019

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.15 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on enzyme activities of rhizosphere soil of maize plants grown in an organic field.

Treatments	Acid phosphatase ($\mu\text{M g}^{-1} \text{hr}^{-1}$)	Alkaline phosphatase ($\mu\text{M g}^{-1} \text{hr}^{-1}$)	Phytase activity ($\mu\text{M g}^{-1} \text{hr}^{-1}$)	Dehydrogenase activity (ppm)
Soil	370 \pm 1.2e	530 \pm 1.8g	6237 \pm 500c	8.63 \pm 0.92b
Soil + At	483 \pm 1.0b	741 \pm 0.7d	12272 \pm 2063ab	10.5 \pm 0.07ab
Soil + An	481 \pm 5b	742 \pm 0.9d	11968 \pm 1496b	10.0 \pm 0.05ab
Soil + FC	505 \pm 1.2b	754 \pm 1.3c	12529 \pm 1224ab	11.3 \pm 0.05ab
Soil + RP	425 \pm 2.7d	703 \pm 2.9e	8735 \pm 126c	9.94 \pm 2.8ab
Soil + RP + At	567 \pm 2.0a	792 \pm 2.1b	14843 \pm 97a	12.2 \pm 0.09a
Soil + RP + An	569 \pm 2.4a	802 \pm 1.1a	14715 \pm 83ab	12.3 \pm 0.09a
Soil + RP + FC	578 \pm 8.7a	796 \pm 0.9ab	14577 \pm 110ab	12.5 \pm 0.1a
DAP	411 \pm 5.1c	615 \pm 5.9f	8175 \pm 203c	9.24 \pm 0.05b
LSD ($P < 0.05$)	4.77	4.54	1650	1.71

Values are Mean \pm SD ($n = 10$). Means sharing a common letter within the column are not significantly different at $P < 0.05$. Pronounced results are represented in bold.

6.2.2 Wheat experiment

In wheat crop field, fungal inoculation alone and along with RP fertilization have significant effects on plant shoot height, shoot and root dry weight, and grain yield (Table 6.16). Effects of fungal inoculation along with RP fertilization were more pronounced compared to other treatments. Fungal inoculation treatments increased the yield of wheat 28 %, RP fertilization 11 %, inoculations along with RP fertilization 38 % and DAP treatments increased the yield 6 % compared to un inoculated control treatments. P uptake in seeds, shoots and roots was increased 44 %, 118 % and 59 % with inoculation, in RP fertilization alone it was 48 %, 81 % and 33 %, in inoculation treatments along with RP fertilization it was increased 76 %, 239 % and 109 % and in DAP treatments 4 %, 23 % and 11% increase in P uptake was observed compared to un-inoculated control (Table 6.17).

With inoculation and RP fertilization treatments, a slight decrease in soil pH was observed (Table 6.18). Organic carbon of soil was significantly improved due to inoculation treatments (39 %) and RP fertilization treatments along with inoculation (54 %) compared to RP fertilization alone (3 %) and chemical fertilizer treatment (13 %) and control soil (Table 6.19). Available P was increased up to 54 % with inoculation and it rose up to 127 % when RP fertilization was done along with inoculation compared to control soil. Increase in available P in inoculation and RP fertilization treatments was significantly higher (127 %) than increase in available P in DAP treatment (12 %) (Table 6.19). Soil total nitrogen was not significantly improved by any treatment. Acid phosphates, alkaline phosphatase, phytase and dehydrogenase enzyme activities were significantly improved in all the treatments but the results were more pronounced with inoculation treatments along with RP fertilization (Table 6.20). Phytase enzyme activities in soil were significantly higher than that of acid and alkaline phosphatase enzyme activities (Table 6.20). Alkaline phosphatase activities were decreased compared to alkaline phosphatase activities in maize rhizospheric soil. Population

density of phosphate-solubilizing fungi in rhizospheric soil after harvesting of the wheat crop was 2.3×10^6 cfu g^{-1} in control treatment, $6.0-7.3 \times 10^6$ cfu g^{-1} in fungal inoculation treatments alone, 4.3×10^6 cfu g^{-1} in RP fertilization treatments alone, $5.7-7.0 \times 10^7$ cfu g^{-1} in inoculation along with RP fertilization and 2.7×10^6 cfu g^{-1} in DAP treatments. Results showed that DAP treatment and RP fertilization alone was not increased the phosphate-solubilizing fungal population as much in soil, as it was significantly increased in fungal inoculation along with RP fertilization treatments.

Table 6.16 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on the growth parameters of wheat plants grown in an organic field.

Treatments	Shoot length (cm)	Shoot dry weight (g)	Root dry weight (g)	Grain yield (ton ha ⁻¹)
Soil	99 ± 8e	1.44 ± 0.10d	0.66 ± 0.07d	3.85 ± 0.18e
Soil + At	115 ± 1d	1.99 ± 0.12bcd	0.94 ± 0.05cd	4.50 ± 0.17bcd
Soil + An	119 ± 2bcd	1.92 ± 0.06cd	1.01 ± 0.05bc	4.30 ± 0.30bcde
Soil + FC	116 ± 2cd	2.15 ± 0.04abc	0.92 ± 0.05cd	4.92 ± 0.22ab
Soil + RP	109 ± 4de	1.70 ± 0.05cd	0.79 ± 0.16cd	4.27 ± 0.18cde
Soil + RP + At	131 ± 3a	2.58 ± 0.20a	1.33 ± 0.14a	4.84 ± 0.16abc
Soil + RP + An	126 ± 4abc	2.54 ± 0.06ab	1.28 ± 0.18ab	5.30 ± 0.30a
Soil + RP + FC	129 ± 2ab	2.62 ± 0.25a	1.27 ± 0.07ab	5.25 ± 0.17a
DAP	100 ± 1e	1.59 ± 0.46d	0.75 ± 0.04cd	4.06 ± 0.31de
LSD ($P < 0.05$)	6.42	0.34	0.176	0.39

Values are Mean ± SD ($n = 10$). Means sharing a common letter within the column are not significantly different at $P < 0.05$. Pronounced results are represented in bold.

Table 6.17 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on P uptake of wheat plants grown in an organic field.

Treatments	Total P (mg kg ⁻¹)		
	Grains	Shoot	Root
Soil	227 ± 10d	38 ± 10d	222 ± 6d
Soil + At	326 ± 17c	63 ± 6bcd	336 ± 17b
Soil + An	313 ± 17c	81 ± 9b	322 ± 10bc
Soil + FC	320 ± 15c	83 ± 11b	352 ± 6b
Soil + RP	336 ± 23bc	69 ± 6bc	295 ± 8c
Soil + RP + At	373 ± 10ab	112 ± 4a	458 ± 8a
Soil + RP + An	384 ± 6a	120 ± 4a	463 ± 6a
Soil + RP + FC	400 ± 4a	129 ± 15a	459 ± 6a
DAP	236 ± 20d	47 ± 6cd	245 ± 26d
LSD (<i>P</i> <0.05)	26	15	21

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.18 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of wheat plants grown in an organic field.

Treatments	pH	EC (mScm ⁻¹)	TDS (ppm)
Soil	8.30 ± 0.02a	0.14 ± 0.01e	0.09 ± 0.007e
Soil + At	7.59 ± 0.04c	0.17 ± 0.02de	0.12 ± 0.010de
Soil + An	7.58 ± 0.02cd	0.22 ± 0.02abc	0.15 ± 0.01abc
Soil + FC	7.56 ± 0.03cde	0.17 ± 0.02de	0.12 ± 0.01de
Soil + RP	8.13 ± 0.02b	0.19 ± 0.02cd	0.13 ± 0.01cd
Soil + RP + At	7.50 ± 0.05cde	0.21 ± 0.02abcd	0.14 ± 0.01bcd
Soil + RP + An	7.46 ± 0.05e	0.24 ± 0.01ab	0.16 ± 0.01ab
Soil + RP + FC	7.47 ± 0.08de	0.25 ± 0.02a	0.17 ± 0.01a
DAP	8.19 ± 0.03b	0.19 ± 0.02bcd	0.13 ± 0.01bcd
LSD (<i>P</i> <0.05)	0.068	0.026	0.017

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.19 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of wheat plants grown in an organic field.

Treatments	Organic carbon (%)	Organic matter (%)	Total P (mg kg ⁻¹)	Available P (mg kg ⁻¹)	Total nitrogen (%)
Soil	0.46 ± 0.04d	0.78 ± 0.06d	204 ± 7d	4.94 ± 0.18e	0.045 ± 0.005a
Soil + At	0.64 ± 0.02ab	1.09 ± 0.04ab	223 ± 6cd	7.23 ± 0.18bc	0.063 ± 0.013a
Soil + An	0.61 ± 0.03abc	1.05 ± 0.05abc	247 ± 9bc	7.59 ± 0.15b	0.056 ± 0.012a
Soil + FC	0.59 ± 0.01bc	1.00 ± 0.02bc	250 ± 8b	7.11 ± 0.18c	0.066 ± 0.011a
Soil + RP	0.47 ± 0.08d	0.80 ± 0.14d	455 ± 6a	5.90 ± 0.18d	0.045 ± 0.009a
Soil + RP + At	0.66 ± 0.02ab	1.13 ± 0.04ab	437 ± 8a	10.95 ± 0.13a	0.060 ± 0.012a
Soil + RP + An	0.69 ± 0.03ab	1.18 ± 0.05ab	434 ± 5a	10.90 ± 0.09a	0.059 ± 0.019a
Soil + RP + FC	0.71 ± 0.04a	1.21 ± 0.06a	443 ± 6a	11.22 ± 0.18a	0.064 ± 0.050a
DAP	0.52 ± 0.02cd	0.88 ± 0.03cd	437 ± 19a	5.51 ± 0.11d	0.046 ± 0.003a
LSD (<i>P</i> <0.05)	0.064	0.108	16	0.265	0.247

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

Table 6.20 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on enzyme activities of rhizosphere soil of wheat plants grown an organic field.

Treatments	Acid phosphatase ($\mu\text{M g}^{-1} \text{hr}^{-1}$)	Alkaline phosphatase ($\mu\text{M g}^{-1} \text{hr}^{-1}$)	Phytase activity ($\mu\text{M g}^{-1} \text{hr}^{-1}$)	Dehydro- genase activity (ppm)
Soil	439 \pm 1.4d	329 \pm 0.90g	6485 \pm 84f	12.30 \pm 0.16e
Soil + At	535 \pm 37b	472 \pm 00.55d	10600 \pm 69c	14.48 \pm 0.07b
Soil + An	564 \pm 3.3ab	474 \pm 0.75cd	10425 \pm 84c	14.45 \pm 0.05b
Soil + FC	575 \pm 0.85a	477 \pm 0.72c	10333 \pm 110c	14.56 \pm 0.06b
Soil + RP	465 \pm 1.2cd	403 \pm 0.36f	8092 \pm 89d	13.94 \pm 0.12c
Soil + RP + At	585 \pm 1.7a	528 \pm 2.2b	12832 \pm 115b	16.91 \pm 0.02a
Soil + RP + An	590 \pm 2.0a	526 \pm 0.4b	12740 \pm 42b	16.96 \pm 0.07a
Soil + RP + FC	596 \pm 0.85a	533 \pm 2.2a	13181 \pm 57a	16.99 \pm 0.16a
DAP	482 \pm 1.2c	444 \pm 2.5e	7679 \pm 161e	13.45 \pm 0.08d
LSD ($P<0.05$)	21	2.56	165	0.168

Values are Mean \pm SD ($n=10$). Means sharing a common letter within the column are not significantly different at $P<0.05$. Pronounced results are represented in bold.

Chapter 7

Effect of Phosphate solubilizing microorganisms at multilocational sites

7.1 Phosphate-solubilizing bacteria as bio-inoculants at multilocal sites

For multilocal study, field trials were conducted at three different sites. These sites come under different agroclimatic regions that have different climatic conditions and soil fertility levels (Table 7.1). The effects of selected PSBs isolates on improvement of crop yield and soil fertility in these different regions were tested. The survival, effect on crop yield and soil fertility in these different agroclimatic regions will provide us the consistent performance of these isolates in different agroclimatic regions.

7.1.1 Maize experiment at multilocal sites with bacterial inoculants

During first year of field study, maize experiment was conducted at all three different sites. Significant improvement in plant shoot height, shoot and root dry weight was observed in all three sites with bacterial inoculation alone or along with RP fertilization. The improvement was significant in inoculation treatments along with RP fertilization compared to individual inoculation and DAP treatments (Table 7.2). A significant increase in maize yield (Fig. 7.1) up to 21 % was observed at all three sites in inoculated and RP fertilized soil, it was increased up to 18 % with inoculation alone, 16 % with RP fertilization alone and 9.0 to 13 % with DAP fertilization compared to un-inoculated control soil. A significant improvement in P uptake in seeds, shoots and roots of maize crop was observed by *P. cyripedii* and *P. plecoglossicida* compared to control treatments (Table 7.3). Stimulatory effects of inoculation treatments along with RP fertilization on P uptake was found to be more pronounced compared to inoculation alone, RP fertilization alone and DAP fertilization. Enhancement in yield and P uptake in maize was more pronounced and significantly higher at sub mountain undulating region compared to central plain region and sub mountain undulating central region.

Physiochemical properties of maize rhizospheric soils were significantly improved in all treatments and the effects were more pronounced when inoculation was done along with RP fertilization. Bacterial inoculation slightly decreased the soil pH in all treatments compared to un-inoculated control in all field trials (Table 7.4). Organic carbon and organic matter was significantly improved due to inoculation and RP fertilization treatments compared to DAP and un-inoculated control treatments (Fig 7.2 and 7.3). During first year in maize crop field experiment, inoculation along with RP fertilization increased the soil organic carbon content 43 %, 39 % and 47 % and DAP treatments increased the soil organic carbon content 3.2 %, 18 % and 20 % at central plain region, sub mountain undulating central region and at sub mountain undulating region, respectively compared to control treatments. Available P significantly increased at central plain region (52 %), sub mountain undulating central region (52 %) and at sub mountain undulating region (42 %) due to inoculation but the effect was more pronounced (113 %, 110 % and 113 %) when RP was supplemented along with inoculation. Significant improvement in total P was observed in RP fertilization and DAP treatments, but available P was significantly improve only in RP fertilization along with inoculation treatments compared to inoculation, RP fertilization and DAP treatment (Table 7.5). Acid phosphatase, alkaline phosphatase, phytase and dehydrogenase enzyme activities were significantly increased in all the treatments compared to control but the results were more pronounced with inoculation treatments along with RP fertilization at all sites (Figure 7.4, 7.5, 7.6 and 7.7). In all the field trials at different sites phytase enzyme activity was higher than acid phosphatase and alkaline phosphatase.

After harvesting of the maize crop, rhizospheric soil was examined for phosphate-solubilizing bacterial population density. Maximum population density of PSBs was observed in inoculation treatments along with RP fertilization compared to other treatments in all field trials. Phosphate-solubilizing bacterial population density at central plain region, sub

mountain undulating central region and sub mountain undulating region was 0.3×10^5 cfu g⁻¹, 0×10^5 cfu g⁻¹ and 1.8×10^6 cfu g⁻¹ in un-inoculated control seed treatment, $5.3-6.7 \times 10^7$ cfu g⁻¹, $3.6-5.7 \times 10^7$ cfu g⁻¹ and $1.0-1.1 \times 10^8$ cfu g⁻¹ in inoculation treatments, 1.7×10^5 cfu g⁻¹, 0.7×10^5 cfu g⁻¹ and 3.5×10^6 cfu g⁻¹ in single RP fertilization treatments, $8.9-9.3 \times 10^7$ cfu g⁻¹, $6.7-7.6 \times 10^7$ cfu g⁻¹ and $2.3-2.5 \times 10^8$ cfu g⁻¹ in inoculation along with RP fertilization and 1.7×10^5 cfu g⁻¹, 1.3×10^5 cfu g⁻¹ and 2.1×10^6 cfu g⁻¹ in DAP treatments. RP fertilization treatments along with bacterial inoculation increased the yield and soil fertility in context to organic carbon, total P, available P, soil enzyme activities and PSBs population significantly, compared to inoculation treatments alone, RP fertilization treatments alone and DAP treatment.

Table 7.1 Some climatic and soil physiochemical properties of experimental sites.

Sites	Agrocilmatic regions	Soil type	Location	Climate	pH	Organic carbon (%)	Available P (mg kg ⁻¹)	Total P (mg kg ⁻¹)	Organic P (mg kg ⁻¹)	Total nitrogen (%)
Patiala	Central plain region	Sandy loam (Typic Ustifluvents)	30.30° N 76.38° E	tropical hot and dry	8.27 ± 0.05	0.33 ± 0.05	3.9 ± 0.17	251 ± 14	138 ± 42	0.020 ± 0.013
Balachaur	Sub mountain undulating central region	Sandy loam (Typic Ustifluvents)	31.07° N 76.32° E	dry sub humid	8.18 ± 0.02	0.31 ± 0.09	3.6 ± 0.16	237 ± 15	124 ± 24	0.019 ± 0.007
Pojewal	Sub mountain undulating region	Loamy sand (Typic Ustorthents)	31.65° N 76.26° E	dry sub humid	8.37 ± 0.02	0.42 ± 0.08	4.3 ± 0.09	245 ± 8.33	207 ± 24	0.035 ± 0.008

Values are Mean ± SD (*n* =3).

Table 7.2 Effect of *Pantoea cypripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on the growth parameters of maize plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	Shoot length (cm)	Shoot dry weight (g)	Root dry weight (g)
Central plain region			
Soil	216 ± 5.3gh	34 ± 1.4gh	9.5 ± 0.30gh
Soil + Pc	231 ± 2.5defgh	42 ± 1.5efgh	11.1 ± 0.40cdefgh
Soil + Pp	234 ± 2.5defg	44 ± 1.8defgh	11.0 ± 0.42defgh
Soil + BC	235 ± 1.5def	46 ± 1.0defgh	11.2 ± 0.34cdefgh
Soil + RP	233 ± 3.9defg	51 ± 5.3bcdef	9.95 ± 0.67gh
Soil + RP + Pc	247 ± 1.5abcd	59 ± 3.5abcd	12.6 ± 1.56bcdef
Soil + RP + Pp	248 ± 6.4abcd	62 ± 7.5abc	12.8 ± 0.98abcde
Soil + RP + BC	249 ± 9.2abcd	63 ± 4.4ab	13.0 ± 0.52abcd
DAP	233 ± 6.4defg	39 ± 1.0fgh	11.1 ± 0.08defgh
Sub mountain undulating central region			
Soil	214 ± 6.3h	32 ± 1.7h	8.71 ± 0.10h
Soil + Pc	228 ± 4.7efgh	41 ± 2.7fgh	9.61 ± 0.34gh
Soil + Pp	229 ± 11efgh	41 ± 3.9fgh	9.86 ± 0.10fgh
Soil + BC	226 ± 6.0fgh	42 ± 3.6efgh	10.2 ± 0.09fgh
Soil + RP	232 ± 6.1defgh	39 ± 2.1fgh	9.80 ± 0.27fgh
Soil + RP + Pc	239 ± 7.0def	49 ± 6.3bcdefg	11.4 ± 0.57cdefgh
Soil + RP + Pp	244 ± 3.0bcde	57 ± 2.5abcde	11.6 ± 1.35cdefg
Soil + RP + BC	246 ± 4.7bcde	58 ± 10abcd	12.1 ± 1.3bcdef
DAP	231 ± 4.7defgh	42 ± 9.4fgh	9.53 ± 0.31gh
Sub mountain undulating region			
Soil	225 ± 3.3fgh	36 ± 1.9fgh	9.70 ± 1.0gh
Soil + Pc	236 ± 4.7def	47 ± 1.3cdefg	11.3 ± 0.3cdefgh
Soil + Pp	238 ± 3.1def	47 ± 10cdefg	11.3 ± 0.3cdefgh
Soil + BC	239 ± 3.5cdef	51 ± 2.6bcdef	11.6 ± 0.9cdefg
Soil + RP	237 ± 3.0def	51 ± 5.1bcdef	10.0 ± 0.5gh
Soil + RP + Pc	257 ± 4.7abc	63 ± 6.6ab	13.7 ± 1.0abc
Soil + RP + Pp	265 ± 6.1a	69 ± 2.1a	14.2 ± 0.7ab
Soil + RP + BC	260 ± 4.7ab	70 ± 2.5a	15.0 ± 0.81a
DAP	239 ± 6.3cdef	49 ± 1.5bcdefg	10.6 ± 0.8efgh
LSD (P<0.05)	9.33	7.80	1.31

Values are Mean ± SD ($n = 10$). Means sharing a common letter within the column are not significantly different at $P < 0.05$. Pronounced results are represented in bold.

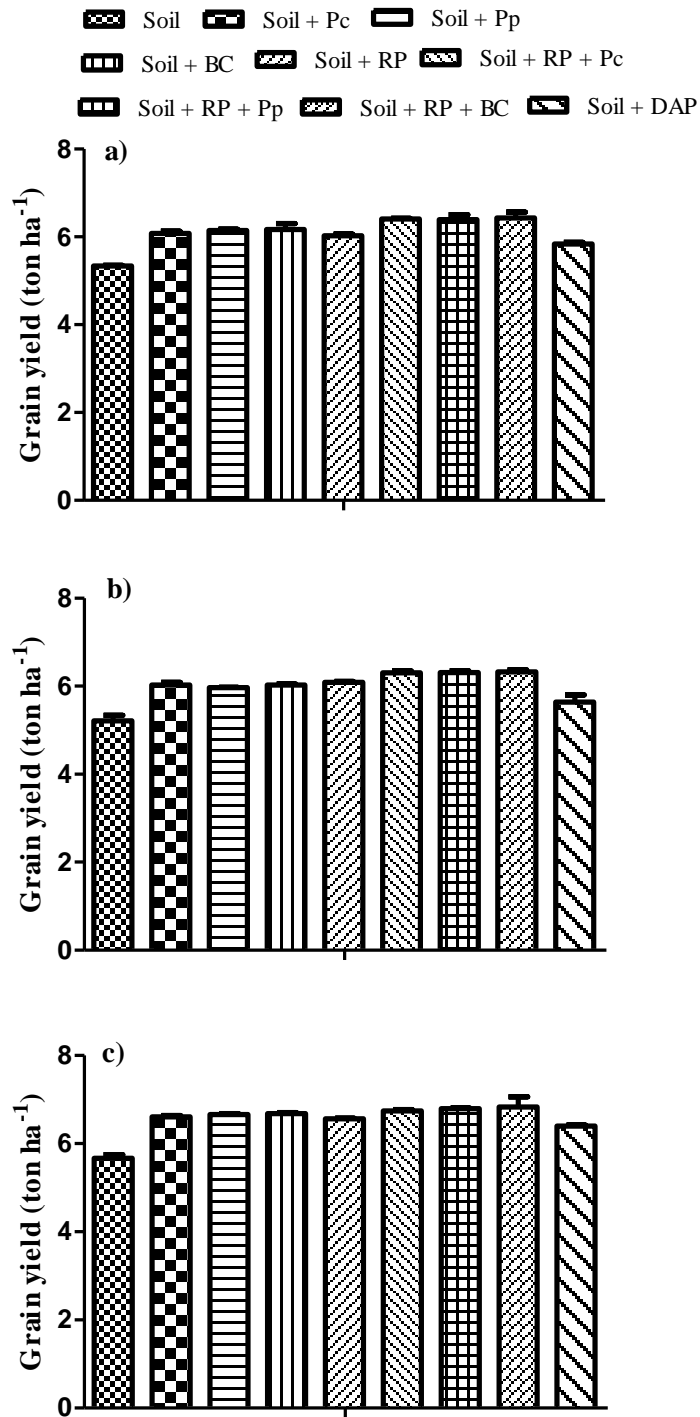


Fig. 7.1 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on grain yield of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

Table 7.3 Effect of *Pantoea cypripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on P uptake of maize plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	Grains (mg kg ⁻¹)	Shoot (mg kg ⁻¹)	Root (mg kg ⁻¹)
Central plain region			
Soil	136 ± 8.7jk	129 ± 4jk	123 ± 5ij
Soil + Pc	169 ± 6.4efghi	155 ± 15cdefghij	166 ± 4defgh
Soil + Pp	166 ± 4.2fghij	156 ± 6cdefghij	168 ± 13defgh
Soil + BC	170 ± 4.2efghi	158 ± 4bcdefghi	170 ± 4defg
Soil + RP	169 ± 6.4efghi	152 ± 13defghijk	133 ± 11ghij
Soil + RP + Pc	204 ± 8.3abcd	175 ± 7abcdef	213 ± 6abc
Soil + RP + Pp	209 ± 6.4abc	177 ± 2abcde	216 ± 11abc
Soil + RP + BC	206 ± 13abcd	179 ± 8abcd	218 ± 6abc
DAP	161 ± 9ghij	145 ± 8ghijk	159 ± 6efghij
Sub mountain undulating central region			
Soil	129 ± 13k	125 ± 11k	120 ± 4j
Soil + Pc	161 ± 9ghij	150 ± 13efghijk	151 ± 17efghij
Soil + Pp	159 ± 9hijk	147 ± 10efghijk	156 ± 15efghij
Soil + BC	162 ± 8ghij	143 ± 13ghijk	155 ± 6efghij
Soil + RP	163 ± 6ghij	145 ± 15ghijk	150 ± 4efghij
Soil + RP + Pc	198 ± 6abcde	163 ± 9abcdeghi	204 ± 8abcd
Soil + RP + Pp	195 ± 8bcdef	168 ± 10abcdefg	206 ± 6abcd
Soil + RP + BC	191 ± 4cdefg	169 ± 6abcdefg	194 ± 6ab
DAP	156 ± 6hijk	137 ± 8hijk	141 ± 11fghij
Sub mountain undulating region			
Soil	143 ± 10ijk	136 ± 9ijk	127 ± 10hij
Soil + Pc	177 ± 9defgh	162 ± 4abcdeghi	165 ± 10defghi
Soil + Pp	180 ± 17cdefgh	165 ± 10abcdeghi	180 ± 13cdef
Soil + BC	183 ± 17cdefgh	166 ± 4abcdeghi	186 ± 9bcde
Soil + RP	179 ± 13cdefgh	170 ± 11abcdeghi	148 ± 6efghij
Soil + RP + Pc	198 ± 13abcde	186 ± 6ab	220 ± 7abc
Soil + RP + Pp	223 ± 13ab	183 ± 4abc	226 ± 10ab
Soil + RP + BC	227 ± 6a	188 ± 2a	230 ± 6a
DAP	155 ± 13hijk	150 ± 11efghijk	138 ± 6fghij
LSD (P<0.05)	16	15	22

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

Table 7.4 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of maize plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	pH	EC (mScm ⁻¹)	TDS (ppm)
Central plain region			
Soil	8.24 ± 0.03b	0.16 ± 0.02fg	0.11 ± 0.010de
Soil + Pc	8.11 ± 0.02cd	0.21 ± 0.02abcdef	0.14 ± 0.013abcd
Soil + Pp	8.03 ± 0.02de	0.22 ± 0.025abcdef	0.15 ± 0.017abcd
Soil + BC	8.02 ± 0.03ef	0.23 ± 0.03abcdef	0.15 ± 0.017abcd
Soil + RP	7.80 ± 0.02hijkl	0.21 ± 0.02abcdef	0.14 ± 0.013abcd
Soil + RP + Pc	7.73 ± 0.02lmn	0.24 ± 0.03abcde	0.16 ± 0.020abc
Soil + RP + Pp	7.74 ± 0.01klmn	0.23 ± 0.02abcde	0.16 ± 0.010abcd
Soil + RP + BC	7.75 ± 0.05jklmn	0.22 ± 0.02abcdef	0.15 ± 0.014abcd
DAP	8.15 ± 0.06bc	0.23 ± 0.03abcdef	0.15 ± 0.018abcd
Sub mountain undulating central region			
Soil	8.19 ± 0.02bc	0.13 ± 0.02g	0.09 ± 0.010e
Soil + Pc	7.87 ± 0.02gh	0.19 ± 0.02bcdefg	0.13 ± 0.010bcde
Soil + Pp	7.85 ± 0.01hi	0.18 ± 0.02defg	0.12 ± 0.010cde
Soil + BC	7.85 ± 0.01hi	0.19 ± 0.01cdefg	0.13 ± 0.007bcde
Soil + RP	7.82 ± 0.02hijk	0.19 ± 0.03defg	0.13 ± 0.017bcde
Soil + RP + Pc	7.72 ± 0.01lmn	0.22 ± 0.01abcdef	0.15 ± 0.007abcd
Soil + RP + Pp	7.70 ± 0.01mn	0.21 ± 0.02abcdef	0.14 ± 0.010abcd
Soil + RP + BC	7.69 ± 0.05n	0.23 ± 0.02abcdef	0.15 ± 0.014abcd
DAP	7.98 ± 0.03ef	0.20 ± 0.02bcdef	0.13 ± 0.014abcd
Sub mountain undulating region			
Soil	8.33 ± 0.03a	0.18 ± 0.02efg	0.12 ± 0.02cde
Soil + Pc	7.84 ± 0.02hij	0.26 ± 0.03abc	0.17 ± 0.02ab
Soil + Pp	7.85 ± 0.01hi	0.24 ± 0.03abcde	0.16 ± 0.02abcd
Soil + BC	7.81 ± 0.06hijk	0.27 ± 0.02abc	0.17 ± 0.01ab
Soil + RP	7.94 ± 0.02fg	0.19 ± 0.02bcdefg	0.13 ± 0.01bcde
Soil + RP + Pc	7.78 ± 0.02ijklm	0.27 ± 0.03a	0.18 ± 0.02a
Soil + RP + Pp	7.76 ± 0.01jklmn	0.26 ± 0.03ab	0.17 ± 0.02ab
Soil + RP + BC	7.75 ± 0.03klmn	0.26 ± 0.01ab	0.17 ± 0.01ab
DAP	8.01 ± 0.04ef	0.25 ± 0.02abcd	0.17 ± 0.01abc
LSD (P<0.05)	0.044	0.035	0.024

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

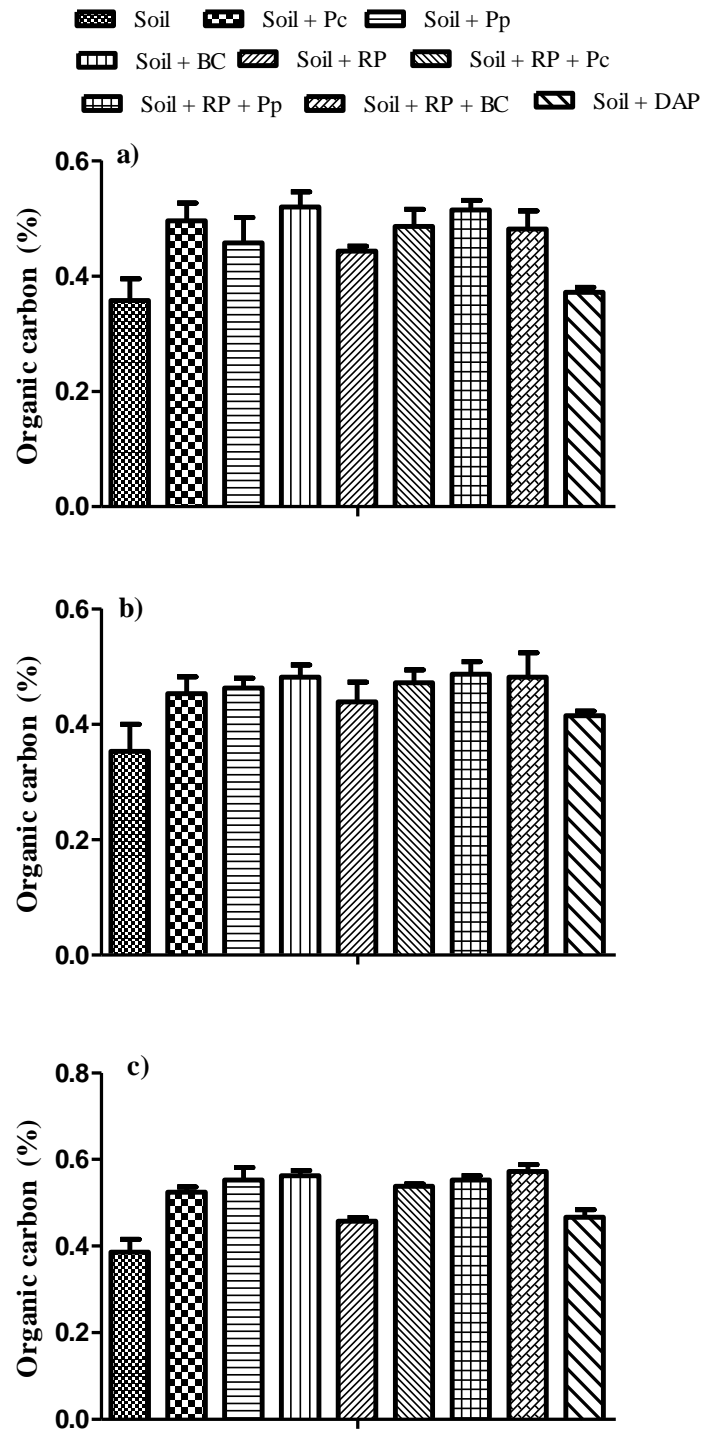


Fig. 7.2 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on organic carbon of rhizosphere soil of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

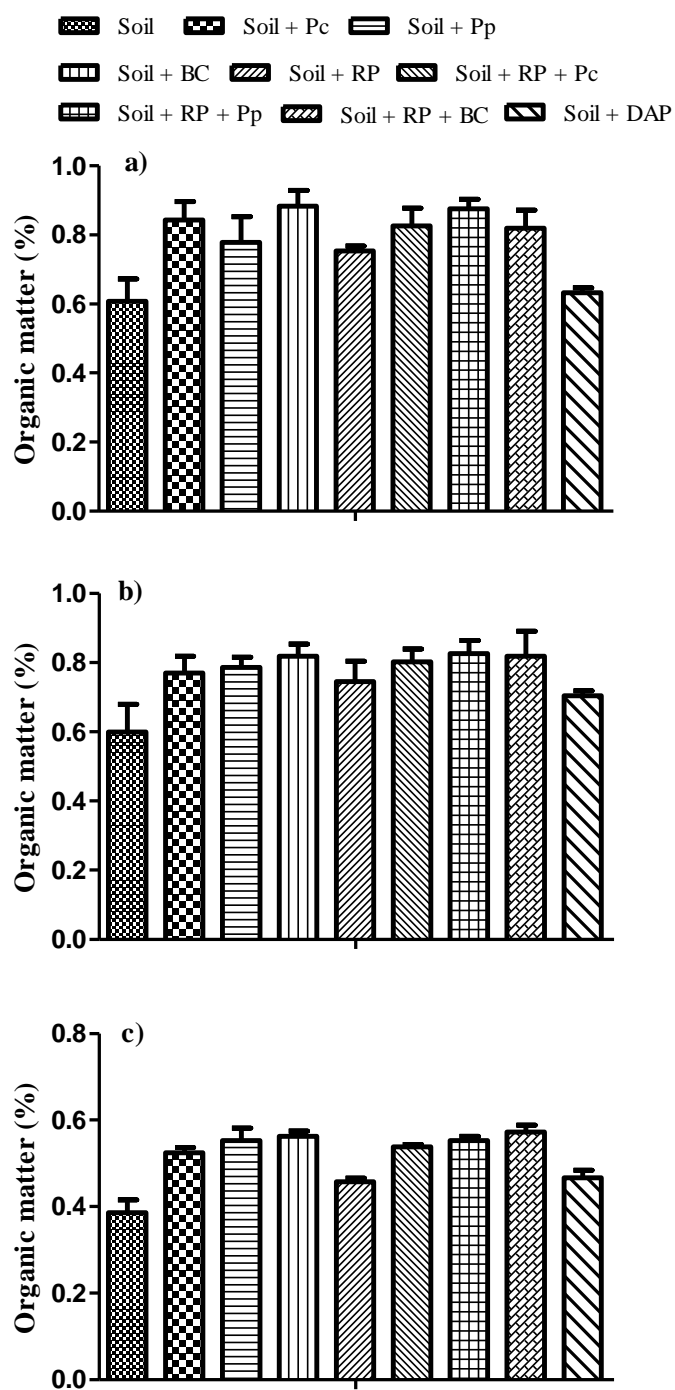


Fig. 7.3 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on organic matter of rhizosphere soil of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) (Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

Table 7.5 Effect of *Pantoea cypripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of maize plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	Total P (mg kg ⁻¹)	Available P (mg kg ⁻¹)	Total nitrogen (%)
Central plain region			
Soil	251 ± 15e	3.37 ± 0.19jk	0.031 ± 0.005a
Soil + Pc	256 ± 6e	4.74 ± 0.12efgh	0.034 ± 0.008a
Soil + Pp	260 ± 9e	4.64 ± 0.13efghi	0.036 ± 0.010a
Soil + BC	251 ± 2e	4.84 ± 0.13defgh	0.037 ± 0.004a
Soil + RP	483 ± 8abc	5.11 ± 0.13cdef	0.035 ± 0.010a
Soil + RP + Pc	494 ± 17a	7.09 ± 0.31b	0.036 ± 0.013a
Soil + RP + Pp	493 ± 10a	7.16 ± 0.14b	0.031 ± 0.010a
Soil + RP + BC	490 ± 21ab	7.26 ± 0.90b	0.031 ± 0.010a
DAP	494 ± 6a	4.11 ± 0.07ghij	0.036 ± 0.005a
Sub mountain undulating central region			
Soil	227 ± 15e	3.22 ± 0.17k	0.028 ± 0.005a
Soil + Pc	240 ± 10e	4.33 ± 0.15fghi	0.034 ± 0.008a
Soil + Pp	244 ± 6e	4.41 ± 0.18fghi	0.031 ± 0.005a
Soil + BC	250 ± 7e	4.40 ± 0.09fghi	0.031 ± 0.013a
Soil + RP	447 ± 21bcd	4.89 ± 0.09cdefg	0.036 ± 0.005a
Soil + RP + Pc	436 ± 15d	6.57 ± 0.18b	0.028 ± 0.013a
Soil + RP + Pp	447 ± 15cd	6.75 ± 0.24b	0.034 ± 0.008a
Soil + RP + BC	445 ± 21cd	6.85 ± 0.26b	0.036 ± 0.005a
DAP	504 ± 13a	3.87 ± 0.22ijk	0.031 ± 0.005a
Sub mountain undulating region			
Soil	237 ± 8e	4.01 ± 0.17hijk	0.034 ± 0.002a
Soil + Pc	241 ± 4e	5.62 ± 0.16cde	0.039 ± 0.004a
Soil + Pp	256 ± 2e	5.63 ± 0.05cd	0.040 ± 0.001a
Soil + BC	261 ± 6e	5.66 ± 0.10c	0.039 ± 0.013a
Soil + RP	465 ± 9abcd	5.29 ± 0.07cde	0.042 ± 0.017a
Soil + RP + Pc	480 ± 35abc	8.07 ± 0.22a	0.039 ± 0.009a
Soil + RP + Pp	504 ± 17a	8.48 ± 0.33a	0.048 ± 0.010a
Soil + RP + BC	497 ± 6a	8.53 ± 0.10a	0.041 ± 0.002a
DAP	487 ± 8abc	4.94 ± 0.09cdefg	0.039 ± 0.005a
LSD (P<0.05)	22	0.43	0.01

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

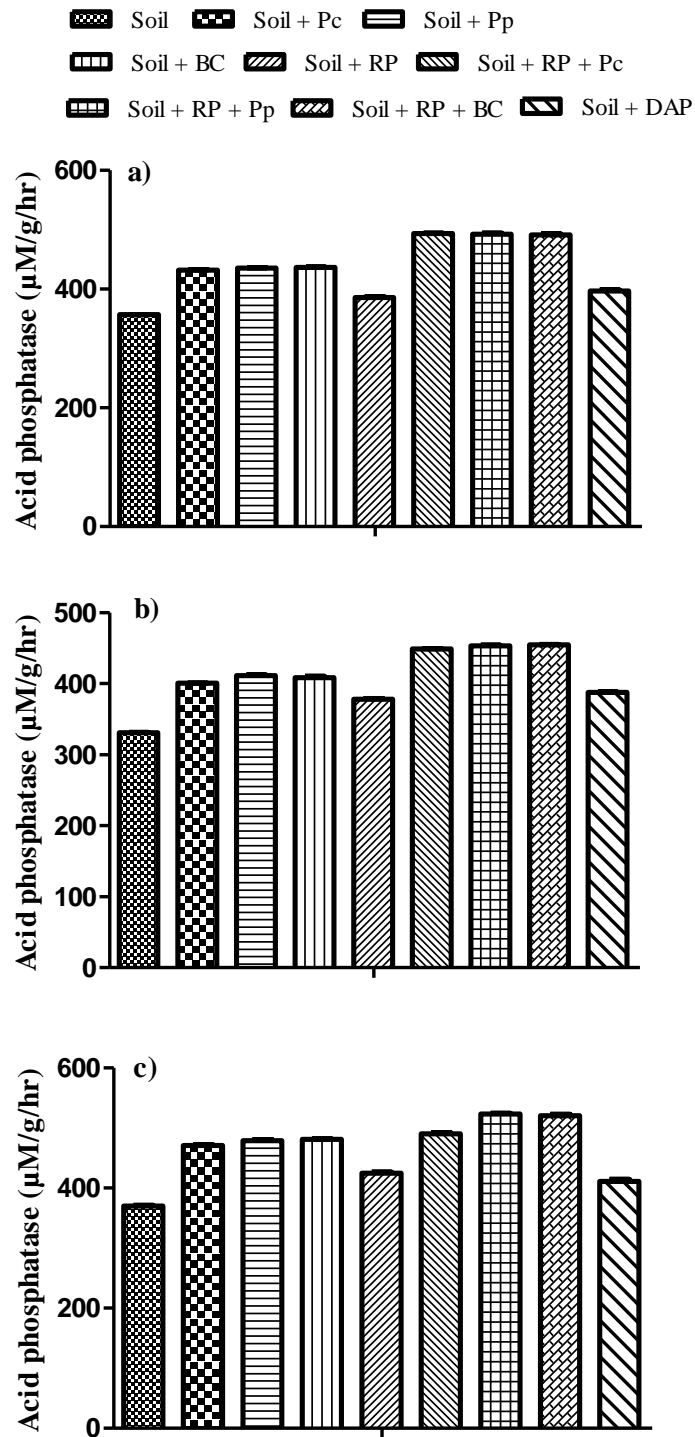


Fig. 7.4 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on acid phosphatase enzyme activities of rhizosphere soil of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

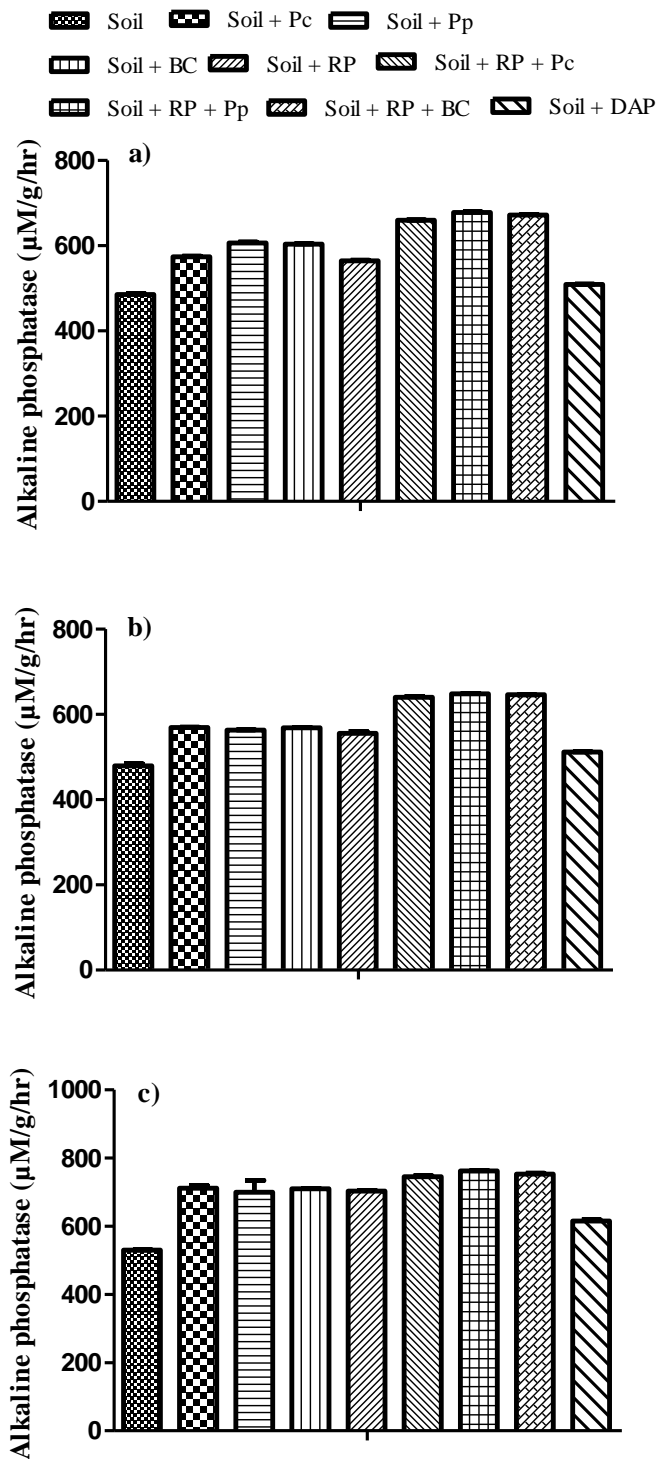


Fig. 7.5 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on alkaline phosphatase enzyme activities of rhizosphere soil of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

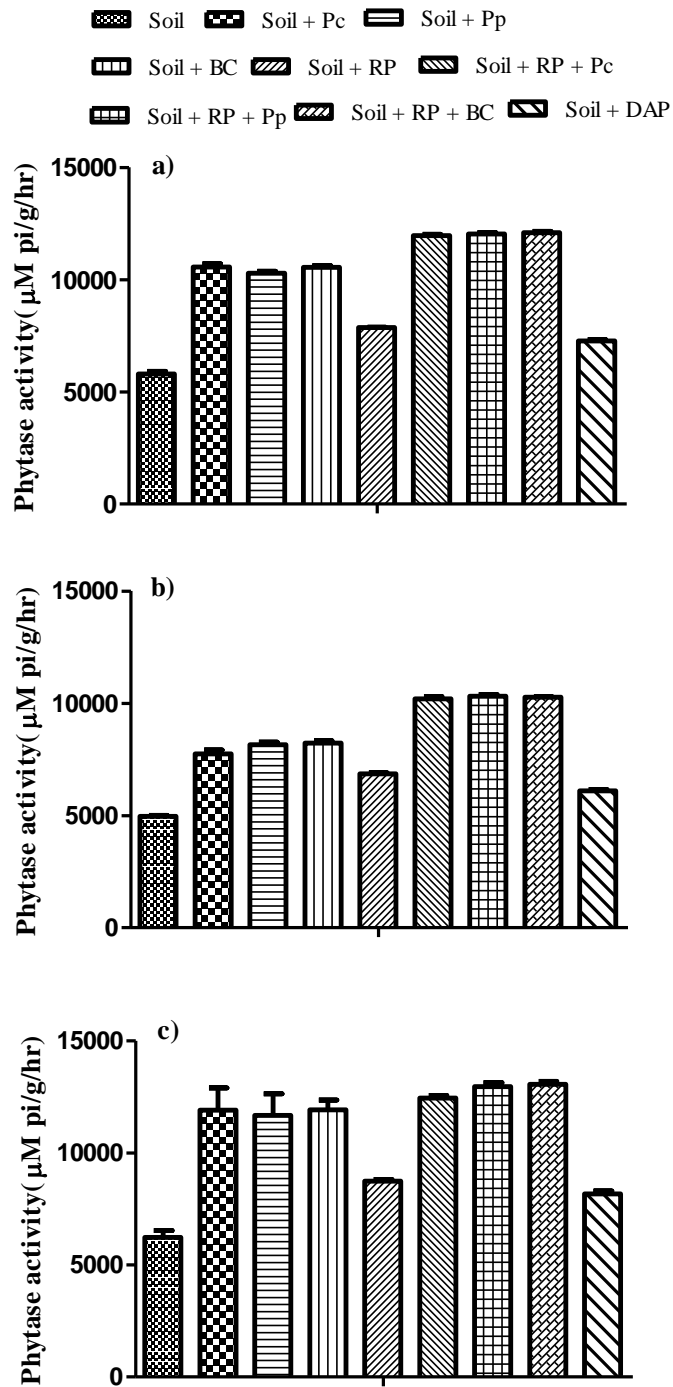


Fig. 7.6 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on phytase enzyme activities of rhizosphere soil of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

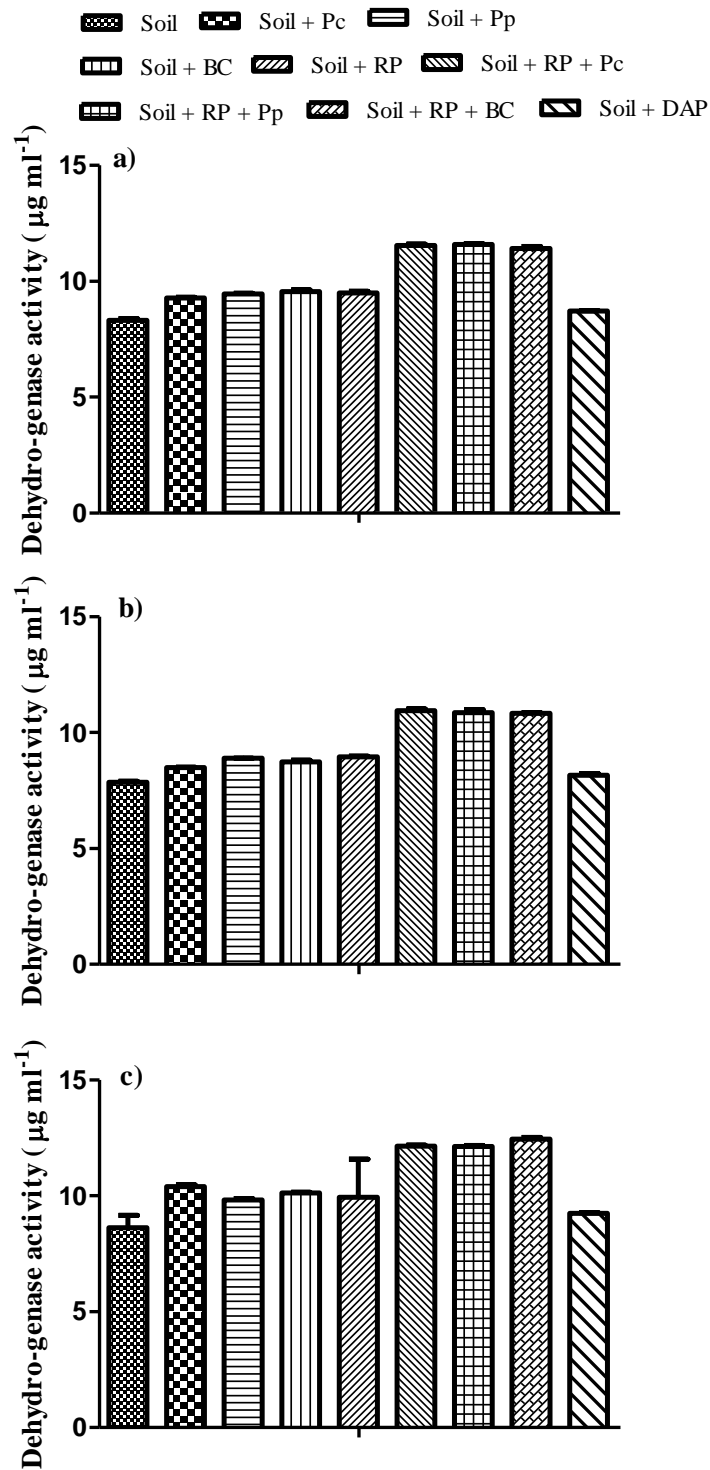


Fig. 7.7 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on dehydro-genase enzyme activities of rhizosphere soil of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

7.1.2 Wheat experiment at multilocational sites with bacterial inoculants

To test the effect of previously (in maize experiment) added RP on second year crop, wheat experiment was conducted at all the three sites. Plant shoot height, shoot and root dry weight at all three different sites were significantly improved with bacterial inoculation alone or along with RP fertilization (Table 7.6). A significant increase in wheat grain yield (Fig. 7.8) (16-20 %) was observed in all the three sites due to inoculation along with RP fertilization treatments, the yield was up to 14 % with inoculation treatments, 13 % with RP fertilization treatments and 6 to 10 % with DAP treatment compared to control soil. Significant improvement in total P uptake in seeds, shoots and roots of wheat crop was observed by introduction of *P. cyripedii* and *P. plecoglossicida* during second year of field study compared to control soil (Table 7.7). Stimulatory effects of inoculation treatments along with RP fertilization on yield and P uptake was found to be more pronounced compared to inoculation alone, RP fertilization alone and DAP treatment. Enhancement in yield and P uptake in wheat was more pronounced at sub mountain undulating region compared to central plain region and sub mountain undulating central region.

Physiochemical properties of wheat rhizospheric soils were significantly improved in all the treatments but the effects were more significant when inoculation was done along with RP fertilization. Bacterial inoculation slightly decreased the soil pH in all the treatments compared to un-inoculated control seed treatments in all field trials (Table 7.8). Inoculation along with RP fertilization increased the soil organic carbon content 25 %, 26 % and 33 % and DAP treatments increased the soil organic carbon content 3 %, 11 % and 13 % at central plain region, sub mountain undulating central region and at sub mountain undulating region compared to control treatment respectively (Fig. 7.9). Organic matter in all the inoculation and RP fertilization treatments was significantly improved, compared to DAP and control treatment (Fig 7.10). Available P level was significantly increased at central plain region (42

%), sub mountain undulating central region (38 %) and at sub mountain undulating region (41 %) due to inoculation but the effect was more pronounced (85 %, 84 % and 86 %) when RP was supplemented along with inoculation compared to control. Acid phosphatase, alkaline phosphatase, phytase and dehydrogenase enzyme activities were more pronounced in inoculation treatments along with RP fertilization compared to other treatments (Figure 7.11, 7.12, 7.13 and 7.14). In all the field trials at different sites phytase enzyme activities were higher than acid phosphatase and alkaline phosphatase enzyme activities.

Population density of phosphate-solubilizing bacteria was tested after harvesting of the wheat crop, at central plain region, sub mountain undulating central region and sub mountain undulating region and it was 1.7×10^5 cfu g⁻¹, 0.6×10^5 cfu g⁻¹ and 2.0×10^6 in control treatments, $7.7-8.4 \times 10^7$ cfu g⁻¹, $6.6-6.9 \times 10^7$ cfu g⁻¹ and $2.1-2.2 \times 10^8$ cfu g⁻¹ in inoculation treatments, 4×10^5 cfu g⁻¹, 2.7×10^5 cfu g⁻¹ and 4.1×10^6 cfu g⁻¹ in RP fertilization treatments, $9.8 \times 10^7-1.3 \times 10^8$ cfu g⁻¹, $8.4-9.6 \times 10^7$ cfu g⁻¹ and $3.6-4.1 \times 10^8$ cfu g⁻¹ in inoculation along with RP fertilization and 2.3×10^5 cfu g⁻¹, 2×10^5 cfu g⁻¹ and 2.1×10^6 cfu g⁻¹ in DAP treatments. There is no significant effect of DAP and RP fertilization treatments alone on increased in population density of PSBs.

Table 7.6 Effect of *Pantoea cypripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on the growth parameters of wheat plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	Shoot length (cm)	Shoot dry weight (g)	Root dry weight (g)
Central plain region			
Soil	96 ± 1.5mn	1.36 ± 0.03ef	0.54 ± 0.03hi
Soil + Pc	101 ± 0.9hijklm	1.6 ± 0.14cdef	0.64 ± 0.18defghi
Soil + Pp	104 ± 1.6ghijklm	1.73 ± 0.07cde	0.63 ± 0.10defghi
Soil + BC	105 ± 1.7fghijklm	1.74 ± 0.05cde	0.65 ± 0.07defghi
Soil + RP	108 ± 2.0efghijk	1.60 ± 0.02cdef	0.75 ± 0.08cdefgh
Soil + RP + Pc	114 ± 1.2abcdef	2.36 ± 0.03a	0.81 ± 0.01bcde
Soil + RP + Pp	116 ± 2.2abcde	2.39 ± 0.02a	0.83 ± 0.02bcde
Soil + RP + BC	117 ± 4.6abcd	2.35 ± 0.07a	0.85 ± 0.01bcd
DAP	99 ± 0.8jklmn	1.58 ± 0.02cdef	0.62 ± 0.03efghi
Sub mountain undulating central region			
Soil	92 ± 3.4n	1.24 ± 0.08f	0.49 ± 0.08i
Soil + Pc	101 ± 2.8hijklm	1.46 ± 0.09def	0.57 ± 0.05ghi
Soil + Pp	99 ± 1.9jklmn	1.53 ± 0.06cdef	0.56 ± 0.07ghi
Soil + BC	101 ± 1.2hijklm	1.56 ± 0.05cdef	0.58 ± 0.04fghi
Soil + RP	106 ± 2.7fghijkl	1.52 ± 0.03cdef	0.65 ± 0.04defghi
Soil + RP + Pc	108 ± 1.5efghijk	2.16 ± 0.01ab	0.74 ± 0.01cdefgh
Soil + RP + Pp	110 ± 1.4cdefgh	2.17 ± 0.01ab	0.75 ± 0.04cdefgh
Soil + RP + BC	111 ± 1.7bcdefg	2.18 ± 0.07ab	0.76 ± 0.05cdefg
DAP	98 ± 2.3lmn	1.38 ± 0.13ef	0.54 ± 0.03ghi
Sub mountain undulating region			
Soil	99 ± 8.1klmn	1.44 ± 0.10def	0.66 ± 0.07defghi
Soil + Pc	104 ± 5.8ghijklm	1.82 ± 0.03bcd	0.79 ± 0.07cdef
Soil + Pp	104 ± 2.0ghijklm	1.86 ± 0.01bc	0.84 ± 0.08bcd
Soil + BC	109 ± 3.8defghij	1.87 ± 0.07bc	0.82 ± 0.07bcde
Soil + RP	109 ± 3.8defghijkl	1.70 ± 0.05cde	0.79 ± 0.16cdef
Soil + RP + Pc	119 ± 0.9abc	2.56 ± 0.16a	0.93 ± 0.05abc
Soil + RP + Pp	120 ± 1.4ab	2.54 ± 0.16a	1.03 ± 0.06ab
Soil + RP + BC	122 ± 2.2a	2.55 ± 0.22a	1.12 ± 0.003a
DAP	100 ± 1.1ijklmn	1.58 ± 0.46cdef	0.75 ± 0.04cdefgh
LSD (P<0.05)	4.85	0.20	0.111

Values are Mean ± SD (*n* =10). Means sharing a common letter within the column are not significantly different at *P*<0.05. Pronounced results are represented in bold.

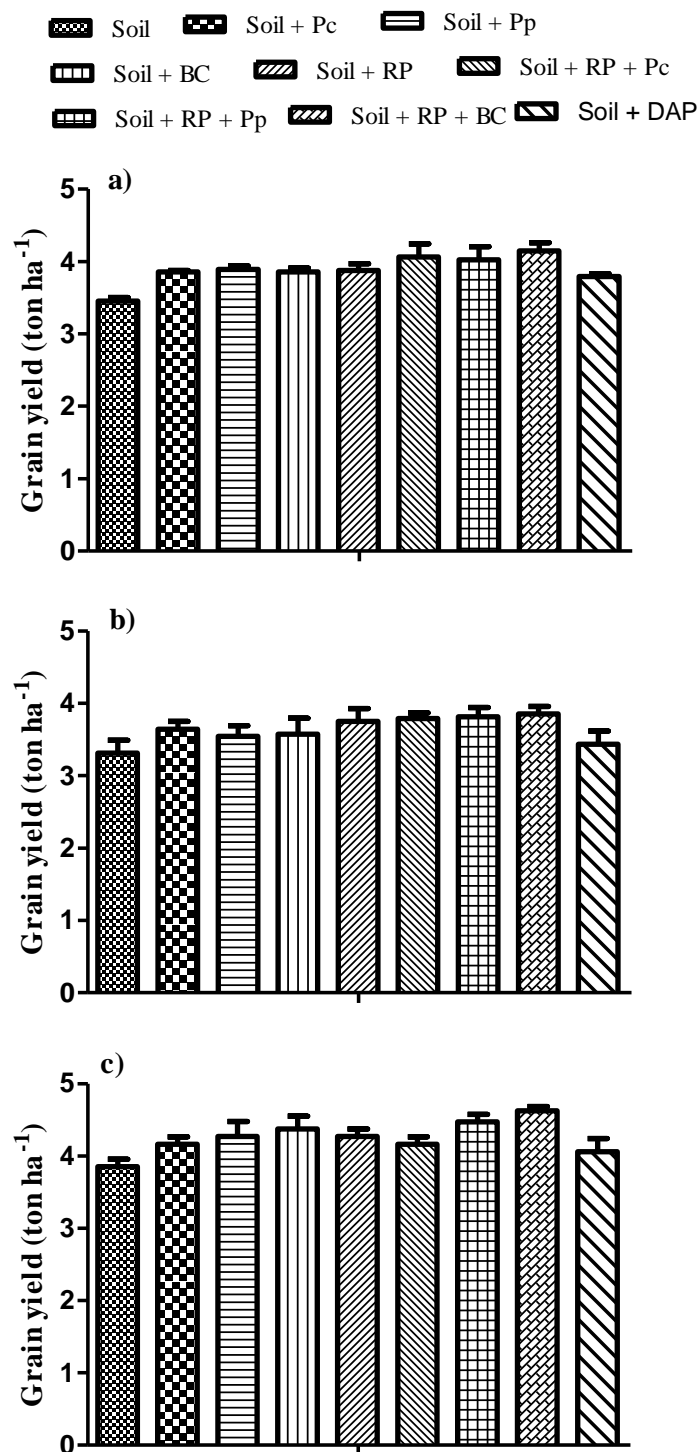


Fig. 7.8 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on grain yield of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

Table 7.7 Effect of *Pantoea cypripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on P uptake of wheat plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	Grains (mg kg ⁻¹)	Shoot (mg kg ⁻¹)	Root (mg kg ⁻¹)
Central plain region			
Soil	218 ± 2jk	28 ± 4gh	191 ± 13i
Soil + Pc	243 ± 13hijk	38 ± 2fgh	258 ± 8efg
Soil + Pp	240 ± 6hijk	36 ± 9fgh	256 ± 10efg
Soil + BC	247 ± 19ghijk	34 ± 6fgh	259 ± 13efg
Soil + RP	298 ± 10cdef	41 ± 8fgh	280 ± 9def
Soil + RP + Pc	333 ± 4abc	77 ± 6bc	359 ± 10c
Soil + RP + Pp	330 ± 10cdefg	83 ± 13b	365 ± 9c
Soil + RP + BC	336 ± 13abc	84 ± 10b	368 ± 9bc
DAP	237 ± 4hijk	31 ± 6fgh	250 ± 11fgh
Sub mountain undulating central region			
Soil	213 ± 5k	26 ± 10h	184 ± 13i
Soil + Pc	236 ± 9hijk	33 ± 8fgh	242 ± 23fgh
Soil + Pp	237 ± 18hijk	34 ± 6fgh	245 ± 15fgh
Soil + BC	238 ± 13hijk	37 ± 11fgh	248 ± 13fgh
Soil + RP	277 ± 5defgh	31 ± 9fgh	275 ± 8def
Soil + RP + Pc	318 ± 10bcde	69 ± 9bcde	347 ± 10c
Soil + RP + Pp	320 ± 18bcde	72 ± 9bcd	348 ± 6c
Soil + RP + BC	325 ± 11abcd	75 ± 8bcd	343 ± 14c
DAP	222 ± 16jk	29 ± 11gh	216 ± 17hi
Sub mountain undulating region			
Soil	227 ± 10ijk	38 ± 10fgh	222 ± 6hi
Soil + Pc	263 ± 10fghij	44 ± 6fgh	265 ± 9def
Soil + Pp	294 ± 5cdefg	52 ± 6defg	301 ± 6d
Soil + BC	273 ± 15efghi	54 ± 4cdef	302 ± 6d
Soil + RP	336 ± 23abc	69 ± 6bcde	295 ± 8de
Soil + RP + Pc	372 ± 10a	109 ± 6a	405 ± 10ab
Soil + RP + Pp	350 ± 11ab	93 ± 6ab	429 ± 8a
Soil + RP + BC	366 ± 18ab	112 ± 4a	430 ± 13a
DAP	236 ± 20hijk	47 ± 6efgh	245 ± 26fgh
LSD (P<0.05)	26	13	20

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

Table 7.8 Effect of *Pantoea cypripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of wheat plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	pH	EC (mScm ⁻¹)	TDS (ppm)
Central plain region			
Soil	8.23 ± 0.02b	0.17 ± 0.01efghi	0.11 ± 0.007fghijkl
Soil + Pc	7.62 ± 0.03hijkl	0.20 ± 0.02bcdef	0.14 ± 0.010bcdefg
Soil + Pp	7.66 ± 0.02fghi	0.20 ± 0.02bcdef	0.13 ± 0.013bcdefg
Soil + RP	7.65 ± 0.03ghij	0.22 ± 0.02abcd	0.15 ± 0.010abcde
Soil + BC	8.14 ± 0.05c	0.21 ± 0.02abcde	0.14 ± 0.013abcdef
Soil + RP + Pc	7.60 ± 0.02ijklm	0.24 ± 0.02ab	0.16 ± 0.010ab
Soil + RP + Pp	7.59 ± 0.04jklm	0.25 ± 0.01a	0.17 ± 0.007a
Soil + RP + BC	7.59 ± 0.02jklm	0.24 ± 0.01ab	0.16 ± 0.009abc
DAP	8.17 ± 0.02bc	0.20 ± 0.02bcdef	0.14 ± 0.010bcdefg
Sub mountain undulating central region			
Soil	8.19 ± 0.02bc	0.12 ± 0.01i	0.08 ± 0.007j
Soil + Pc	7.73 ± 0.02ef	0.17 ± 0.02defgh	0.12 ± 0.010efghi
Soil + Pp	7.74 ± 0.03e	0.15 ± 0.01ghi	0.10 ± 0.007hij
Soil + BC	7.73 ± 0.02ef	0.17 ± 0.01efgh	0.11 ± 0.004fghi
Soil + RP	7.97 ± 0.02d	0.16 ± 0.02fghi	0.11 ± 0.010ghij
Soil + RP + Pc	7.70 ± 0.01efg	0.20 ± 0.02bcdef	0.14 ± 0.010bcdefg
Soil + RP + Pp	7.67 ± 0.02fgh	0.23 ± 0.02abc	0.15 ± 0.013abcd
Soil + RP + BC	7.63 ± 0.01ghijk	0.22 ± 0.01abc	0.15 ± 0.007abcd
DAP	7.98 ± 0.01d	0.19 ± 0.02cdefg	0.13 ± 0.010defgh
Sub mountain undulating region			
Soil	8.30 ± 0.02a	0.14 ± 0.01hi	0.09 ± 0.007ij
Soil + Pc	7.67 ± 0.02fgh	0.14 ± 0.02hi	0.09 ± 0.010ij
Soil + Pp	7.61 ± 0.01hijklm	0.15 ± 0.01ghi	0.10 ± 0.007hij
Soil + BC	7.65 ± 0.01ghij	0.22 ± 0.02abcd	0.15 ± 0.014abcde
Soil + RP	8.13 ± 0.02c	0.19 ± 0.01cdefg	0.13 ± 0.007cdefgh
Soil + RP + Pc	7.56 ± 0.03lm	0.19 ± 0.02bcdefg	0.13 ± 0.01bcdefgh
Soil + RP + Pp	7.55 ± 0.01m	0.16 ± 0.01fghi	0.11 ± 0.004ghij
Soil + RP + BC	7.58 ± 0.03klm	0.22 ± 0.01abc	0.15 ± 0.007abcd
DAP	8.19 ± 0.03bc	0.19 ± 0.02bcdefg	0.13 ± 0.010bcdefgh
LSD (P<0.05)	0.036	0.023	0.015

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

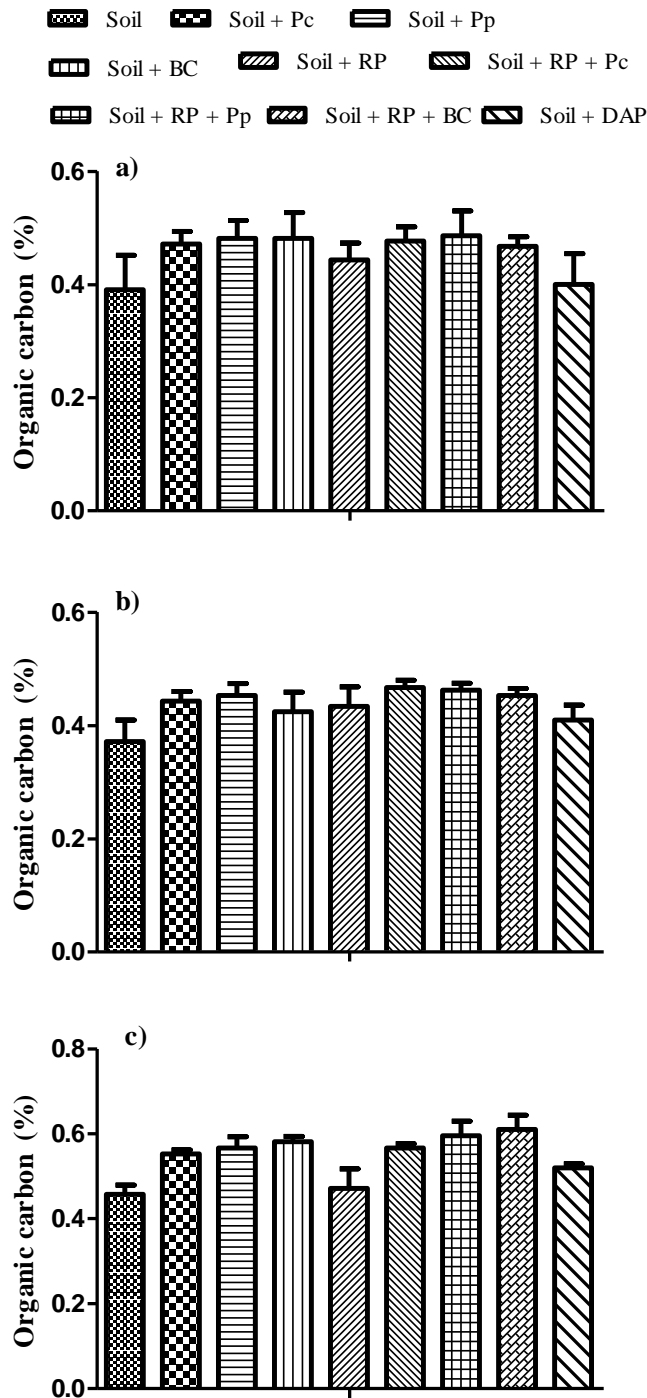


Fig. 7.9 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on organic carbon of rhizosphere soil characteristics of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

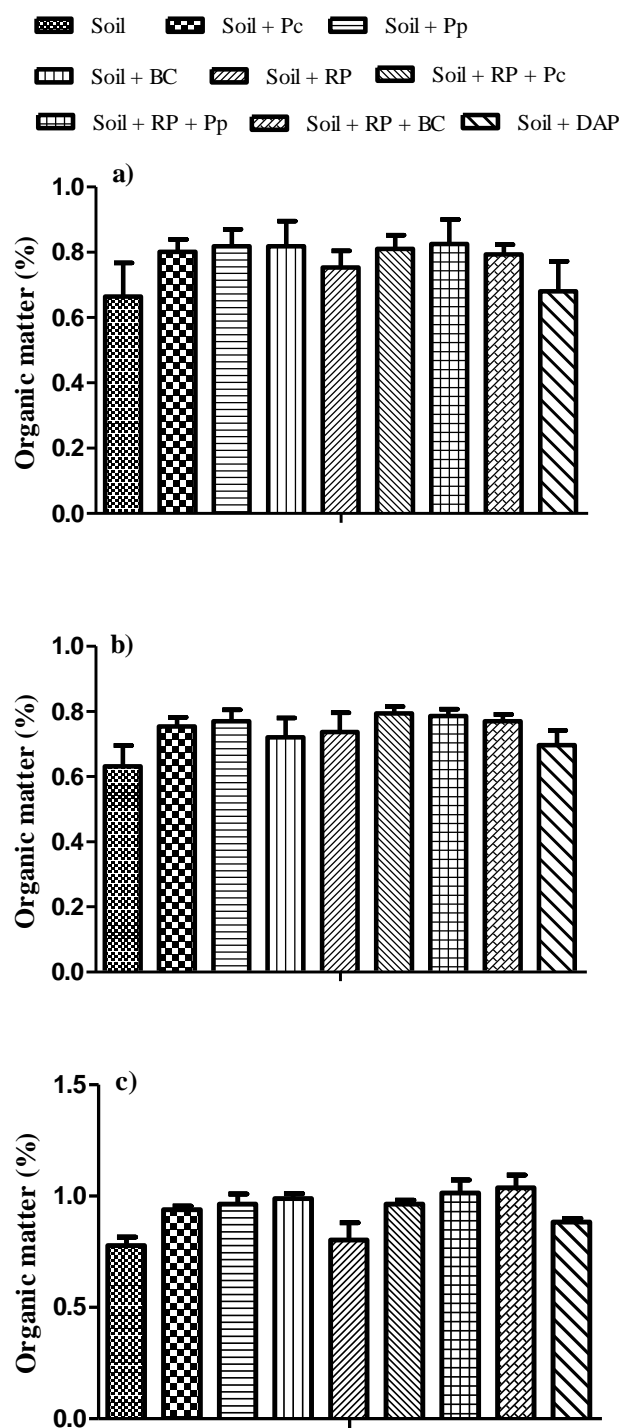


Fig. 7.10 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on organic matter of rhizosphere soil characteristics of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

Table 7.9 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of wheat plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	Total P (mg kg ⁻¹)	Available P (mg kg ⁻¹)	Total nitrogen (%)
Central plain region			
Soil	225 ± 13bc	3.86 ± 0.09gh	0.045 ± 0.005a
Soil + Pc	238 ± 17bc	5.45 ± 0.21cde	0.039 ± 0.010a
Soil + Pp	233 ± 8bc	5.42 ± 0.31cde	0.045 ± 0.010a
Soil + BC	245 ± 8bc	5.46 ± 0.07cde	0.045 ± 0.005a
Soil + RP	468 ± 15a	5.05 ± 0.34de	0.048 ± 0.005a
Soil + RP + Pc	465 ± 16a	7.10 ± 0.07b	0.056 ± 0.005a
Soil + RP + Pp	466 ± 15a	7.16 ± 0.07b	0.050 ± 0.015a
Soil + RP + BC	463 ± 6 a	7.06 ± 0.09b	0.050 ± 0.008a
DAP	461 ± 15a	4.39 ± 0.09fg	0.053 ± 0.010a
Sub mountain undulating central region			
Soil	213 ± 6bc	3.70 ± 0.11h	0.028 ± 0.010a
Soil + Pc	211 ± 19bc	5.10 ± 0.26de	0.031 ± 0.013a
Soil + Pp	206 ± 17bc	4.93 ± 0.07def	0.028 ± 0.010a
Soil + BC	204 ± 8c	4.97 ± 0.09def	0.042 ± 0.008a
Soil + RP	434 ± 15a	4.87 ± 0.24ef	0.031 ± 0.005a
Soil + RP + Pc	429 ± 8a	6.82 ± 0.13b	0.034 ± 0.008a
Soil + RP + Pp	426 ± 6a	6.81 ± 0.18b	0.036 ± 0.010a
Soil + RP + BC	431 ± 16a	6.77 ± 0.20b	0.045 ± 0.024a
DAP	440 ± 6a	4.02 ± 0.13gh	0.045 ± 0.017a
Sub mountain undulating region			
Soil	204 ± 7c	4.94 ± 0.18def	0.045 ± 0.005a
Soil + Pc	234 ± 25bc	6.72 ± 0.18b	0.050 ± 0.008a
Soil + Pp	245 ± 8bc	6.94 ± 0.18b	0.055 ± 0.011a
Soil + BC	250 ± 8b	6.88 ± 0.39b	0.051 ± 0.009a
Soil + RP	455 ± 6a	5.90 ± 0.18c	0.045 ± 0.010a
Soil + RP + Pc	445 ± 8a	9.11 ± 0.11a	0.061 ± 0.006a
Soil + RP + Pp	434 ± 28a	8.81 ± 0.11a	0.060 ± 0.016a
Soil + RP + BC	441 ± 8a	9.18 ± 0.29a	0.062 ± 0.028a
DAP	437 ± 19a	5.50 ± 0.11cd	0.046 ± 0.003a
LSD (P<0.05)	22	0.31	0.019

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

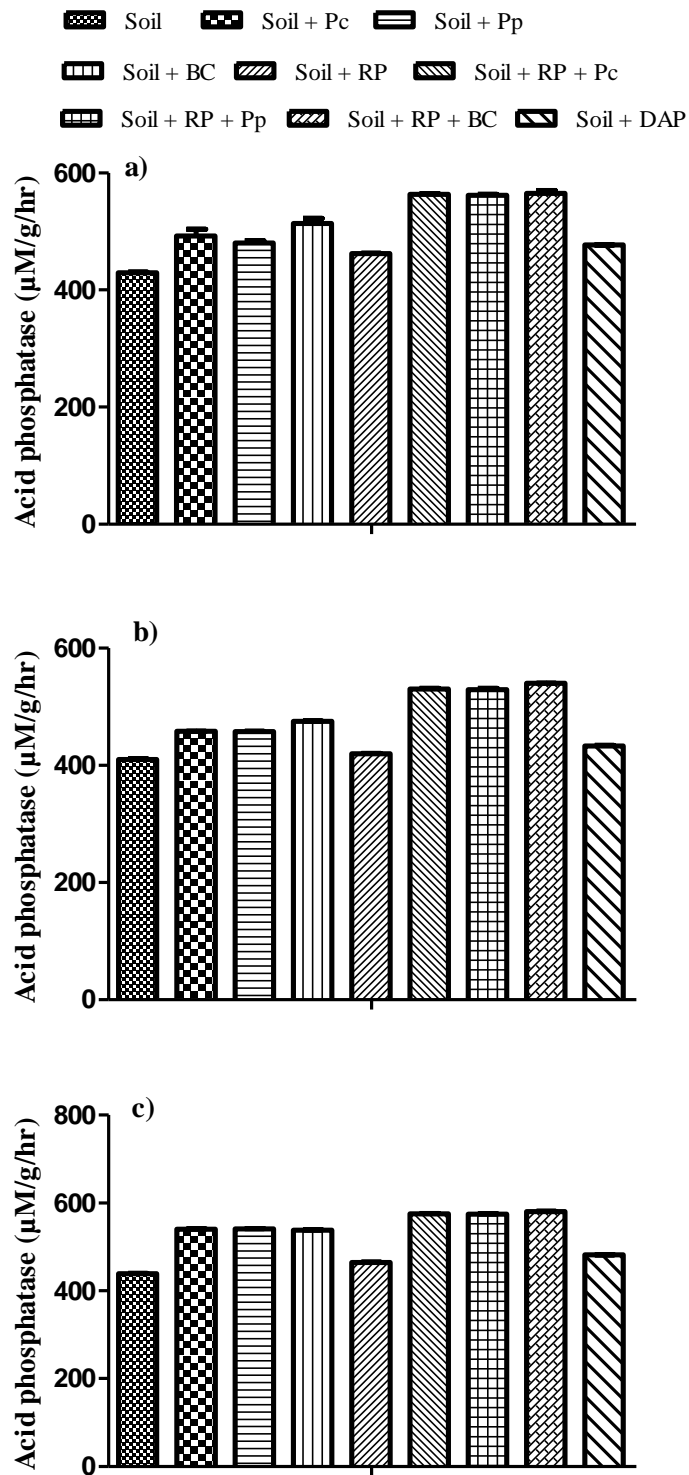


Fig. 7.11 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on acid phosphatase enzyme activities of rhizosphere soil of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

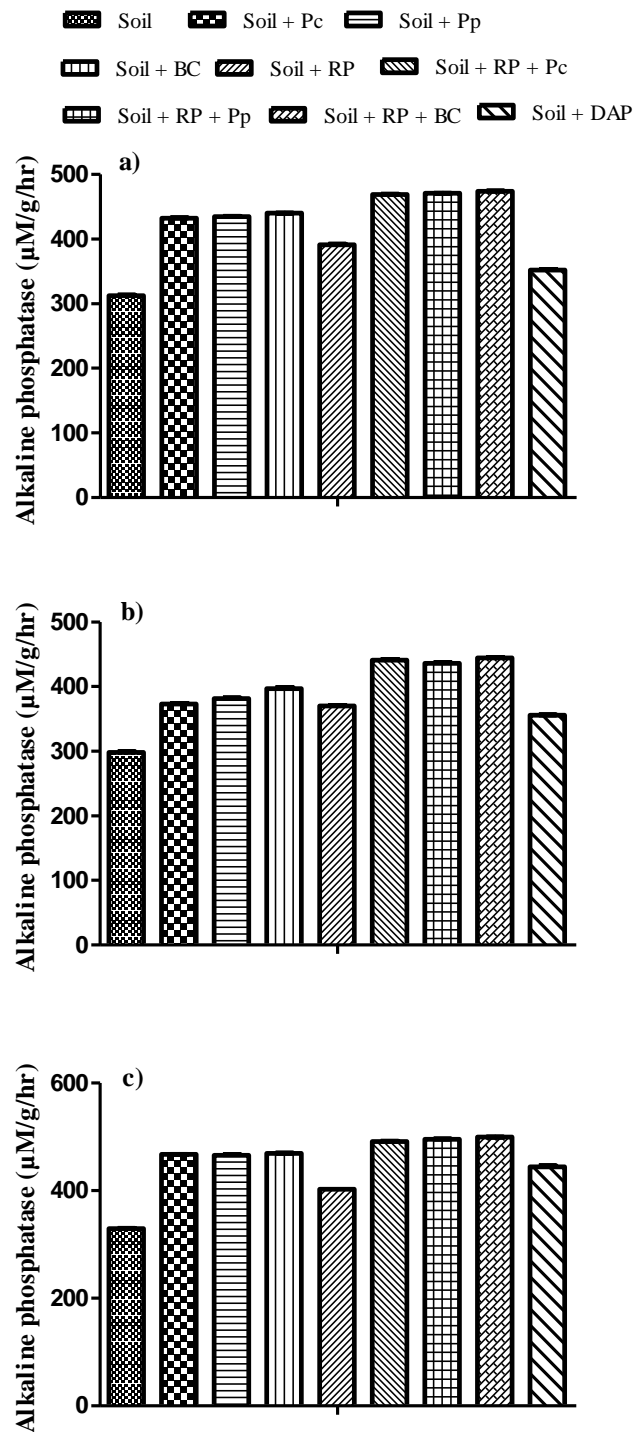


Fig. 7.12 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on alkaline phosphatase enzyme activities of rhizosphere soil of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

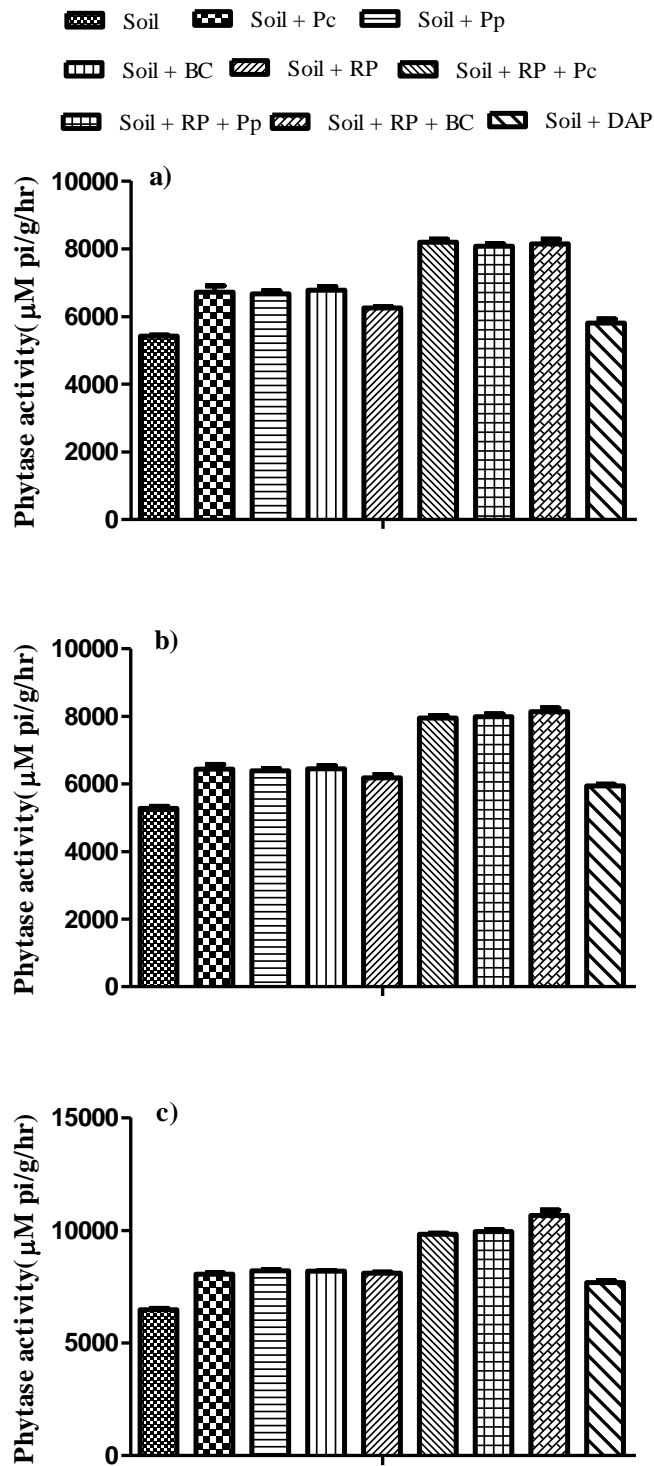


Fig. 7.13 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on phytase enzyme activities of rhizosphere soil of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

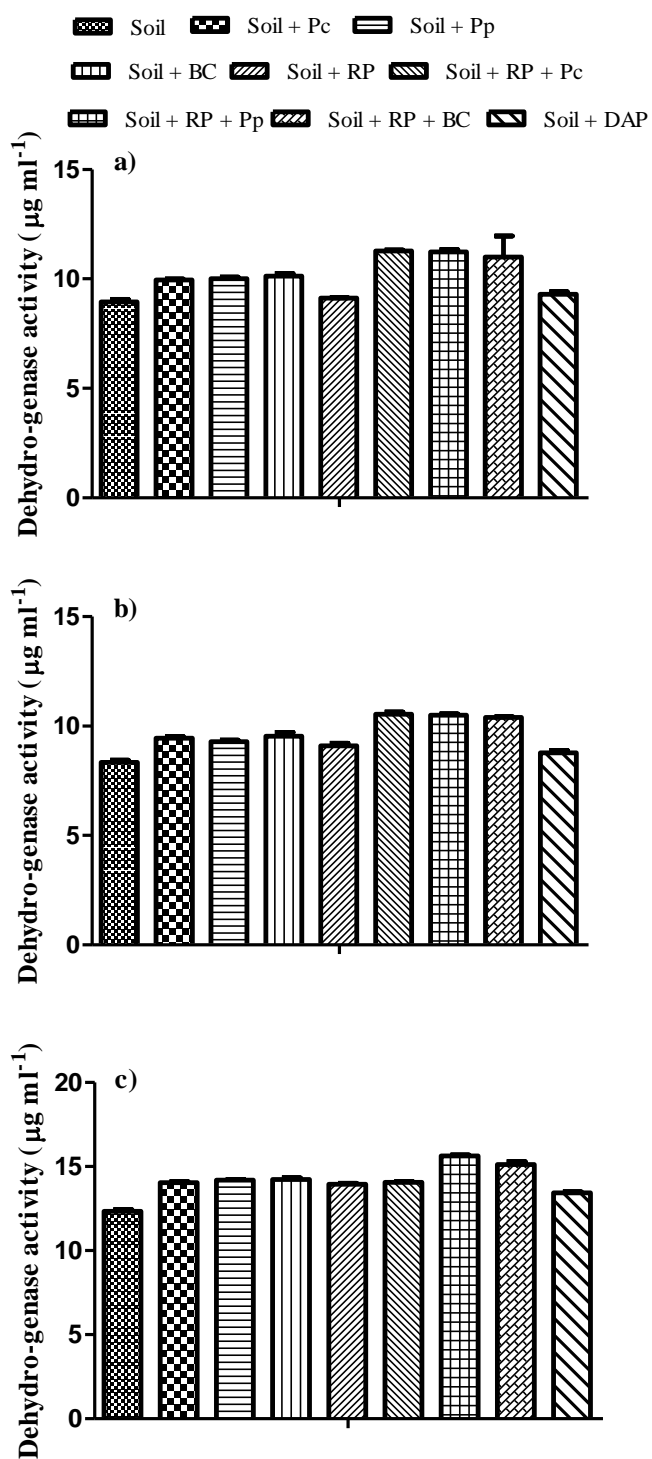


Fig. 7.14 Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) alone or along with RP fertilization and chemical fertilizer (DAP) on dehydro-genase enzyme activities of rhizosphere soil of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

7.2 Phosphate-solubilizing fungi as bio-inoculants at multilocational sites

7.2.1 Maize crop

Maize plant growth parameters such as plant shoot height, shoot and root dry weight, yield and P uptake at all three different sites were significantly improved with fungal (*Aspergillus tubingensis* or *Aspergillus niger*) inoculation alone or along with RP fertilization (Table 7.10 and 7.11; Fig 7.15). Maize grain yield was increased up to 25 % at all three sites in inoculated and RP fertilization treatments, it was up to 19 % with inoculation treatments, 16 % with RP fertilization treatments and 9.0-13 % with DAP treatment compared to control treatments. Enhancement in yield and P uptake in maize was more pronounced at sub mountain undulating region compared to central plain region and sub mountain undulating central region.

Physiochemical properties of maize rhizospheric soils such as pH, organic carbon, organic matter, total P and available P were significantly improved in all the treatments, but the effects were more pronounced when inoculation was done along with RP fertilization. Fungal inoculation slightly decreased the soil pH in all the treatments compared to control in all field trials (Table 7.12). A significant improvement in organic carbon and organic matter in all the fungal inoculation and RP fertilization treatments was observed compared to DAP and control soil (Fig 7.16 and 7.17). At central plain region, sub mountain undulating central region and at sub mountain undulating region, organic carbon content was improved 44 %, 42 % and 48 % with fungal inoculation along with RP fertilization and 3.2 %, 18 % and 20 % with DAP treatments respectively, compared to control (Fig 7.16). Increase in available P level at central plain region was 84 %, at sub mountain undulating central region 80 % and at sub mountain undulating region it was 87 % due to inoculation but the effect was more pronounced (154 %, 161 % and 146 %) when RP was supplemented along with inoculation

compared to control. Total P of soil was improved significantly with RP fertilization and DAP treatments but available P was significantly improved only in RP fertilization along with inoculation treatments compared to inoculation treatments alone, RP fertilization treatments alone and DAP treatment (Table 7.13). Acid phosphatase, alkaline phosphatase, phytase and dehydrogenase enzyme activities were significantly increased in all the treatments compared to control treatment at different sites (Figure 7.18, 7.19, 7.20 and 7.21). In all the field trials at different sites, phytase enzyme activities were higher than acid phosphatase and alkaline phosphatase.

Population density of P-solubilizing fungi was tested after harvesting of the maize crop, at central plain region, sub mountain undulating central region and sub mountain undulating region and it was 1.3×10^5 cfu g⁻¹, 0.7×10^5 cfu g⁻¹ and 2.0×10^6 control treatment, $5.3-6.7 \times 10^6$ cfu g⁻¹, $4.3-6 \times 10^6$ cfu g⁻¹ and $5.3-7.0 \times 10^6$ cfu g⁻¹ in fungal inoculation treatments alone, 3×10^5 cfu g⁻¹, 2.7×10^5 cfu g⁻¹ and 3.3×10^6 cfu g⁻¹ in RP fertilization treatments alone, $7.7-9.0 \times 10^6$ cfu g⁻¹, $7.0-8.0 \times 10^6$ cfu g⁻¹ and $3.0-4.7 \times 10^7$ cfu g⁻¹ in inoculation along with RP fertilization and 2×10^5 cfu g⁻¹, 1.3×10^5 cfu g⁻¹ and 2.3×10^6 cfu g⁻¹ in DAP treatment.

Table 7.10 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on the growth parameters of maize plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	Shoot length (cm)	Shoot dry weight (g)	Root dry weight (g)
Central plain region			
Soil	217 ± 5jk	34 ± 1.4ij	9.49 ± 0.3fgh
Soil + At	245 ± 5 defgh	56 ± 1.4cdefg	12.9 ± 0.3abcdef
Soil + An	247 ± 11defgh	57 ± 1.3cdef	13.5 ± 0.2abcd
Soil + FC	246 ± 3defgh	61 ± 7.8bcdef	13.9 ± 0.9abc
Soil + RP	233 ± 4ghij	51 ± 5.3efgh	9.95 ± 0.7h
Soil + RP + At	259 ± 7abcde	65 ± 5.8abcde	14.6 ± 0.2ab
Soil + RP + An	262 ± 5abcd	68 ± 6.0abc	14.2 ± 1.0abc
Soil + RP + FC	257 ± 4abcde	67 ± 8.6abcd	14.4 ± 0.6abc
DAP	233 ± 6ghij	39 ± 2.0hij	11.1 ± 0.1bcdefg
Sub mountain undulating central region			
Soil	214 ± 6k	32 ± 1.7j	8.72 ± 0.1gh
Soil + At	238 ± 8fghi	53 ± 1.7defgh	12.07 ± 1.6abcdefg
Soil + An	245 ± 5defgh	55 ± 5.0cdefg	12.3 ± 0.2abcdefg
Soil + FC	243 ± 8efghi	52 ± 8.1efgh	12.6 ± 0.3abcdefg
Soil + RP	232 ± 6ghijk	39 ± 2.1hij	9.80 ± 0.3defgh
Soil + RP + At	250 ± 3defg	62 ± 3.5bcdef	13.0 ± 0.2abcdef
Soil + RP + Pp	253 ± 6bcdef	59 ± 2.1cdef	13.3 ± 0.1abcdef
Soil + RP + FC	255 ± 4abcdef	60 ± 3.9bcdef	13.4 ± 0.2abcde
DAP	231 ± 5hijk	42 ± 9.4ghij	9.5 ± 0.3efgh
Sub mountain undulating region			
Soil	225 ± 3.3ijk	36 ± 1.9il	9.70 ± 1.0efgh
Soil + At	258 ± 4.7abcde	58 ± 1.3cdef	14.2 ± 0.9abc
Soil + An	250 ± 3defg	64 ± 6.0bcde	15.0 ± 0.9a
Soil + FC	252 ± 6.3cdef	67 ± 1.0abcd	14.8 ± 1.1a
Soil + RP	238 ± 3.0fghi	51 ± 5.1efgh	10.0 ± 0.5efgh
Soil + RP + At	271 ± 3.0ab	74 ± 0.7ab	15.3 ± 0.4a
Soil + RP + An	270 ± 1.8abc	75 ± 1.2ab	15.8 ± 0.9a
Soil + RP + FC	272 ± 4.7a	78 ± 2.6a	15.3 ± 1.2a
DAP	239 ± 6.3fghi	49 ± 1.5fghi	10.6 ± 0.8cdefg
LSD (P<0.05)	9.48	7.50	2.017

Values are Mean ± SD ($n = 10$). Means sharing a common letter within the column are not significantly different at $P < 0.05$. Pronounced results are represented in bold.

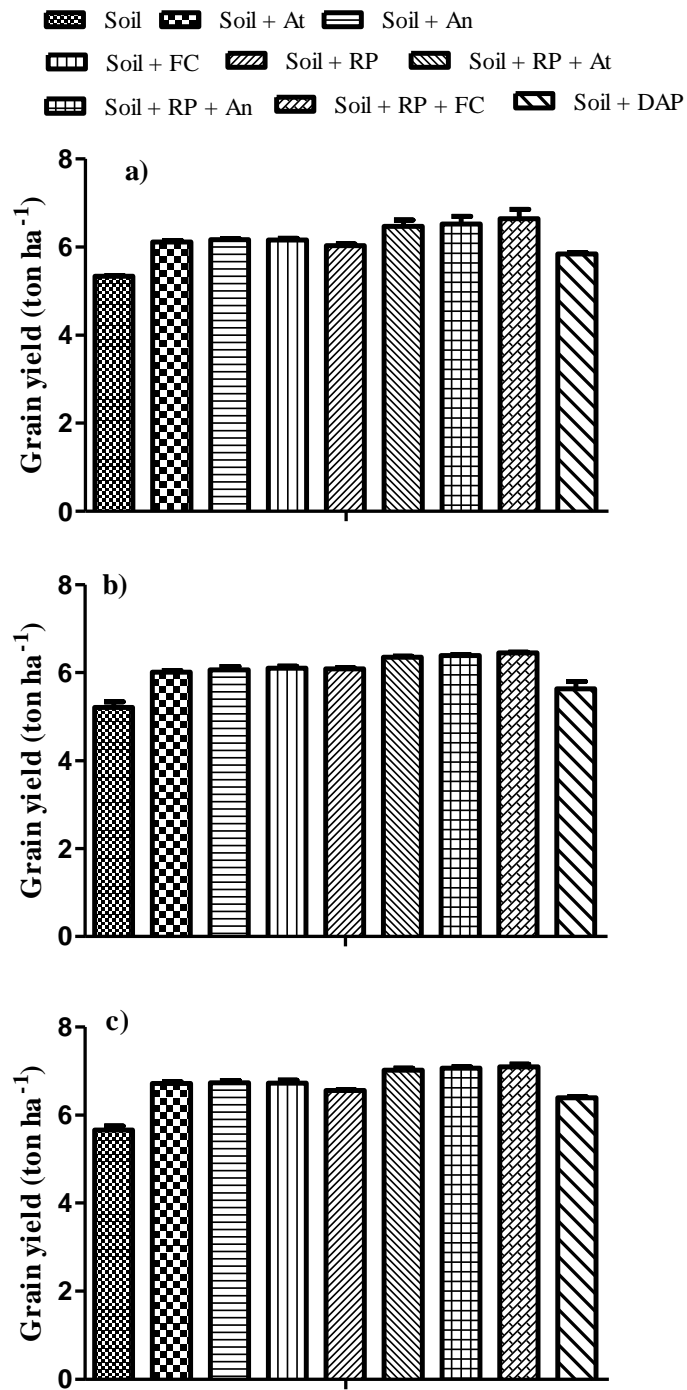


Fig. 7.15 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on the grain yield of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

Table 7.11 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on P uptake of maize plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	Grains (mg kg ⁻¹)	Shoot (mg kg ⁻¹)	Root (mg kg ⁻¹)
Central plain region			
Soil	136 ± 9ij	129 ± 4hi	123 ± 5l
Soil + At	179 ± 8cdefghij	166 ± 7bcdefgh	172 ± 6efgh
Soil + An	172 ± 10defghij	163 ± 13bcdefgh	169 ± 10efghi
Soil + FC	145 ± 17ghij	169 ± 6bcdefg	173 ± 5efgh
Soil + RP	169 ± 6efghij	152 ± 13defghi	133 ± 1ijkl
Soil + RP + At	223 ± 10abc	184 ± 6abcde	220 ± 8abcd
Soil + RP + An	222 ± 9abcd	188 ± 10abcd	219 ± 9abcd
Soil + RP + FC	228 ± 13abc	184 ± 10abcde	225 ± 8abc
DAP	161 ± 9ghij	145 ± 8fghi	159 ± 6fghijk
Sub mountain undulating central region			
Soil	129 ± 13j	125 ± 11i	120 ± 4l
Soil + At	168 ± 9fghij	155 ± 10defghi	165 ± 13efghij
Soil + An	170 ± 4defghij	159 ± 5cdefghi	162 ± 8efghij
Soil + FC	141 ± 8hij	169 ± 15bcdefg	169 ± 10efghi
Soil + RP	163 ± 6ghij	145 ± 15fghi	150 ± 4ghijkl
Soil + RP + At	218 ± 6abcdef	170 ± 8bcdefg	218 ± 6abcd
Soil + RP + An	218 ± 9abcdef	176 ± 13abcdef	211 ± 10bcd
Soil + RP + FC	220 ± 4abcde	175 ± 15abcdef	212 ± 13abcd
DAP	156 ± 6abc	137 ± 8ghi	141 ± 11hijkl
Sub mountain undulating region			
Soil	143 ± 10hij	136 ± 9ghi	127 ± 10kl
Soil + At	187 ± 11bcdefghi	172 ± 4bcdefg	176 ± 9efg
Soil + An	197 ± 17abcdefg	177 ± 10abcdef	194 ± 24cde
Soil + FC	191 ± 4bcdefgh	176 ± 19abcdef	191 ± 4def
Soil + RP	179 ± 13cdefghij	170 ± 11bcdefg	148 ± 6ghijkl
Soil + RP + At	230 ± 15abc	212 ± 17a	229 ± 18ab
Soil + RP + An	238 ± 15ab	197 ± 15abc	236 ± 10ab
Soil + RP + FC	248 ± 17a	201 ± 17ab	244 ± 15a
DAP	155 ± 13ghij	150 ± 11efghi	139 ± 6ijkl
LSD (P<0.05)	27	19	17

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

Table 7.12 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of maize plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	pH	EC (mScm ⁻¹)	TDS (ppm)
Central plain region			
Soil	8.24 ± 0.03b	0.16 ± 0.02gh	0.11 ± 0.010fg
Soil + At	7.93 ± 0.02ef	0.21 ± 0.02cdefg	0.14 ± 0.014cdefg
Soil + An	7.91 ± 0.02f	0.23 ± 0.02cdefg	0.15 ± 0.012cdef
Soil + FC	7.82 ± 0.03g	0.22 ± 0.02cdefg	0.15 ± 0.014cdef
Soil + RP	7.80 ± 0.02gh	0.21 ± 0.02cdefg	0.14 ± 0.013cdef
Soil + RP + At	7.71 ± 0.01jklm	0.26 ± 0.04abcde	0.17 ± 0.027bcde
Soil + RP + An	7.65 ± 0.01mn	0.28 ± 0.02abc	0.19 ± 0.010bc
Soil + RP + FC	7.61 ± 0.02n	0.32 ± 0.02ab	0.21 ± 0.013ab
DAP	8.15 ± 0.06c	0.23 ± 0.03cdefg	0.15 ± 0.018cdef
Sub mountain undulating central region			
Soil	8.19 ± 0.02bc	0.13 ± 0.015h	0.08 ± 0.01g
Soil + At	7.82 ± 0.02g	0.19 ± 0.015defgh	0.13 ± 0.01defg
Soil + An	7.80 ± 0.01ghi	0.19 ± 0.015efgh	0.13 ± 0.01defg
Soil + FC	7.81 ± 0.01gh	0.19 ± 0.03defgh	0.3 ± 0.02defg
Soil + RP	7.82 ± 0.02g	0.19 ± 0.025efgh	0.13 ± 0.02defg
Soil + RP + At	7.69 ± 0.02klm	0.23 ± 0.015cdefg	0.15 ± 0.01cdef
Soil + RP + An	7.64 ± 0.02mn	0.23 ± 0.020cdefg	0.16 ± 0.01cdef
Soil + RP + FC	7.69 ± 0.02klm	0.23 ± 0.02cdefg	0.15 ± 0.01cdef
DAP	7.98 ± 0.03de	0.20 ± 0.02defgh	0.13 ± 0.01defg
Sub mountain undulating region			
Soil	8.33 ± 0.03a	0.18 ± 0.02fgh	0.12 ± 0.01efg
Soil + At	7.77 ± 0.01ghij	0.25 ± 0.02bcdef	0.17 ± 0.02bcde
Soil + An	7.74 ± 0.01hijk	0.23 ± 0.03cdefg	0.16 ± 0.02cdef
Soil + FC	7.81 ± 0.01gh	0.23 ± 0.05cdefg	0.15 ± 0.03cdef
Soil + RP	7.94 ± 0.02def	0.19 ± 0.02defgh	0.13 ± 0.01defg
Soil + RP + At	7.67 ± 0.03lmn	0.32 ± 0.04ab	0.22 ± 0.02ab
Soil + RP + An	7.70 ± 0.02klm	0.27 ± 0.04abcd	0.18 ± 0.02bcd
Soil + RP + FC	7.73 ± 0.04ijkl	0.33 ± 0.4a	0.22 ± 0.02a
DAP	8.01 ± 0.04d	0.25 ± 0.02bcdef	0.17 ± 0.01bcde
LSD (P<0.05)	0.037	0.039	0.028

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

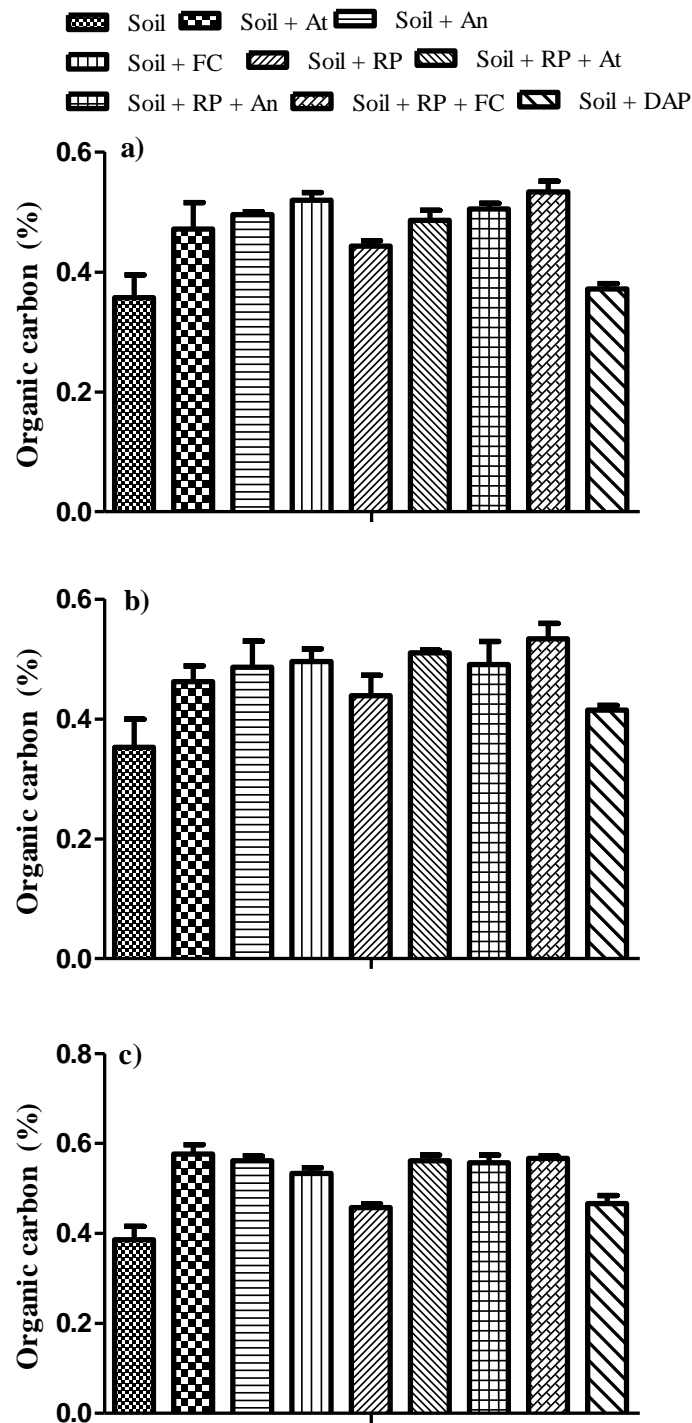


Fig. 7.16 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on organic carbon of rhizosphere soil of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

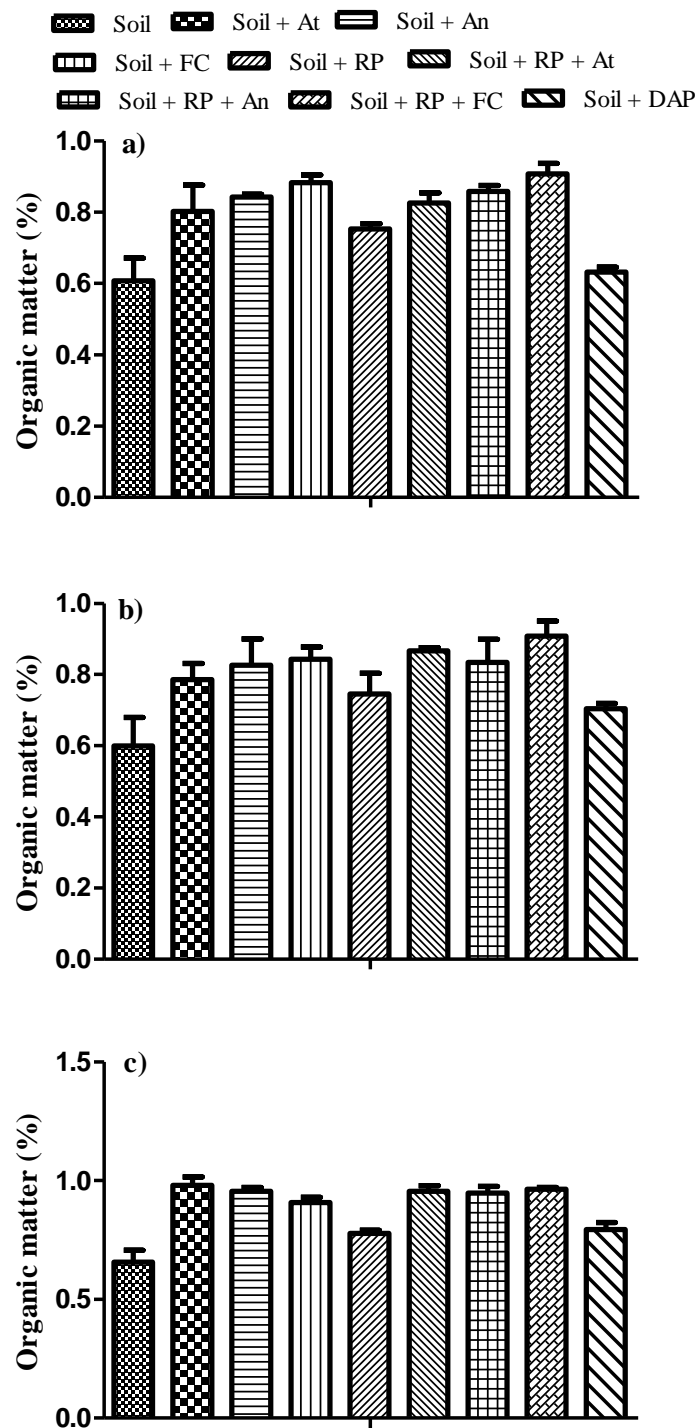


Fig. 7.17 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on organic matter of rhizosphere soil of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

Table 7.13 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of maize plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	Total P (mg kg ⁻¹)	Available P (mg kg ⁻¹)	Total nitrogen (%)
Central plain region			
Soil	251 ± 15e	3.37 ± 0.19ij	0.031 ± 0.005a
Soil + At	254 ± 17e	5.83 ± 0.15de	0.034 ± 0.008a
Soil + An	265 ± 19e	6.19 ± 0.21d	0.036 ± 0.010a
Soil + FC	256 ± 2e	5.51 ± 0.28efg	0.036 ± 0.013a
Soil + RP	483 ± 8abcd	5.11 ± 0.13fg	0.035 ± 0.009a
Soil + RP + At	480 ± 6abcd	8.45 ± 0.29b	0.036 ± 0.013a
Soil + RP + An	504 ± 4ab	8.51 ± 0.42b	0.034 ± 0.008a
Soil + RP + FC	468 ± 6abcd	8.46 ± 0.39b	0.034 ± 0.015a
DAP	494 ± 6abc	4.11 ± 0.07h	0.036 ± 0.005a
Sub mountain undulating central region			
Soil	227 ± 15e	3.22 ± 0.17j	0.028 ± 0.005a
Soil + At	247 ± 9e	5.61 ± 0.13def	0.034 ± 0.008a
Soil + An	230 ± 19e	5.81 ± 0.09de	0.034 ± 0.015a
Soil + FC	245 ± 8e	5.79 ± 0.03de	0.042 ± 0.008a
Soil + RP	447 ± 21d	4.89 ± 0.09g	0.036 ± 0.005a
Soil + RP + At	462 ± 22cd	8.34 ± 0.29b	0.036 ± 0.005a
Soil + RP + An	484 ± 30abcd	8.42 ± 0.40b	0.034 ± 0.008a
Soil + RP + FC	476 ± 13abcd	8.27 ± 0.20b	0.031 ± 0.010a
DAP	504 ± 13abc	3.87 ± 0.22hi	0.031 ± 0.005a
Sub mountain undulating region			
Soil	237 ± 8e	4.02 ± 0.17h	0.035 ± 0.002a
Soil + At	248 ± 2e	7.51 ± 0.14c	0.045 ± 0.018a
Soil + An	250 ± 3e	7.23 ± 0.09c	0.042 ± 0.008a
Soil + FC	250 ± 4e	7.37 ± 0.07c	0.039 ± 0.005a
Soil + RP	465 ± 9bcd	5.29 ± 0.07efg	0.042 ± 0.017a
Soil + RP + At	459 ± 9cd	9.96 ± 0.12a	0.053 ± 0.010a
Soil + RP + An	508 ± 8a	9.86 ± 0.05a	0.050 ± 0.008a
Soil + RP + FC	469 ± 9abcd	9.91 ± 0.15a	0.048 ± 0.013a
DAP	487 ± 8abcd	4.94 ± 0.10g	0.039 ± 0.005a
LSD (P<0.05)	21	0.33	0.02

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

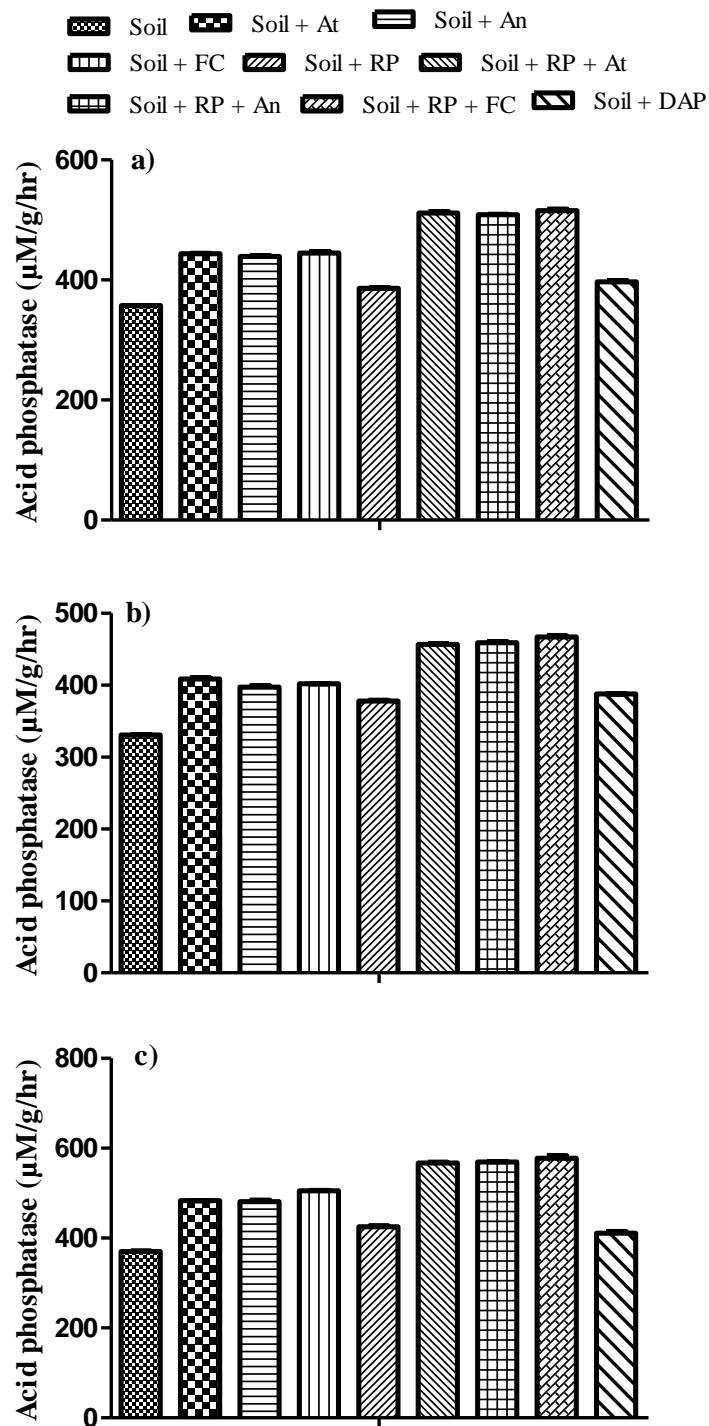


Fig. 7.18 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on acid phosphatase enzyme activities of rhizosphere soil of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

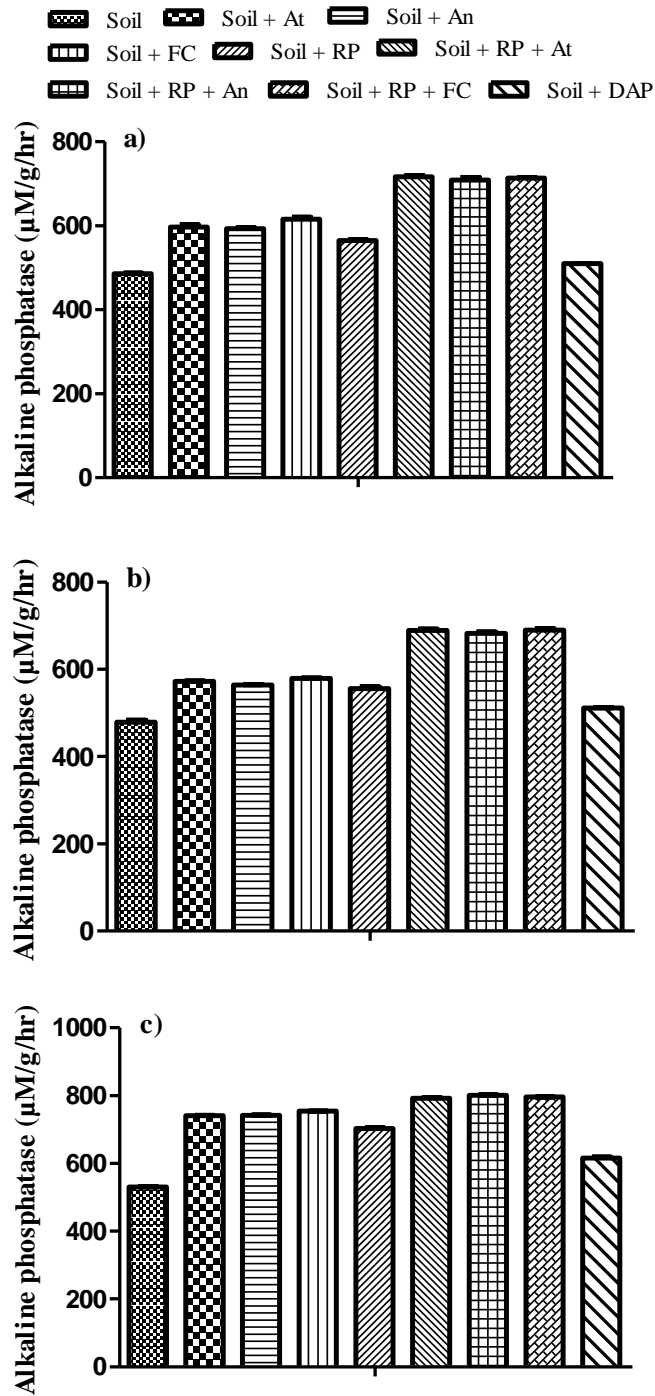


Fig. 7.19 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on alkaline phosphatase enzyme activities of rhizosphere soil of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

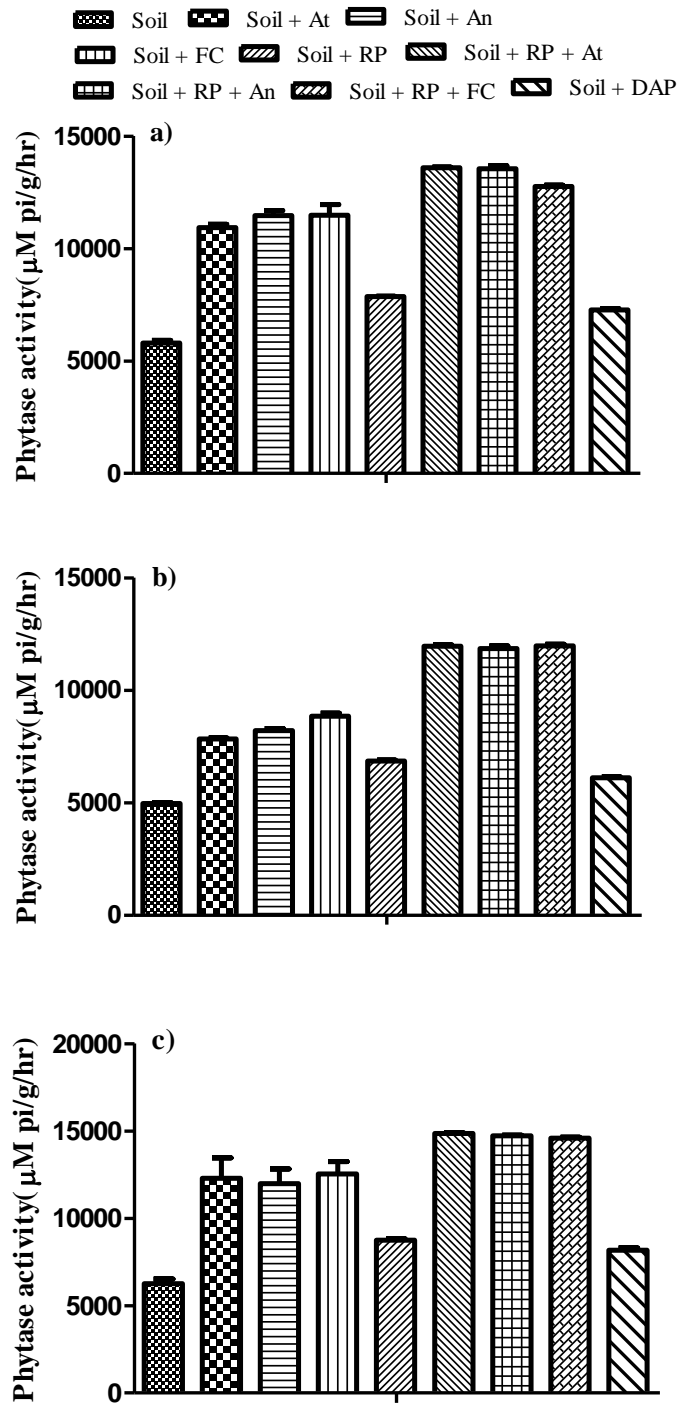


Fig. 7.20 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on phytase enzyme activities of rhizosphere soil of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

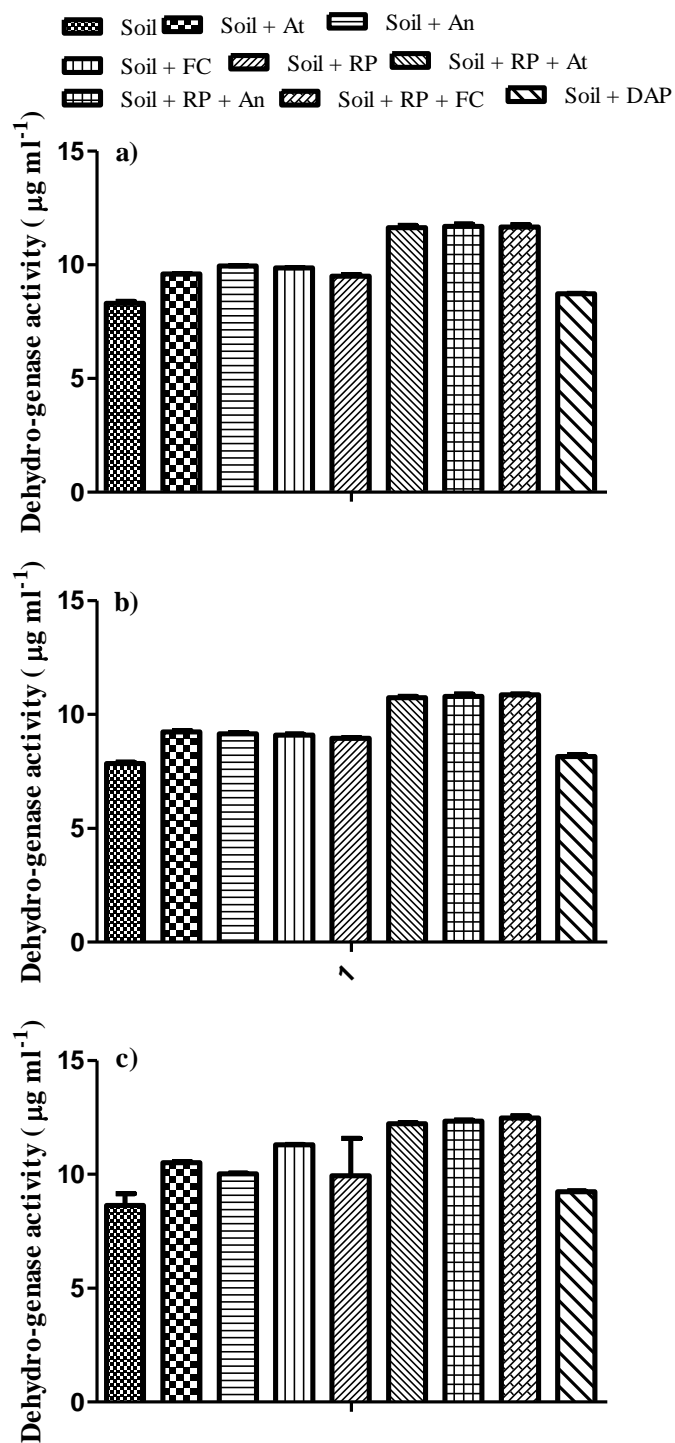


Fig. 7.21 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on dehydro-genase enzyme activities of rhizosphere soil of maize plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

7.2.2 Wheat crop

Inoculation with *A. tubingensis* and *A. niger* alone or along with RP fertilization showed positive effects on plant growth promotion such as plant shoot height, shoot and root dry weight, yield and P uptake (Table 7.14 and 7.15; Fig. 7.22). Increase in yield up to 38 % was observed at all three sites in inoculated and RP fertilized soil treatments, it was up to 28 % with inoculation, 13 % with RP fertilization and 6 to 10 % with DAP treatment compared to control treatments. Stimulatory effects of inoculation treatments along with RP fertilization on growth parameters, yield and P uptake was found to be more pronounced compared to inoculation, RP fertilization and DAP treatments.

Soil pH slightly decreases in all the treatments compared to un-inoculated control in all field trials (Table 7.16). Results showed that there was a significant improvement in organic carbon and organic matter of the soil in all the inoculation and RP fertilization treatments compared to DAP and control treatments (Fig 7.23 and 7.24). During second year of field study on wheat crop, inoculation along with RP fertilization increased the soil organic carbon content 48 %, 48 % and 54 % and DAP treatments increased the soil organic carbon content 3 %, 11 % and 13 % at central plain region, sub mountain undulating central region and at sub mountain undulating region compared to control treatments, respectively. Available P level was significantly increased at central plain region (86 %), sub mountain undulating central region (77 %) and at sub mountain undulating region (54 %) due to inoculation but the effect was more pronounced (151 %, 149 % and 127 %) when RP was supplemented along with inoculation compared to control (Table 7.17). Acid phosphatase, alkaline phosphatase, phytase and dehydrogenase enzyme activities were significantly increased in all the treatments compared to control, but the results were more pronounced with inoculation treatments along with RP fertilization (Fig. 7.25, 7.26, 7.27 and 7.28). In all the field trials at different sites phytase enzyme activities were higher than acid phosphatase and alkaline

phosphatase enzyme activities. It was also observed that alkaline phosphatase activities were slightly decreased, as compared to the alkaline phosphatase activities in maize crop field at all different sites.

After harvesting of the wheat crop, rhizospheric soil was examined for phosphate-solubilizing fungal population density, and it was found that, at central plain region, sub mountain undulating central region and sub mountain undulating region PSFs population density was 1.5×10^5 cfu g⁻¹, 1.3×10^5 cfu g⁻¹ and 2.3×10^6 in control treatment, $6.5-8.0 \times 10^5$ cfu g⁻¹, $6.6-6.7 \times 10^5$ cfu g⁻¹ and $6.0-7.3 \times 10^6$ cfu g⁻¹ in inoculation treatments, 4×10^5 cfu g⁻¹, 3.0×10^5 cfu g⁻¹ and 4.3×10^6 cfu g⁻¹ in RP fertilization treatments, $8.5-9.0 \times 10^6$ cfu g⁻¹, $7.3-9.0 \times 10^6$ cfu g⁻¹ and $5-7.7 \times 10^7$ cfu g⁻¹ in inoculation along with RP fertilization and 2.5×10^5 cfu g⁻¹, 2×10^5 cfu g⁻¹ and 2.7×10^5 cfu g⁻¹ in DAP treatment. Field study of wheat crop at different sites showed that, there was a significant improvement in crop yield, total P uptake and soil fertility in RP fertilization treatments along with inoculation compared to inoculation alone and DAP treatment.

Table 7.14 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on the growth parameters of wheat plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	Shoot length (cm)	Shoot dry weight (g)	Root dry weight (g)
Central plain region			
Soil	96 ± 1.5no	1.36 ± 0.03ij	0.54 ± 0.03gh
Soil + At	110 ± 1.5fghijk	1.89 ± 0.03defgh	0.74 ± 0.10efgh
Soil + An	111 ± 3.8efghij	1.93 ± 0.25cdefg	0.79 ± 0.07efg
Soil + FC	113 ± 1.7efghij	2.01 ± 0.18bcdef	0.77 ± 0.11efgh
Soil + RP	108 ± 2.0hijklm	1.60 ± 0.02fghij	0.75 ± 0.08efgh
Soil + RP + At	116 ± 4.3defghi	2.37 ± 0.06abc	1.16 ± 0.10abcd
Soil + RP + An	119 ± 2.4bcdef	2.46 ± 0.11ab	1.20 ± 0.10abcd
Soil + RP + FC	126 ± 4.7abcd	2.48 ± 0.11ab	1.24 ± 0.11abc
DAP	99 ± 0.8klmno	1.58 ± 0.02fghij	0.62 ± 0.03gh
Sub mountain undulating central region			
Soil	92 ± 3.4o	1.24 ± 0.08j	0.49 ± 0.08h
Soil + At	107 ± 1.4ijklm	1.67 ± 0.04fghij	0.61 ± 0.05gh
Soil + An	109 ± 1.3ghijkl	1.69 ± 0.03efghij	0.65 ± 0.14fgh
Soil + FC	105 ± 1.4jklmn	0.72 ± 0.03efghi	0.70 ± 0.10efg
Soil + RP	106 ± 2.7ijklmn	1.52 ± 0.03ghij	0.65 ± 0.04fgh
Soil + RP + At	114 ± 4.6efghij	2.17 ± 0.26abcde	0.96 ± 0.02cde
Soil + RP + Pp	117 ± 3.4cdefgh	2.21 ± 0.10abcd	0.93 ± 0.03def
Soil + RP + FC	120 ± 2.2bcde	2.15 ± 0.03abcde	0.97 ± 0.02cde
DAP	98 ± 2.3mno	1.38 ± 0.13ij	0.54 ± 0.03gh
Sub mountain undulating region			
Soil	99 ± 8lmno	1.44 ± 0.10hij	0.66 ± 0.07fgh
Soil + At	115 ± 1efghij	1.99 ± 0.12cdef	0.94 ± 0.05def
Soil + An	119 ± 2cdefg	1.92 ± 0.06cdefg	1.01 ± 0.05bcde
Soil + FC	116 ± 2cdefgh	2.15 ± 0.04abcde	0.92 ± 0.05def
Soil + RP	109 ± 4ghijkl	1.70 ± 0.05efghij	0.79 ± 0.16efg
Soil + RP + At	131 ± 3a	2.58 ± 0.20a	1.33 ± 0.14a
Soil + RP + An	126 ± 4abc	2.54 ± 0.06a	1.28 ± 0.18ab
Soil + RP + FC	129 ± 2ab	2.62 ± 0.25a	1.27 ± 0.07ab
DAP	100 ± 1klmno	1.59 ± 0.46fghij	0.75 ± 0.04efgh
LSD (P<0.05)	5.26	0.24	0.15

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

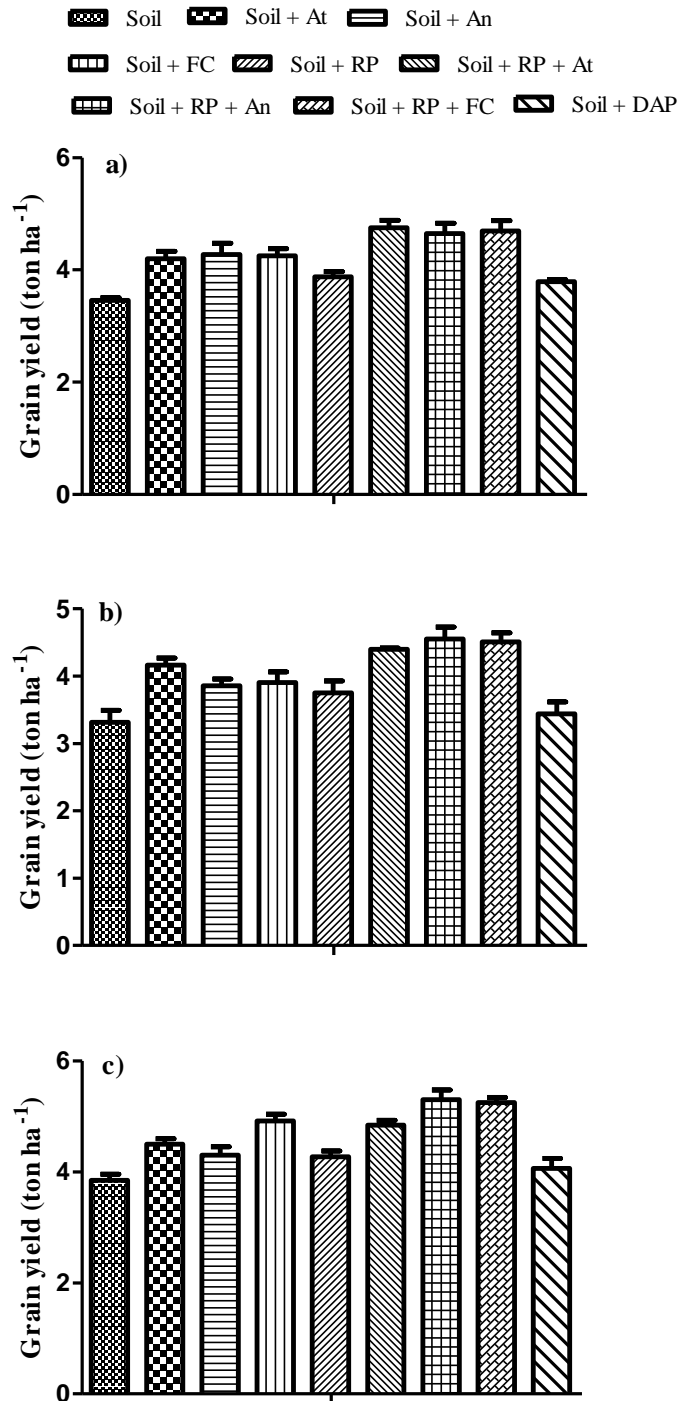


Fig. 7.22 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on the grain yield of wheat plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Table 7.15 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on P uptake of wheat plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	Grains (mg kg ⁻¹)	Shoot (mg kg ⁻¹)	Root (mg kg ⁻¹)
Central plain region			
Soil	218 ± 2l	29 ± 4l	191 ± 13k
Soil + At	295 ± 8fghi	66 ± 8defghi	293 ± 6fghi
Soil + An	286 ± 13ghi	72 ± 13defg	283 ± 8ghi
Soil + FC	272 ± 20ijk	68 ± 6defghi	301 ± 6efg
Soil + RP	298 ± 10efghi	41 ± 8hijkl	280 ± 9ghi
Soil + RP + At	344 ± 6cd	86 ± 17cde	369 ± 10bcd
Soil + RP + An	350 ± 8bcd	91 ± 15bcd	375 ± 14bc
Soil + RP + FC	345 ± 11cd	90 ± 15cd	397 ± 16b
DAP	237 ± 4jkl	31 ± 6kl	250 ± 11hij
Sub mountain undulating central region			
Soil	213 ± 5l	26 ± 10l	184 ± 13k
Soil + At	275 ± 4ij	48 ± 9ghijkl	290 ± 9fghi
Soil + An	286 ± 10ghi	54 ± 4fghijkl	279 ± 4gh
Soil + FC	270 ± 13ijk	61 ± 6cdefghijk	288 ± 6f
Soil + RP	277 ± 5hi	37 ± 8jkl	275 ± 8ghi
Soil + RP + At	343 ± 13cd	66 ± 4defghij	375 ± 8cde
Soil + RP + An	336 ± 13cde	69 ± 2defgh	366 ± 15bcd
Soil + RP + FC	340 ± 6cd	72 ± 2defg	372 ± 6bc
DAP	222 ± 16l	29 ± 11l	216 ± 17jk
Sub mountain undulating region			
Soil	227 ± 10l	38 ± 10ijkl	222 ± 6jk
Soil + At	326 ± 17def	63 ± 6defghij	336 ± 17cdef
Soil + An	313 ± 17defgh	81 ± 9def	322 ± 10defg
Soil + FC	320 ± 15defg	83 ± 11cdef	352 ± 6bcd
Soil + RP	336 ± 23cde	69 ± 6defgh	295 ± 8efgh
Soil + RP + At	373 ± 10abc	112 ± 4abc	458 ± 8a
Soil + RP + An	384 ± 6ab	120 ± 4ab	463 ± 6a
Soil + RP + FC	400 ± 4a	129 ± 15a	459 ± 6a
DAP	236 ± 20kl	47 ± 6ghijkl	245 ± 26ij
LSD (P<0.05)	19	15	24

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

Table 7.16 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of wheat plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	pH	EC (mScm ⁻¹)	TDS (ppm)
Central plain region			
Soil	8.23 ± 0.02ab	0.17 ± 0.01efgh	0.11 ± 0.007efgh
Soil + At	7.60 ± 0.02defghi	0.22 ± 0.02abcde	0.15 ± 0.010abcde
Soil + An	7.56 ± 0.02fghi	0.23 ± 0.02abc	0.15 ± 0.010abc
Soil + FC	7.58 ± 0.04fghi	0.23 ± 0.02abc	0.15 ± 0.013abc
Soil + RP	8.14 ± 0.05b	0.21 ± 0.02abcdef	0.14 ± 0.013abcdef
Soil + RP + At	7.49 ± 0.07ghi	0.25 ± 0.02ab	0.17 ± 0.010ab
Soil + RP + An	7.53 ± 0.12ghi	0.25 ± 0.03a	0.17 ± 0.018a
Soil + RP + FC	7.53 ± 0.11ghi	0.24 ± 0.02abc	0.16 ± 0.010abc
DAP	8.17 ± 0.02ab	0.20 ± 0.02abcdef	0.14 ± 0.010abcdef
Sub mountain undulating central region			
Soil	8.19 ± 0.02ab	0.12 ± 0.01h	0.08 ± 0.007h
Soil + At	7.71 ± 0.02def	0.16 ± 0.02fgh	0.11 ± 0.010fgh
Soil + An	7.74 ± 0.02d	0.17 ± 0.02defg	0.12 ± 0.014defg
Soil + FC	7.74 ± 0.02de	0.20 ± 0.02bcdef	0.13 ± 0.010bcdef
Soil + RP	7.97 ± 0.02c	0.16 ± 0.02fgh	0.11 ± 0.010fgh
Soil + RP + At	7.60 ± 0.07defghi	0.22 ± 0.02abcd	0.15 ± 0.010abcd
Soil + RP + An	7.63 ± 0.02defg	0.20 ± 0.01abcdef	0.13 ± 0.007abcdef
Soil + RP + FC	7.62 ± 0.04defgh	0.23 ± 0.02abc	0.17 ± 0.014abc
DAP	7.98 ± 0.01c	0.19 ± 0.02cdef	0.13 ± 0.010adefg
Sub mountain undulating region			
Soil	8.30 ± 0.02a	0.14 ± 0.01gh	0.09 ± 0.01gh
Soil + At	7.59 ± 0.04efghi	0.17 ± 0.02defg	0.12 ± 0.01defg
Soil + An	7.58 ± 0.02fghi	0.22 ± 0.02abcde	0.15 ± 0.01abcde
Soil + FC	7.56 ± 0.03fghi	0.17 ± 0.02defg	0.12 ± 0.01defg
Soil + RP	8.13 ± 0.02b	0.19 ± 0.02cdefg	0.13 ± 0.01cdefg
Soil + RP + At	7.50 ± 0.05ghi	0.21 ± 0.02abcdef	0.14 ± 0.01abcdef
Soil + RP + An	7.46 ± 0.05i	0.24 ± 0.01abc	0.16 ± 0.01abc
Soil + RP + FC	7.47 ± 0.08hi	0.25 ± 0.02a	0.17 ± 0.01a
DAP	8.19 ± 0.03ab	0.19 ± 0.02cdef	0.13 ± 0.01cdef
LSD (P<0.05)	0.075	0.026	0.0178

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

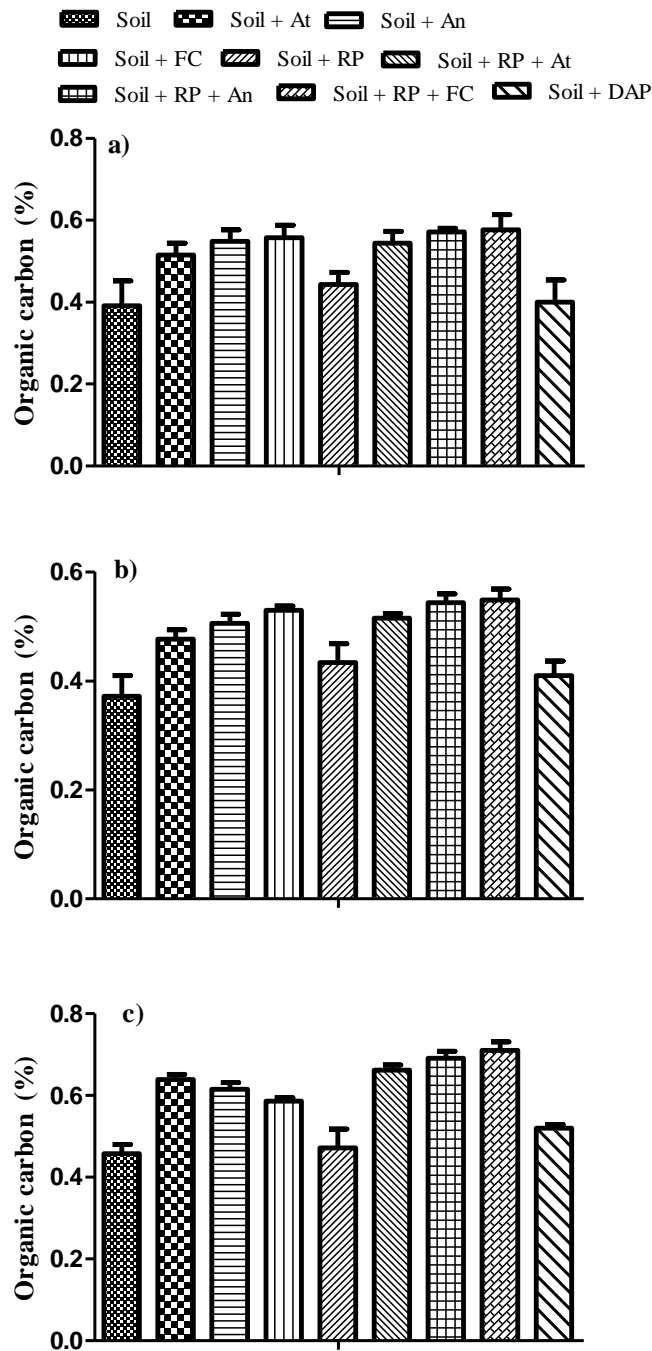


Fig. 7.23 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on organic carbon of rhizosphere soil characteristics of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

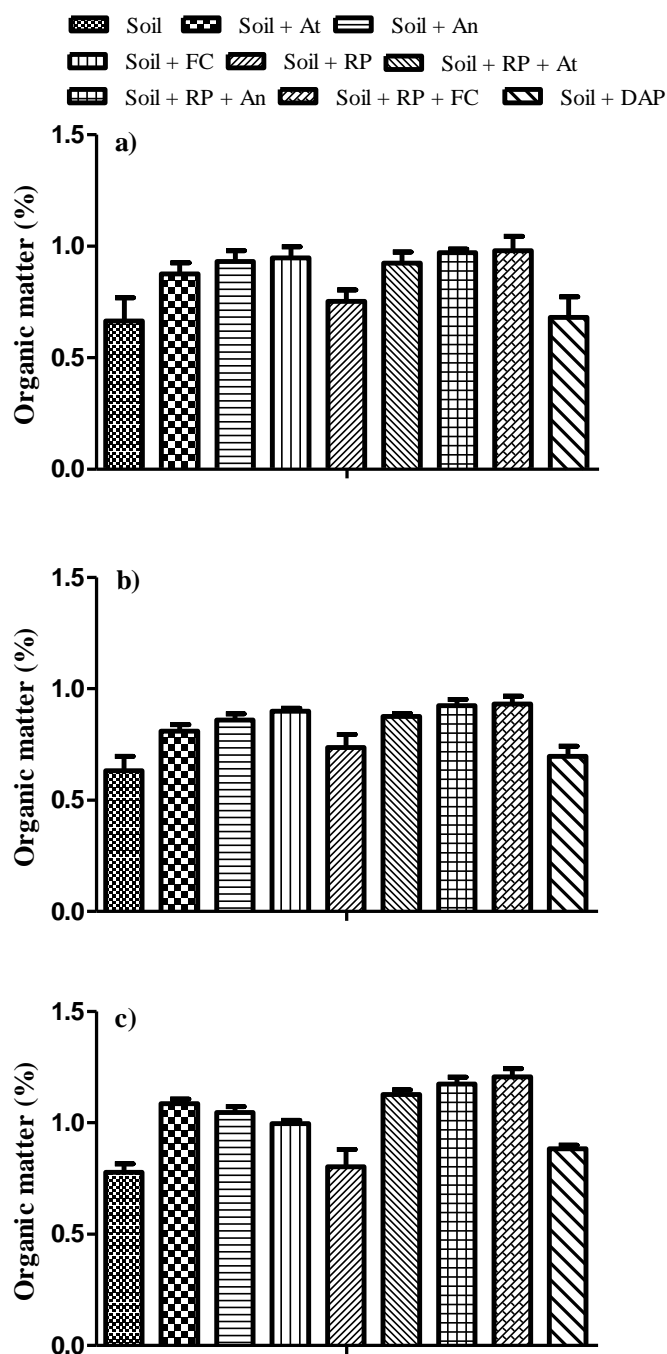


Fig. 7.24 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on organic matter of rhizosphere soil characteristics of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

Table 7.17 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on rhizosphere soil characteristics of wheat plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab)

Treatments	Total P (mg kg ⁻¹)	Available P (mg kg ⁻¹)	Total nitrogen (%)
Central plain region			
Soil	225 ± 13cdefg	3.86 ± 0.09n	0.045 ± 0.005a
Soil + At	245 ± 8cd	6.84 ± 0.07fgh	0.050 ± 0.008a
Soil + An	237 ± 4cde	6.95 ± 0.16fg	0.048 ± 0.013a
Soil + FC	227 ± 6cdef	7.17 ± 0.09ef	0.050 ± 0.015a
Soil + RP	468 ± 15a	5.05 ± 0.34jk	0.048 ± 0.005a
Soil + RP + At	452 ± 20ab	9.49 ± 0.27bc	0.053 ± 0.010a
Soil + RP + An	445 ± 8ab	9.59 ± 0.09bc	0.042 ± 0.008a
Soil + RP + FC	459 ± 9a	9.67 ± 0.15b	0.056 ± 0.005a
DAP	461 ± 15a	4.39 ± 0.09lm	0.053 ± 0.010a
Sub mountain undulating central region			
Soil	213 ± 6defg	3.70 ± 0.11n	0.028 ± 0.010a
Soil + At	205 ± 17efg	6.46 ± 0.07h	0.036 ± 0.005a
Soil + An	191 ± 8g	6.56 ± 0.09gh	0.039 ± 0.013a
Soil + FC	195 ± 8fg	6.55 ± 0.11gh	0.039 ± 0.010a
Soil + RP	434 ± 15ab	4.87 ± 0.24kl	0.031 ± 0.005a
Soil + RP + At	441 ± 8ab	8.73 ± 0.09d	0.039 ± 0.005a
Soil + RP + An	425 ± 8b	9.21 ± 0.11bcd	0.042 ± 0.008a
Soil + RP + FC	448 ± 10ab	9.17 ± 0.08cd	0.048 ± 0.005a
DAP	440 ± 6ab	4.02 ± 0.13mn	0.045 ± 0.017a
Sub mountain undulating region			
Soil	204 ± 7efg	4.94 ± 0.18k	0.045 ± 0.005a
Soil + At	223 ± 6cdefg	7.23 ± 0.18ef	0.063 ± 0.013a
Soil + An	247 ± 9c	7.59 ± 0.15e	0.056 ± 0.012a
Soil + FC	250 ± 8c	7.11 ± 0.18ef	0.066 ± 0.011a
Soil + RP	455 ± 6ab	5.90 ± 0.18i	0.045 ± 0.009a
Soil + RP + At	437 ± 8ab	10.95 ± 0.13a	0.060 ± 0.012a
Soil + RP + An	434 ± 5ab	10.90 ± 0.09a	0.059 ± 0.019a
Soil + RP + FC	443 ± 6ab	11.22 ± 0.18a	0.064 ± 0.050a
DAP	437 ± 19ab	5.51 ± 0.11ij	0.046 ± 0.003a
LSD (P<0.05)	17	0.25	0.023

Values are Mean ± SD (n =10). Means sharing a common letter within the column are not significantly different at P<0.05. Pronounced results are represented in bold.

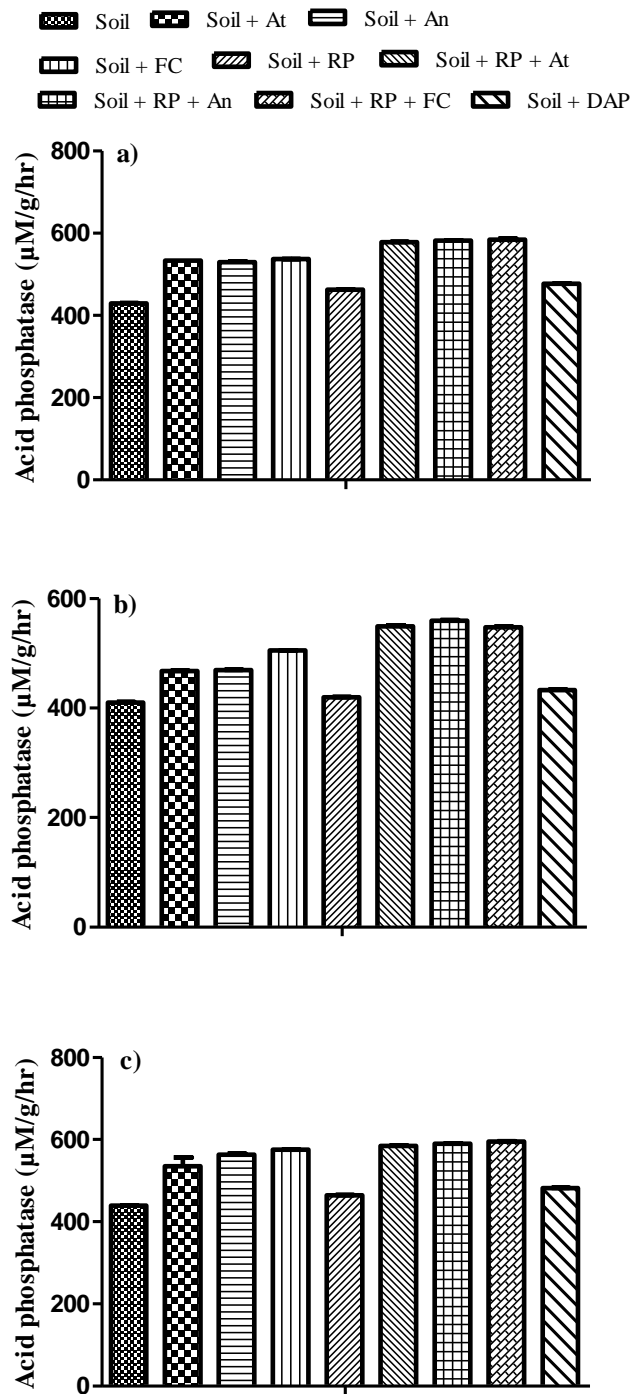


Fig. 7.25 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on acid phosphatase enzyme activities of rhizosphere soil of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

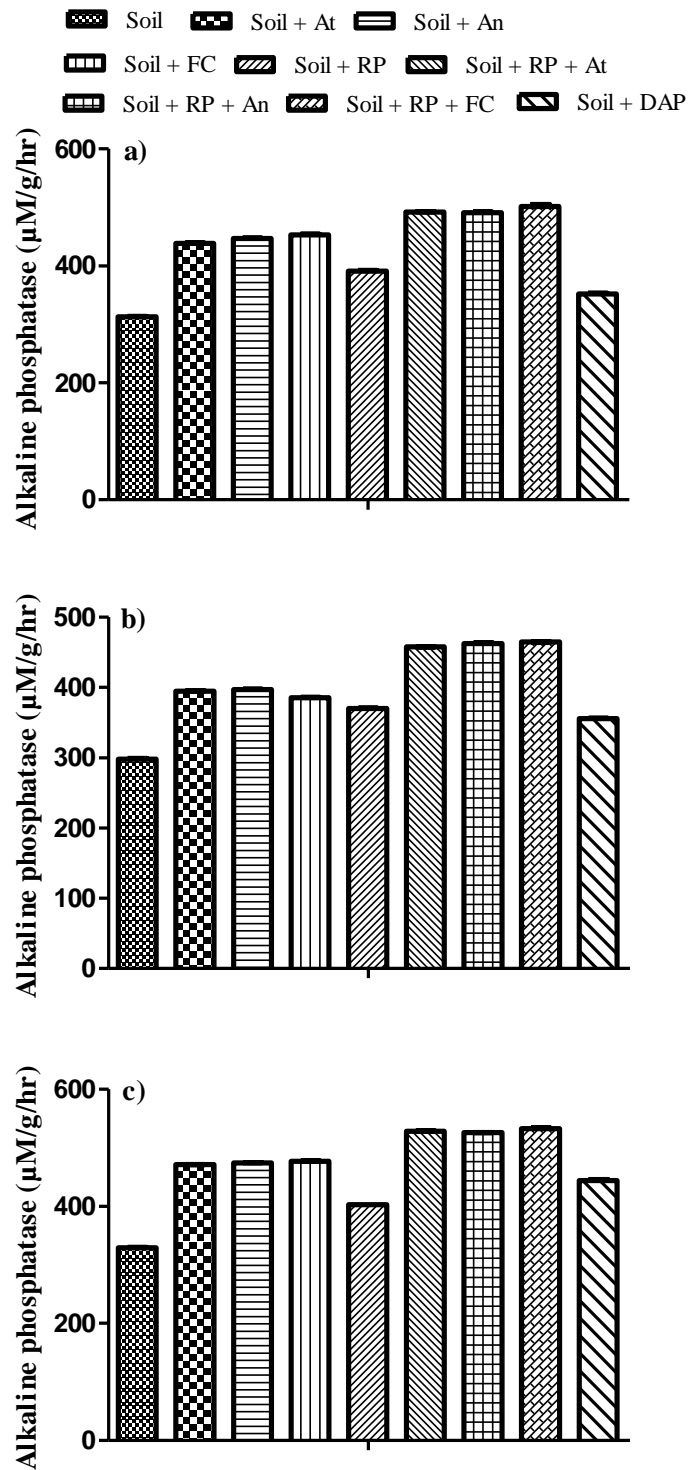


Fig. 7.26 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on alkaline phosphatase enzyme activities of rhizosphere soil of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

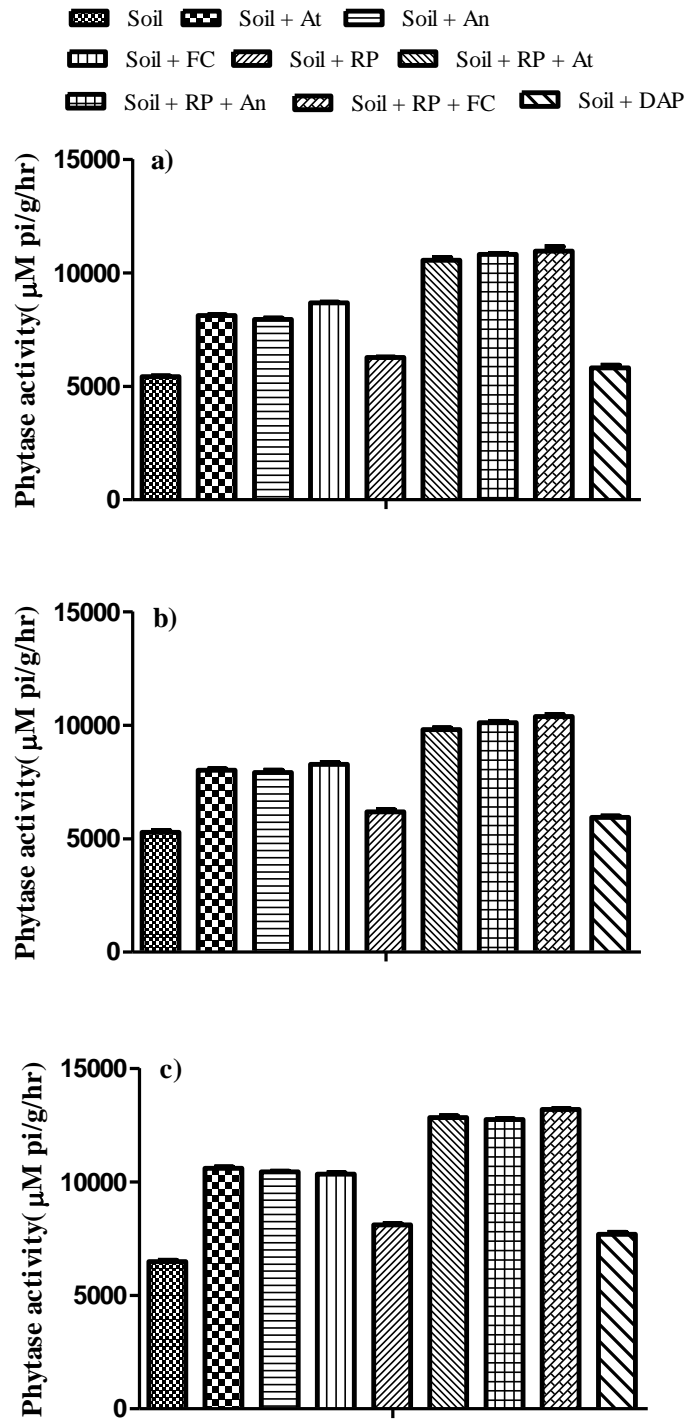


Fig. 7.27 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on phytase enzyme activities of rhizosphere soil of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

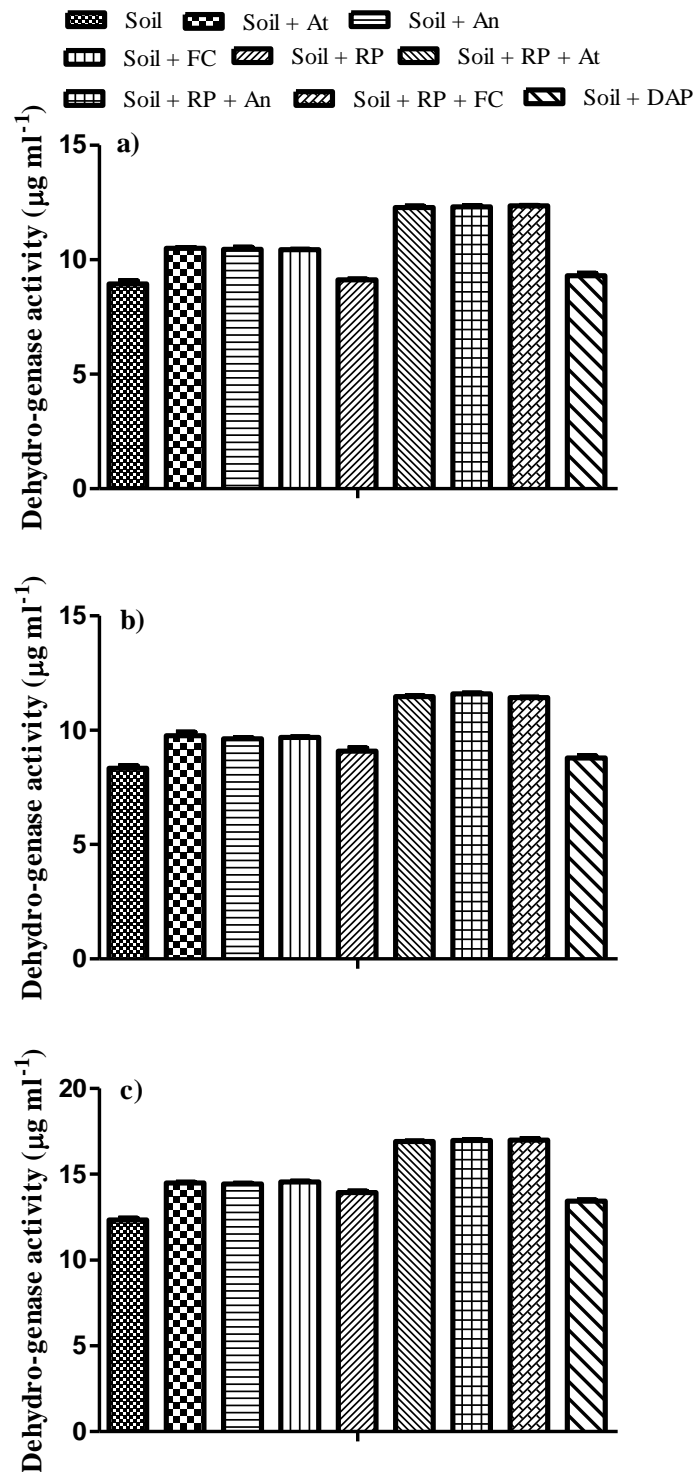


Fig. 7.28 Effect of *Aspergillus tubingensis* (At) and *Aspergillus niger* (An) alone or along with RP fertilization and chemical fertilizer (DAP) on dehydro-genase enzyme activities of rhizosphere soil of wheat plants grown in a) Central plain region (Thapar university, Patiala, Punjab), b) Sub mountain undulating central region (Balachaur, Punjab) and c) Sub mountain undulating region (Pojewal, Punjab).

Chapter 8

Development of inoculum formulations

8.1 Development of phosphate-solubilizing bacterial inoculum formulations and test their shelf life and phosphate solubilization efficiency

For sustainable agriculture, substitution of high priced chemical fertilizers with eco-friendly biofertilizers is the most desired practice. The microbial inoculation in the form of seed inoculation has been proved beneficial for the maintenance of soil fertility, but the use of suitable carrier, capable of supporting high viable microbial population for a prolonged duration is most important. Keeping the above factors in view, inoculum formulations of selected phosphate-solubilizing bacterial isolates *P. cyripedii* and *P. plecoglossicida* were developed. For the inoculum formulations development, rock phosphate, fly ash, charcoal and vermiculite were used as a carrier materials. Efforts were being made to check compatibility of these carrier materials with PSB isolates, and there support as a carrier material for the viability of bacterial isolates at different temperatures for a long period of storage. The final moisture content was adjusted to 30 % of the carrier materials before packing into separate bags.

Results in Fig 8.1, 8.2, 8.3 and 8.4 showed the effect of different temperature over time and carrier material types on population of bacteria in four carrier materials. The initial population density was 10.24 - 10.51 log cfu g⁻¹ of carrier material for *P. cyripedii* and *P. plecoglossicida*. As the bacterial population in logarithmic scale (Fig. 8.1, 8.2, 8.3 and 8.4) in carrier materials at 37 °C showed an upward trend up to 15 days of incubation after that bacterial population in all the carrier materials was started decreased (about one logarithmic unit in one month) while there was no increase in bacterial population stored at 4 °C.

At the end of 270 days of storage at 4 °C, the logarithm of population in carrier rock phosphate was up to 7.0 cfu g⁻¹, in fly ash 5.61 cfu g⁻¹, in charcoal 6.23 cfu g⁻¹ and in vermiculite 5.59 cfu g⁻¹ respectively. Similarly at 37 °C, after 270 days of storage, the mean

logarithm of population in carrier rock phosphate was 5.53 cfu g⁻¹, in fly ash 4.57 cfu g⁻¹, in charcoal 4.49 cfu g⁻¹ and in vermiculite 4.43 cfu g⁻¹ respectively. Results showed that viability of bacterial isolates were higher at 4 °C compared to 37 °C. Among the different carrier materials tested, rock phosphate formulations supported maximum viability of both the isolates up to 270 days of storage periods of 4 °C and 37 °C compared to other carrier materials tested. Purity of carrier materials in context to contamination was tested in control (carriers without bacterial inoculation) of all carriers after regular interval of one month and it was observed that control of each carrier material was free from any microbial load up to 270 days of storage at 4 °C and 37 °C. The loss in moisture content up 6-7 % was observed at 37 °C and there was no loss in moisture content of all the formulations at 4 °C.

Rock phosphate as a carrier material support maximum viability for bacterial isolates, at 37 °C and 4 °C, the 270 days old inoculum formulations of RP was tested for their ability to P solubilization and plant growth promotion activities and compared with fresh culture. Results in the Table 8.1 and 8.2 showed that P solubilization, and plant growth promotion activities of both the inoculums formulations at 4 °C and 37 °C was comparable to the fresh culture activities. Results suggested that long time storage of these formulations at 4 °C and 37 °C in which RP was used as carrier material were maintained their P solubilization efficiency and plant growth promotion activities and maximum shelf life.

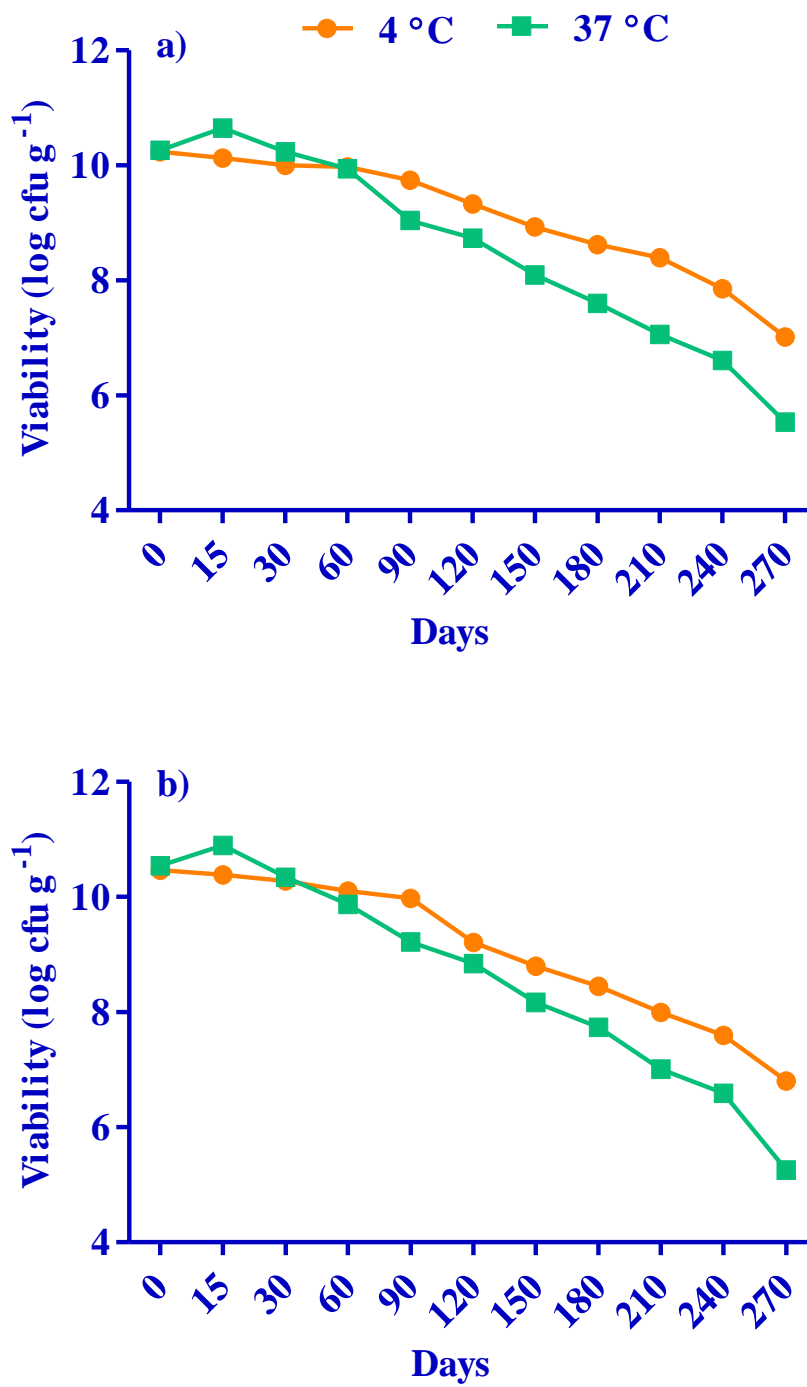


Fig. 8.1 Shelf life of inoculum formulations of a) *Pantoea cyripedii* and b) *Pseudomonas plecoglossicida* in RP at different time intervals (days), at 4 °C and 37 °C temperature.

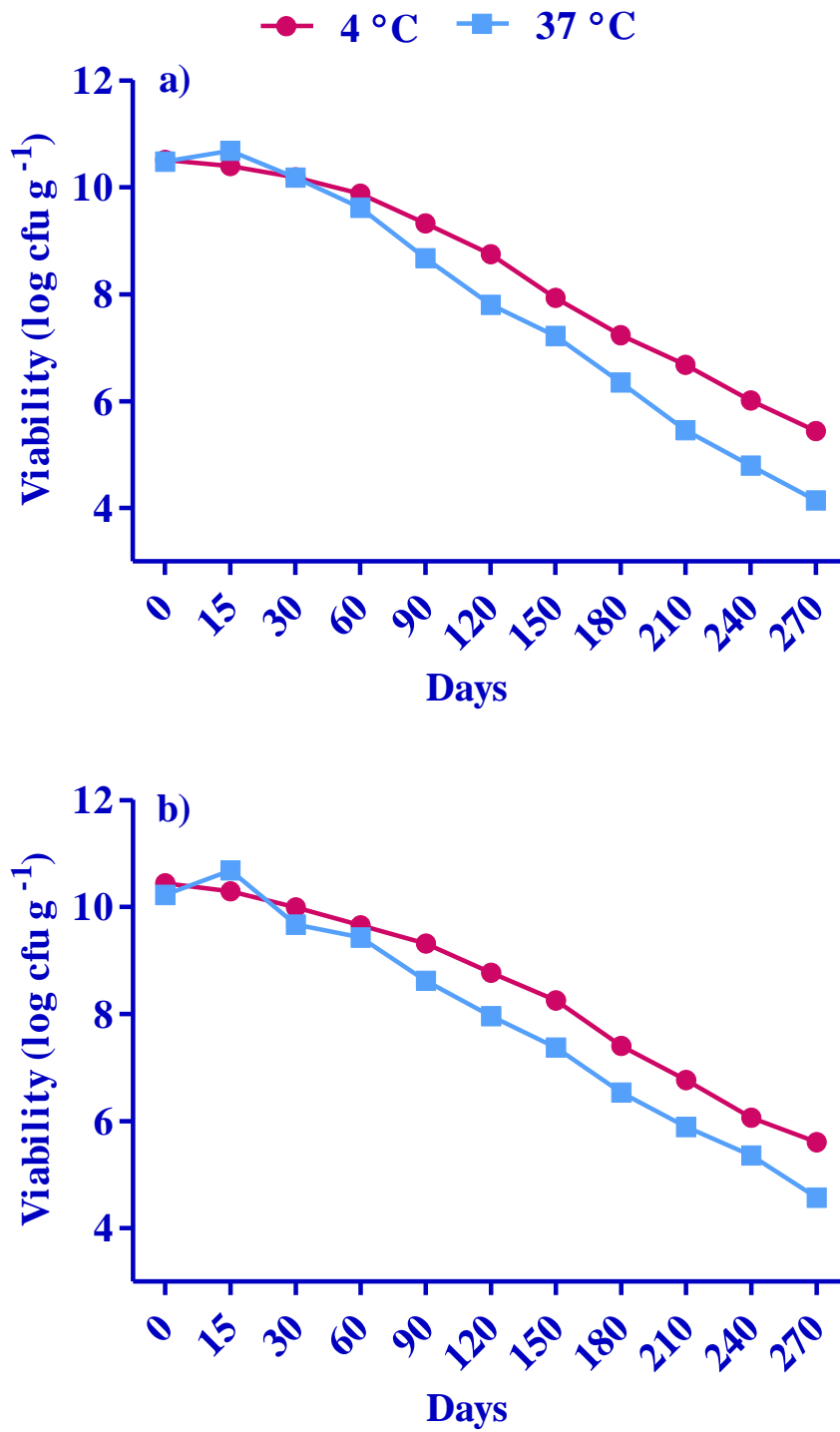


Fig. 8.2 Shelf life of inoculum formulations of a) *Pantoea cyripedii* and b) *Pseudomonas plecoglossicida* in fly ash at different time intervals (days), at 4 °C and 37 °C temperature.

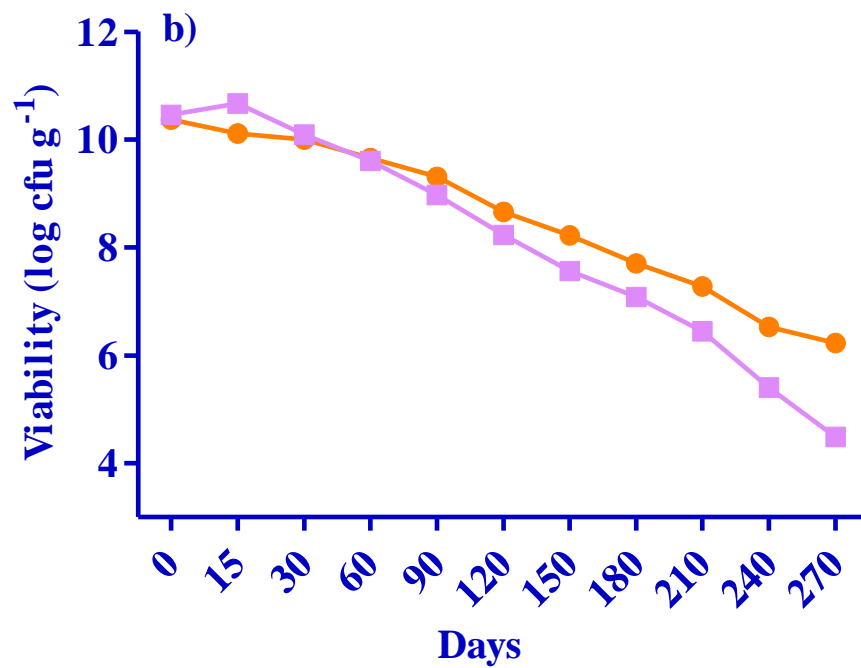
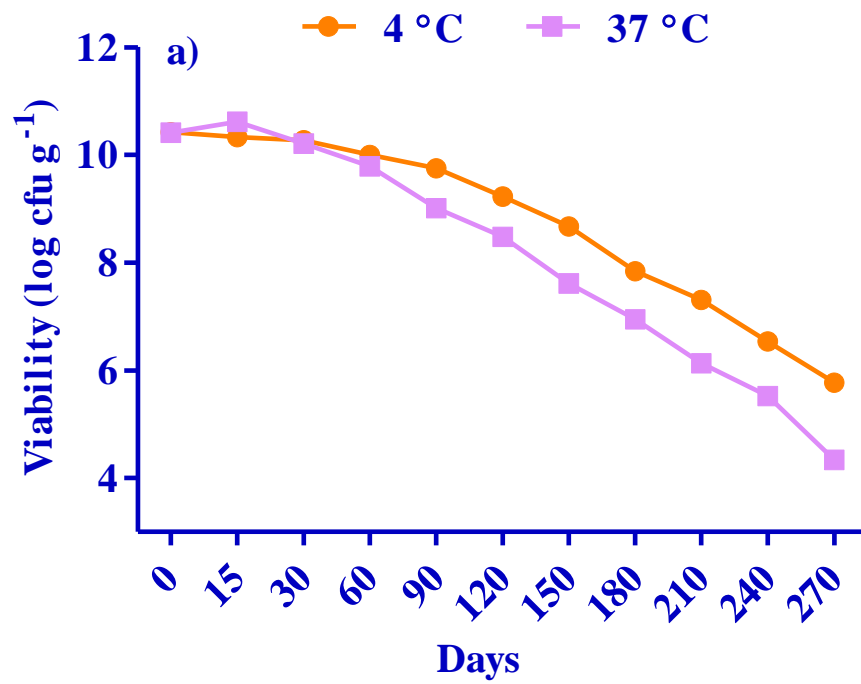


Fig. 8.3 Shelf life of inoculum formulations of a) *Pantoea cyripedii* and b) *Pseudomonas plecoglossicida* in charcoal at different time intervals (days), at 4 °C and 37 °C temperature.

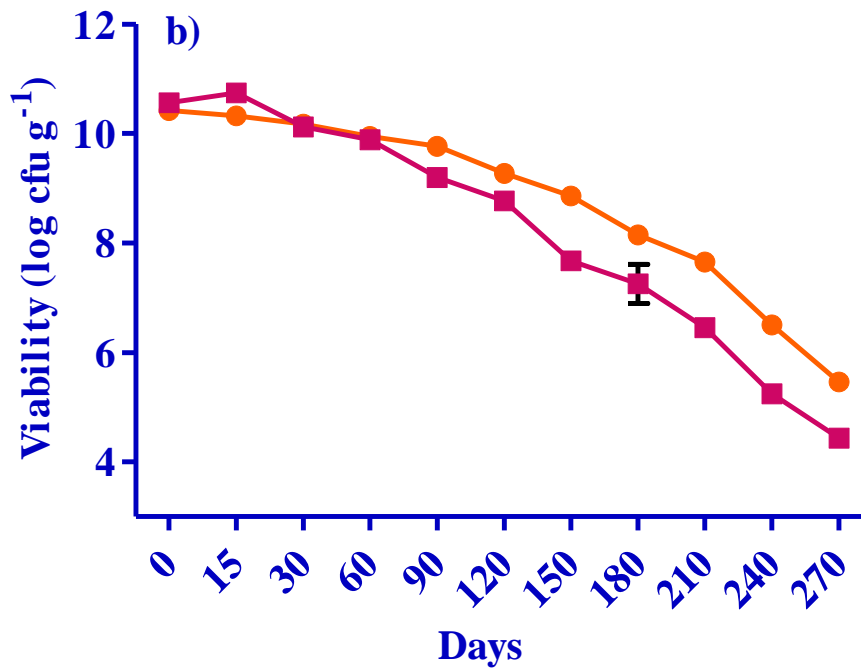
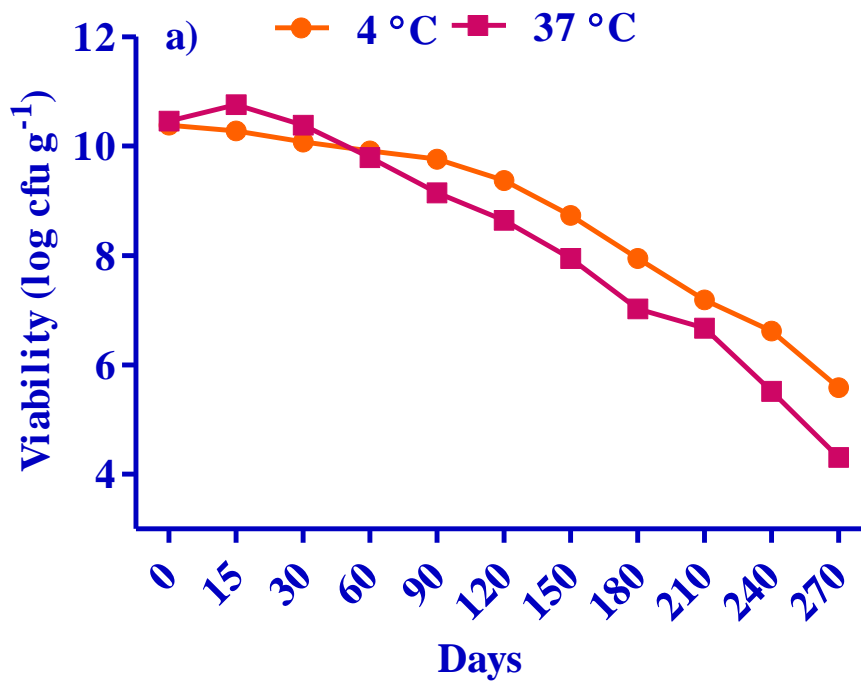


Fig. 8.4 Shelf life of inoculum formulations of a) *Pantoea cypripedii* and b) *Pseudomonas plecoglossicida* in vermiculite at different time intervals (days), at 4 °C and 37 °C temperature.

Table 8.1 Physiological study of bacterial inoculum formulations kept at 4 °C after 270 days of incubation and compare with fresh culture.

Activities	Inoculums formulation		Fresh culture	
	<i>Pantoea cypripedii</i>	<i>Pseudomona Plecoglossicida</i>	<i>Pantoea cypripedii</i>	<i>Pseudomona Plecoglossicida</i>
pH	3.79 ± 0.13	3.73 ± 0.16	3.87 ± 0.07	3.61 ± 0.04
Soluble P (µg ml ⁻¹)	415 ± 4	434 ± 6	426 ± 4	429 ± 4
Enzyme activities				
Acid phosphatase (µM p.NPP/ml/hrs)	91 ± 5	95 ± 4	96 ± 3	97 ± 2
Alkaline phosphatase (µM p.NPP/ml/hrs)	55 ± 6	53 ± 3	59 ± 3	63 ± 4
Phytase (µM pi/ml/hrs)	6762 ± 92	6860 ± 44	6966 ± 50	6847 ± 24
Indole acetic acid (µg ml ⁻¹)				
Without tryptophan	5.8 ± 0.21	6.4 ± 0.08	7.9 ± 0.43	7.1 ± 0.33
With tryptophan	90 ± 0.7	26 ± 0.2	93 ± 0.3	27 ± 0.2
Siderophore	+	+	+	+

Values are Mean ± SD (n =3).

Table 8.2 Physiological study of bacterial inoculum formulations kept at 37 °C after 270 days of incubation and compare with fresh culture.

Activities	Inoculums formulation		Fresh culture	
	<i>Pantoea cypripedii</i>	<i>Pseudomona Plecoglossicida</i>	<i>Pantoea cypripedii</i>	<i>Pseudomona Plecoglossicida</i>
pH	3.76 ± 0.04	3.59 ± 0.04	3.87 ± 0.07	3.61 ± 0.04
Soluble P	415 ± 12	410 ± 2	426 ± 4	429 ± 4
Enzyme activities				
Acid phosphatase (µM p.NPP/ml/hrs)	71 ± 1	81 ± 8	96 ± 3	97 ± 2
Alkaline phosphatase (µM p.NPP/ml/hrs)	46 ± 5	50 ± 5	59 ± 3	63 ± 4
Phytase (µM pi/ml/hrs)	6671 ± 72	6705 ± 83	6966 ± 50	6847 ± 24
Indole acetic acid (µg ml ⁻¹)				
Without tryptophan	4.8 ± 0.39	6.13 ± 0.06	7.9 ± 0.43	7.1 ± 0.33
With tryptophan	87 ± 1.8	22 ± 0.4	93 ± 0.3	27 ± 0.20
Siderophore	+	+	+	+

Values are Mean ± SD (n =3).

8.2 Development of phosphate-solubilizing fungal inoculum formulations and check their shelf life and phosphate solubilization efficiency

Similar to the bacterial isolates, fungal isolates were also subjected to test their viability in different carrier materials such as rock phosphate, fly ash, charcoal and vermiculite to develop the inoculum formulations. The carriers were inoculated with fungal spores and incubated at 37 °C and 4 °C. Viability of fungal inoculums in different carrier materials was tested after regular interval of 30 days. Results in Fig. 8.5, 8.6, 8.7 and 8.8 showed that both the fungal inoculums showed similar trend in decreased of viability rate at different temperature condition over a period of 270 days in different carrier materials. On day first of incubation population density of fungal inoculums in all carrier materials was 8.3 to 8.5 log cfu g⁻¹ at 4 °C and 37 °C. It was observed that up to day 15 of incubation at 37 °C viability of fungal inoculums was slightly increased from its initial values in all the carrier materials. Viability of fungal inoculums was decreased rapidly at 37 °C compared to 4 °C during long period of incubation. After 270 days of incubation, logarithm of population density was reached 6.19 cfu g⁻¹, 5.00 cfu g⁻¹, 5.41 cfu g⁻¹ and 4.88 cfu g⁻¹ in rock phosphate, fly ash, charcoal and vermiculite respectively at 4 °C. At 37 °C, logarithm of population density was reached 4.85 cfu g⁻¹, 4.07 cfu g⁻¹, 4.25 cfu g⁻¹ and 3.97 cfu g⁻¹ in rock phosphate, fly ash, charcoal and vermiculite respectively. Results showed that viability of inoculums was higher at 4 °C as compared to 37 °C. It is evident from the data of present study that, *Aspergillus tubingensis* and *Aspergillus niger* can survive in the selected carrier materials but maximum viability and better survival after 270 days of storage was observed in RP compared to other carrier materials at 4 °C and 37 °C.

The 270 days old fungal inoculum that showed better survival in RP formulation was tested for P solubilization and plant growth promotion activities and compared with fresh culture. Results in the Table 8.3 and 8.4 showed that P solubilization, acid phosphatase, alkaline

phosphatase, phytase enzymes, and plant growth promotion activities such as IAA production and siderophore production of both fungal inoculum formulations at 4 °C and 37 °C were not affected and comparable to the fresh fungal culture activities. Results suggested that long time storage of these fungal inoculum formulations at 4 °C and 37 °C in which RP was used as carrier material were maintained their P solubilization potential, plant growth promotion activities and shelf life.

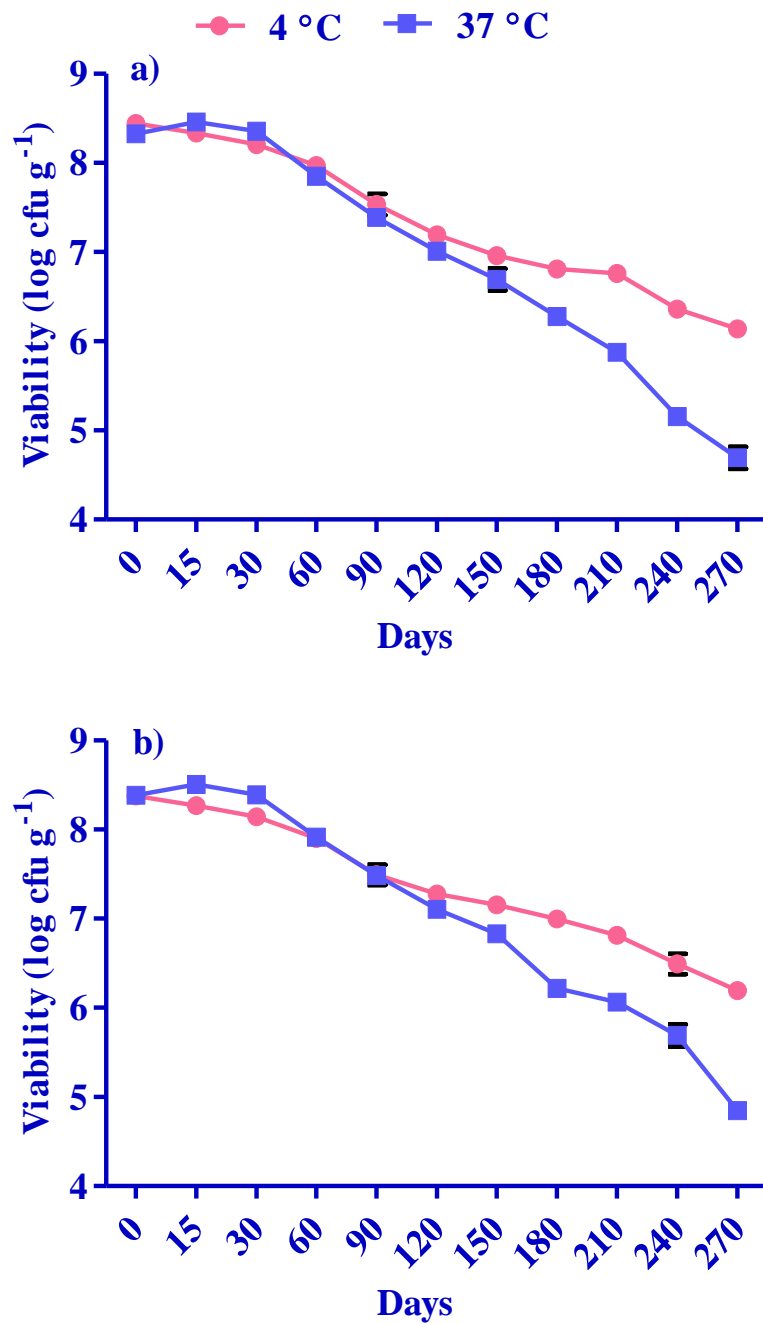


Fig 8.5 Shelf life of inoculum formulations of a) *Aspergillus tubingensis* and b) *Aspergillus niger* in RP at different time intervals (days), at 4 °C and 37 °C temperature.

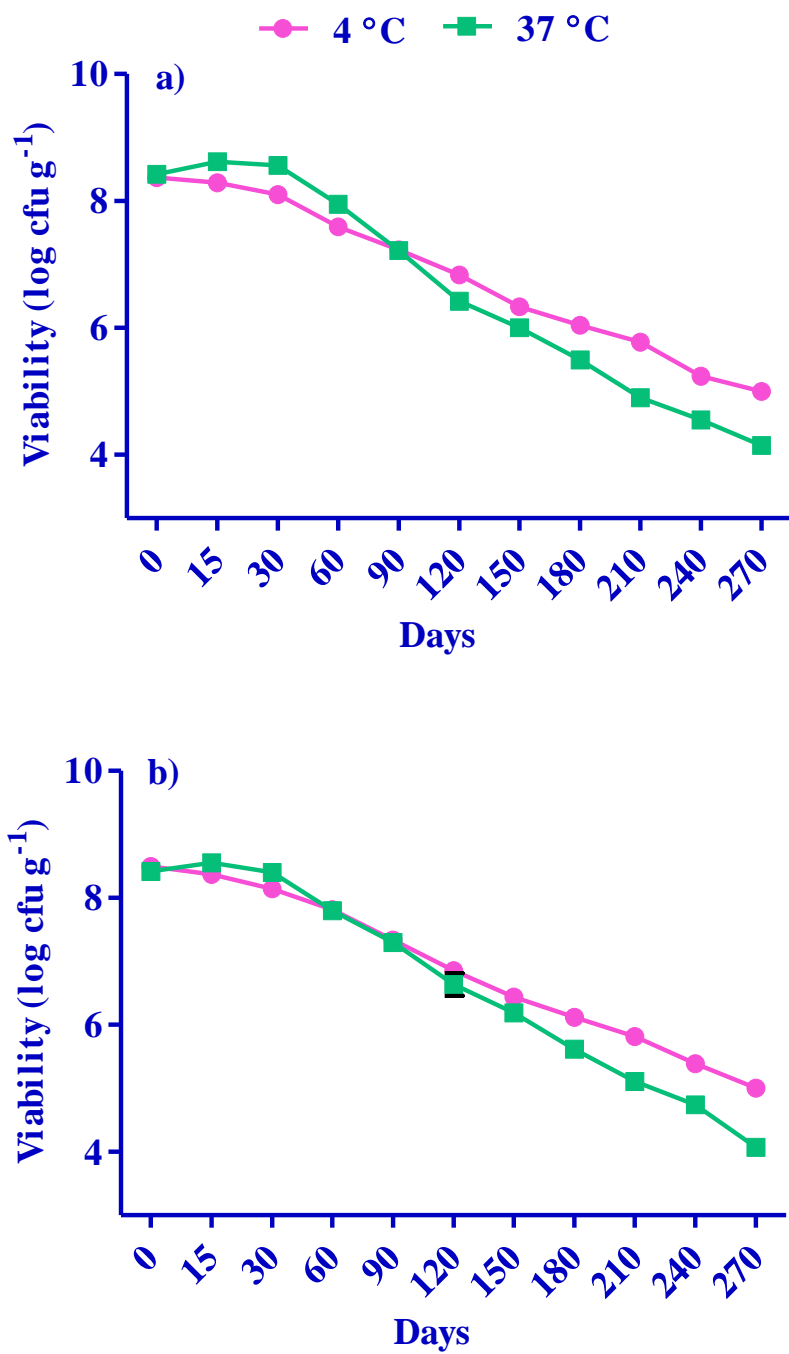


Fig 8.6 Shelf life of inoculum formulations of a) *Aspergillus tubingensis* and b) *Aspergillus niger* in fly ash at different time intervals (days), at 4 °C and 37 °C temperature.

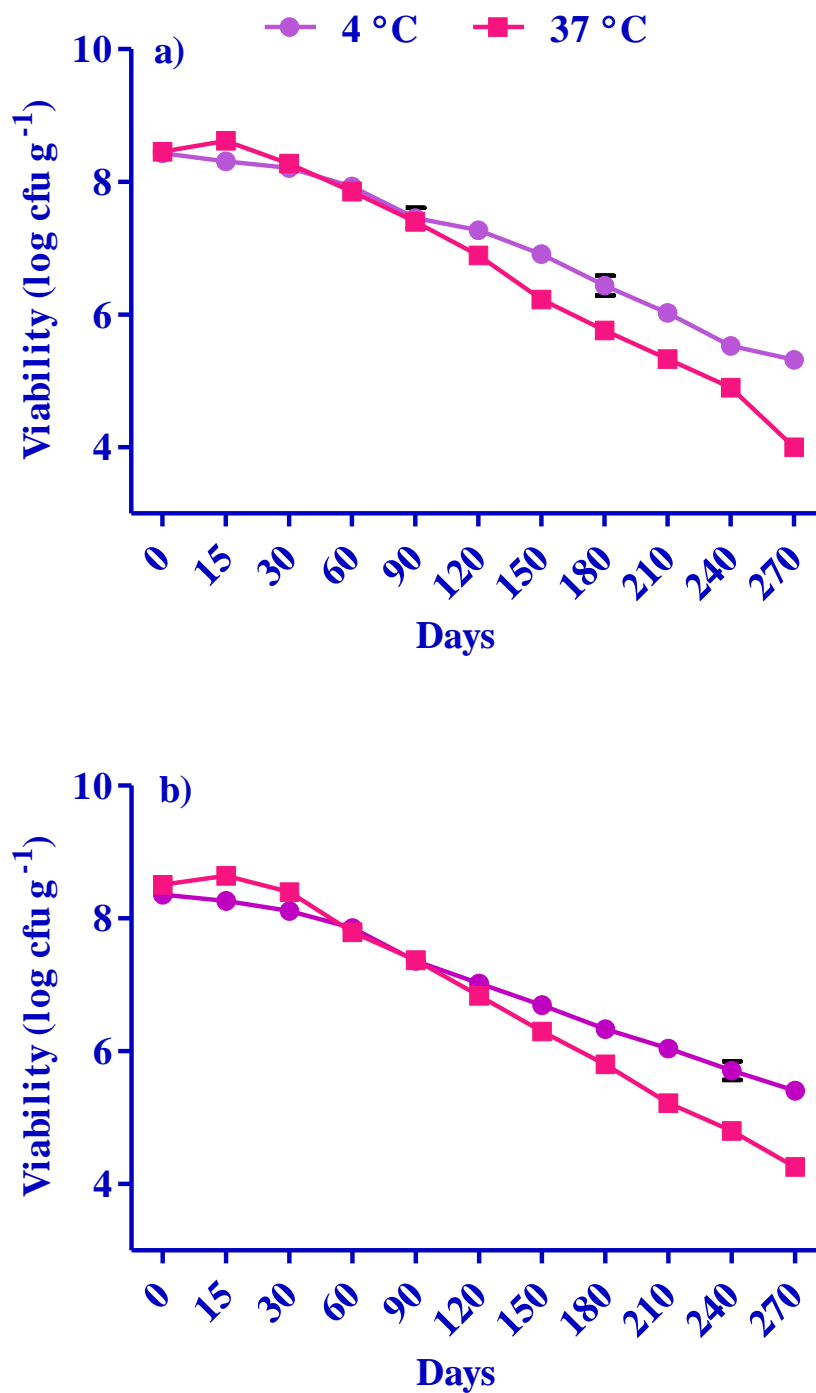


Fig 8.7 Shelf life of inoculum formulations of a) *Aspergillus tubingensis* and b) *Aspergillus niger* in charcoal at different time intervals (days), at 4 °C and 37 °C temperature.

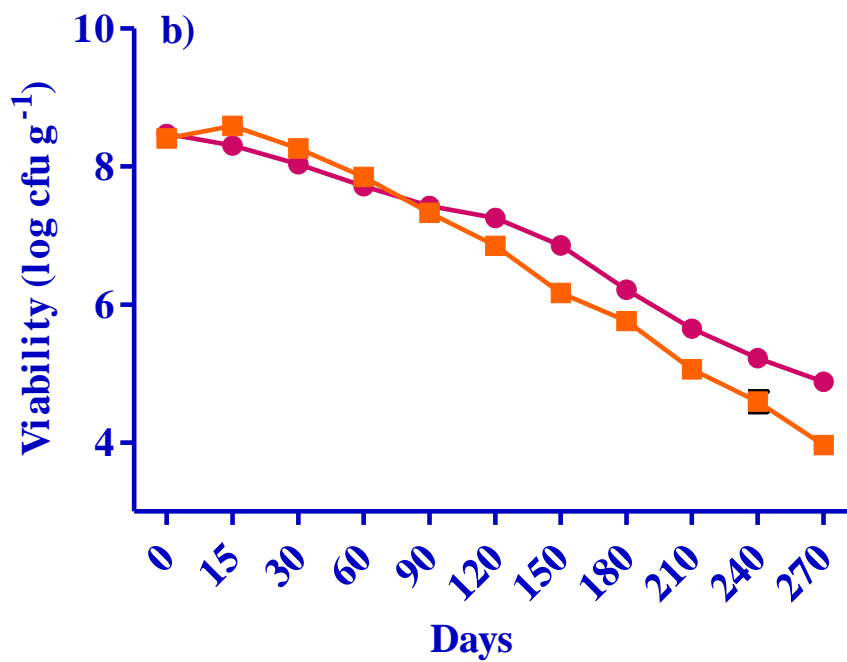
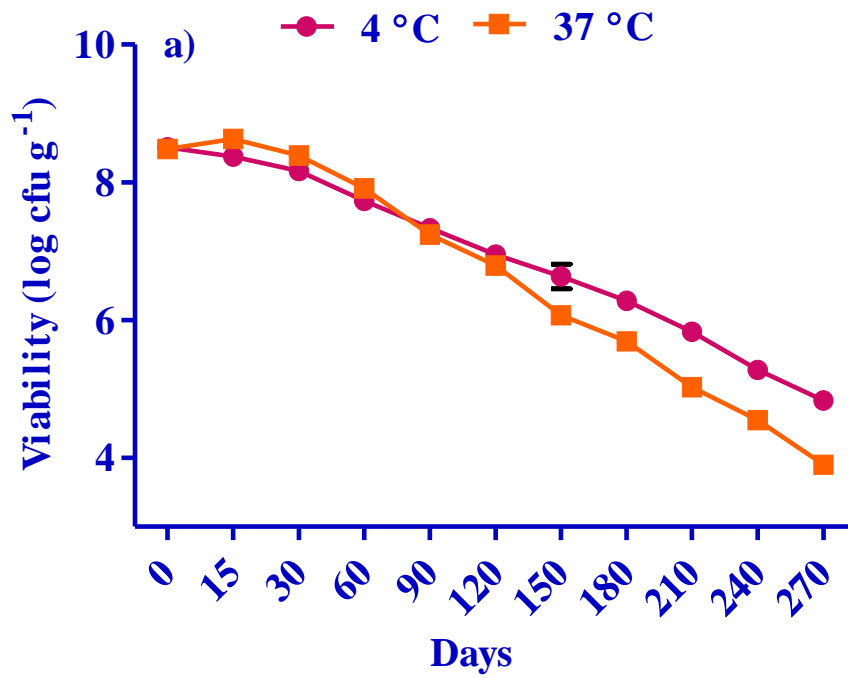


Fig 8.8 Shelf life of inoculum formulations of a) *Aspergillus tubingensis* and b) *Aspergillus niger* in vermiculite at different time intervals (days), at 4 °C and 37 °C temperature.

Table 8.3 physiological study of fungal inoculum formulations kept at 4 °C after 270 days of incubation and compare with fresh culture.

Activities	Inoculum formulations		Fresh culture	
	<i>Aspergillus tubingensis</i>	<i>Aspergillus niger</i>	<i>Aspergillus tubingensis</i>	<i>Aspergillus niger</i>
pH	2.92 ± 0.17	2.59 ± 0.13	2.36 ± 0.15	2.30 ± 0.11
Soluble P	455 ± 6	442 ± 10	458 ± 8	440 ± 2
Dry biomass (gm/100ml)	0.430 ± 0.01	0.425 ± 0.06	0.447 ± 0.02	0.438 ± 0.065
Enzyme activities				
Acid phosphatase (µM p.NPP/ml/hrs)	148 ± 4	140 ± 3	151 ± 3	149 ± 1
Alkaline phosphatase (µM p.NPP/ml/hrs)	71 ± 2	74 ± 1	79 ± 2	76 ± 1
Phytase (µM pi/ml/hrs)	10068 ± 30	10397 ± 29	10113 ± 76	10513 ± 98
Indole acetic acid (µg ml ⁻¹)				
Without tryptophan	17.9 ± 0.30	19.2 ± 0.44	18.6 ± 0.21	20.5 ± 0.20
With tryptophan	47 ± 0.46	55 ± 1.0	51 ± 0.17	57 ± 0.15
Siderophore	+	+	+	+

Values are Mean ± SD (n =3).

Table 8.4 physiological study of fungal inoculum formulations kept at 37 °C after 270 days of incubation and compare with fresh culture.

Activities	Inoculum formulations		Fresh culture	
	<i>Aspergillus tubingensis</i>	<i>Aspergillus niger</i>	<i>Aspergillus tubingensis</i>	<i>Aspergillus niger</i>
pH	2.58 ± 0.07	2.80 ± 0.06	2.36 ± 0.15	2.30 ± 0.11
Soluble P	445 ± 3	436 ± 5	458 ± 8	440 ± 2
Dry biomass (gm/100ml)	0.419 ± 0.02	0.434 ± 0.04	0.0447 ± 0.02	0.438 ± 0.065
Enzyme activities				
Acid phosphatase (µM p.NPP/ml/hrs)	143 ± 3	138 ± 8	151 ± 3	149 ± 1
Alkaline phosphatase (µM p.NPP/ml/hrs)	67 ± 6	69 ± 1	79 ± 2	76 ± 1
Phytase (µM pi/ml/hrs)	9978 ± 48	10195 ± 19	10113 ± 76	10513 ± 98
Indole acetic acid (µg ml ⁻¹)				
Without tryptophan	13.7 ± 0.30	16.0 ± 0.25	18.6 ± 0.21	20.5 ± 0.20
With tryptophan	45 ± 0.21	53 ± 0.24	51 ± 0.17	57 ± 0.15
Siderophore	+	+	+	+

Values are Mean ± SD (n =3).

Chapter 9

Discussion

Discussion

Phosphorus is one the most essential elements for plant growth after nitrogen. However, the availability of this nutrient for plants is limited by different chemical reactions. Phosphorus plays a significant role in several physiological and biochemical plant activities like photosynthesis, transformation of sugar to starch, and transporting of the genetic traits. Sharma (2002) reported that the advantages of feeding the plants with phosphorus are to create deeper and more abundant roots. Phosphorus causes early ripening in plants, decreasing grain moisture, improving crop quality and is the most sensitive nutrient to soil pH (Malakooti 2000). Arpana et al (2002) reported that a great proportion of phosphorus in chemical fertilizer becomes unavailable to the plants after its application in the soil. They referred this to formation of strong bonds between phosphorus with calcium and magnesium in alkaline pH and the same bonds with iron and aluminum in acidic soils. The mobility of this element is very slow in the soil and can not respond to rapid uptake by plants. This causes the creation and development of phosphorus depleted zones near the contact area of roots and soil in rhizosphere. Therefore, plants need an assisting system which could extend beyond the depletion zones and help to absorb the phosphorus from a wider area by developing an extended network around root system (Salehrastin 1999). Bio-fertilizers (phosphate-solubilizing microorganisms) are considered among the most effective plant assistants to supply phosphorus at a favorable level. These fertilizers are produced on the basis of selection of beneficial soil microorganisms which have the highest efficiency to enhance plant growth by providing nutrients in a readily absorbable form. These beneficial microbes are considered as bio-inoculants or bio-fertilizers and they improve the growth and quality of the seeding leading to their better survival (Mohan and Karthkeyan 2011). Application of inoculants provided from these microorganisms enhances an abundant population of active and effective microorganisms to the root activity zone which increases

plant ability to uptake phosphorus for it. Phosphate-solubilizing microorganisms refer to a group of soil microorganisms that as components of phosphorus cycle, can release it from insoluble sources by different mechanisms (Salehrastin 1999). Phosphate-solubilizing fungi and bacteria are known as effective organisms in this process (Reyes et al. 1999).

Isolation of phosphate-solubilizing bacteria and fungi

It is well known that a considerable variety of microorganisms are associated with the plant rhizosphere (Rodriguez and Fraga 1999). Rhizosphere biology is considered to be the most intensive area of research in agriculture (Sachdev and Cameotra 2013). Phosphate-solubilizing microorganisms often constitute maximal 30 % of the culturable population size from the rhizosphere soils. It is suggested that phosphate solubilization by rhizosphere microorganisms is a major mechanism for phosphorus supply (Kucey 1983).

In the present study soil samples from rhizosphere of different crop plants grown in conventional farming and in organic farming and from surface soils were taken to isolates the efficient phosphate-solubilizing microorganisms (bacteria and fungi). When we compare the rhizospheric soil and surface soil, maximum phosphate-solubilizing bacterial and fungal population was came from rhizospheric soils. Root exudates are known to serve as a substantial source of reduced carbon compounds which are released in rhizosphere. Microbes in rhizosphere utilize root exudates as their major nutrient source and this forms the basis of rhizosphere colonization. Maximum exudation occurs near the root tips, so large numbers of bacteria occur near the growing root area. Thus, the rhizospheric zone for the isolation of phosphate-solubilizing microorganisms is very effective (Patel et al. 2008). Rhizospheric microorganisms are known to play a very significant role in plant growth promotion by different mechanisms, one of them being the ability to solubilize mineral phosphate in the rhizosphere, thus making it available for plant uptake (Gyaneshwar et al. 2002). Considerable

higher concentration of phosphate-solubilizing bacteria and fungi (*Penicillium* and *Aspergillus*) with this capacity is commonly found in rhizospheric soil (Suh et al. 1995; Whitelaw et al. 1999). It has been observed by many investigators that, a high proportion of P-solubilizing microorganisms (PSMs) especially bacteria, fungi and actinomycetes reside in the rhizosphere of plants and play an important role in solubilization of bound phosphates, making them available to plants (Gaur 1990). Use of plant growth promoting rhizobacteria (PGPR) for the benefits of agriculture is gaining worldwide importance and acceptance and appears to be the trend for the future (Rai et al. 2006).

Among the rhizospheric soil of conventional farming crop and organic farming crop, population density of PSMs was higher in organic farming soil. System effects of organic and conventional agricultural farming practice on soil biota were reviewed by Mader et al (1996) comparing four different long term field trials at Darmstadt, Germany (Bachinger 1996), Jarna, Sweden (Pettersson et al. 1992), Kaisheim, Germany (Beck 1991) and Therwil, Switzerland. In any case soil microbial activity (dehydrogenase and catallase) in the organic farming treatments were 30-70 % and in the bio-dynamic 40-90 % higher than in respective soils receiving mineral fertilizers only. Organically managed rice fields showed superior microbial population and soil enzyme activities in comparison to conventional managed field (Chhotaray et al. 2011). Araujo et al (2009) also suggested that organic farming practices resulted in higher soil microbial activity measured by soil respiration, and organic carbon. Variation in population density of PSMs might be attributed to many soil factors such as soil nutrients, pH, moisture contents, organic matter and enzyme activities (Ponmurugan and Gopi 2006).

Phosphate-solubilizing bacteria and fungi showing more than 5 mm zone of solubilization were selected for further study. All the bacterial isolates were tested for their P-solubilization activity (PSA) on PKV agar plates and it was ranged up to 2.6 mm. A similar criterion was

followed by Parihar et al (2004) to find out the phosphate solubilizing activity of endophytic bacteria isolated from Sugarcane plant. Similar criteria was also discussed by Fankem et al (2006) to check the PSA of phosphate-solubilizing microorganisms from oil palm tree rhizosphere in Cameroon.

To test the purity and stability, selected bacterial and fungal isolates were point inoculated on BCG PKV agar plates. Change in color of medium from blue to green was observed surrounding the phosphate-solubilizing bacterial and fungal colonies, when point inoculation was done on the Pikovskaya's agar plates containing Bromo Creasol Green as an indicator. The change in color of medium from blue to green indicated the change in pH and this change in medium pH was taken to be directly associated to the process of phosphate solubilization (Fankem et al. 2006).

Out of all bacteria and fungi isolated from the soil, only 32 bacterial isolates and nine fungal isolates showed significant zone of phosphate solubilization. A clear halo zone was formed around the colonies after 3-5 days of incubation on PKV agar plates, indicating phosphate solubilization ability of the bacterial and fungal isolates. These isolates on the basis of halo zone were selected for further screening for TCP and RP solubilization in PKV broth.

TCP and RP solubilization by phosphate-solubilizing microorganisms

Bacterial and fungal isolates selected in the present study were able to solubilized TCP and RP in culture medium and there was a significant increase in P solubilization with reduction in pH of the culture supernatant. Himani and Reddy (2012) showed a significant relationship between quantities of phosphate solubilized and drop in pH of culture filtrate. In case of bacterial isolates, maximum-solubilization occurred at day five of incubation and later it decreased. In case of fungal isolates, maximum solubilization of TCP was observed at day three of incubation and solubilization of RP was observed at day five of incubation. As the

time of incubation increased, pH of the culture filtrate was increased and soluble P levels decreased. Among the microorganisms, it is observed that several fungal strains have a higher solubilizing ability of inorganic insoluble phosphates than bacteria (Rajankar et al. 2007). In both bacterial and fungal isolates, P-solubilization was higher with TCP as compared to RP. Pardhan and Sukla (2005) also reported that *Aspergillus* sp. solubilized 480 $\mu\text{g ml}^{-1}$ of phosphorus from 0.5 % tri-calcium phosphate with decreased in pH from 7.0 to 4.0 in day four and treatment of rock phosphate by *Aspergillus* released only 58 $\mu\text{g ml}^{-1}$ of P in culture medium after day seven of incubation. RP solubilization was lower compared to TCP solubilization by both bacterial and fungal isolates, which may be due to complexity of the RP structure. An extensive range of microorganisms that are able to solubilize various form of soil-bound phosphorus have been reported (Rodriguez and Fraga 1999; Whitelaw 2000), and among them, most predominant and representative ones are *Bacillus* sp. and *Pseudomonas* sp. soil bacteria, and *Penicillium* sp. and *Aspergillus* sp. saprophytic fungi. Nahas (1996) and Kucey et al (1989) showed that, the solubilization of insoluble phosphates depends upon a multitude of factors including decrease in pH, microorganisms and the insoluble phosphates used. The ability of these phosphate-solubilizing microorganisms to release soluble orthophosphate (Pi) from TCP and RP make this phenotype of great potential importance for the development of eco-rational phosphate fertilizer technologies for agriculture (Goldstein et al. 1993). The decrease in P-solubilizing ability after a certain period of incubation may be due to depletion of nutrients, production of certain toxic metabolites in growth medium, or the autolysis of cells. pH of broth was found to be decreased in each isolates with increase in P-solubilization in growth medium. Achal et al (2007) reported that P-solubilizing activity is associated with a drop in pH. Lowering of pH indicated the production of organic acids during the metabolism of glucose, which is considered as a mechanism responsible for the dissolution of insoluble form of phosphate

(Hwangbo et al. 2003). Reddy et al (2002) reported that, *A. tubingensis* and *A. niger* showed a significant decrease in pH with increase of soluble P in culture medium. The P-solubilizing activity is also determined by the biochemical ability of the bacterial and fungal isolates to produce and release organic acids in culture broth. Bacterial and fungal isolates used in this study were produced significant amount of organic acids in TCP and RP amended medium. Gluconic acid and acetic acid were the main organic acids produced by bacterial isolates in TCP amended PKV broth. Gluconic acid was found to be predominant in RP supplemented PKV broth by bacterial isolates. In case of fungal isolates, gluconic acid was found to be the main organic acid in TCP and RP supplemented PKV broth followed by succinic acid, citric acid and oxalic acids. Gluconic acid seems to be the most frequent agent of phosphate solubilization. The major microbiological mean by which the insoluble P compounds are mobilized is by the production of organic acids, accompanied by acidification of the medium (Illmer and Schinner 1992). All the isolates produced acid phosphatase, alkaline phosphatase, and phytase enzymes in TCP and RP amended medium. Several mechanisms like lowering of pH by acid production, ion chelation and exchange reactions in growth environment have been reported to play a role in phosphate solubilization by phosphate-solubilizing microorganisms (Cunningham and Kuiack 1992). Pardhan and Sukla (2005) suggested that considering amount of glucose used in the medium and corresponding efficacy of P-solubilization suggesting the PKV medium as most cost effective without compromising the solubilization. Fungi have been reported to possess greater ability to solubilize rock-phosphate than bacteria. Filamentous fungi are widely used as producers of organic acids, particularly black *Aspergillus* and some species of *Penicillium*. These species have been tested for solubilization of RP and have been reported for various properties of biotechnological importance, such as P-solubilization and P fertilizer (Richa et al. 2007; Pandey et al. 2008). Increased in P concentration in the medium containing phosphate-

solubilizing fungi was related to the secretion of organic acid and nature of organic acids produced in the medium; which should correlate with the pH of the medium (Illmer and Schinner 1992; Illmer et al. 1995). *Aspergillus niger* have been shown to solubilize mineral phosphates by the secretion of organic acid such as gluconic acid, citric acid and oxalic acid (Cerezine et al. 1988). In terms of amount of P released as a fraction of the total P and specific RP solubilizing abilities, fungal species are better than bacterial species, possibly due to secretion of strong acids such as citric acid and oxalic acid in addition to gluconic acid (Cerezine 1988; Cunningham and Kuiuack 1992).

Anderson (1980) reported that phytates (derivatives of inositol hexaphosphates) account for large component of the organic P (some 20-50 % of the total soil organic P), yet appear to be only poorly utilized by plants (Hayes et al. 2000; Richardson et al. 2000). Phytates may readily undergo physical and chemical reactions in soil environments, rendering them unavailable for plant uptake (McKercher and Anderson 1989). Phosphatases (phytase and acid phosphatase) produced by soil microorganisms play a major role in mineralization of organic forms of soil P to release phosphate (Raghothama 1999). Acid phosphatase and phytase enzyme activity were significantly high in P-solubilizing fungi in presence of TCP as well as RP. Relwani et al (2008) suggested that both phosphatase and phytase enzymes play a major role in P-solubilization, apart from other phosphate solubilization mechanisms. The fungi and probably all living organisms, synthesize a number of phosphatase which are necessary to scavenge phosphates from medium containing bound phosphorus. Both acid and alkaline phosphatases exist in soil and are distinguished on the basis of pH ranges at which they are active. These are secreted in response to signals of the absence of available P (Peleg et al. 1996).

Identification of bacterial and fungal isolates

Regarding genetic and phylogenetic characterizations of microorganisms, molecular techniques such as DNA sequencing have been used (Eisen 1995). The sequence of the 16S rRNA gene has been widely used as phylogenetic marker in microbial ecology (Ludwig et al. 1998), since the extent of divergence in the sequence of this gene provides an estimate of the phylogenetic distance existing between different species (Igual et al. 2001). 16S rRNA gene sequence was used as phylogenetic marker because (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes (Patel 2001). 16S rRNA sequence analysis of selected bacterial isolates using Ez Taxon-e database revealed that most closely related type strain for PSB-3 is *Pantoea cyripedii* which showed 98.67% pairwise similarity and 98.5% query coverage similarly. For PSB-5, the close related sequence is *Pseudomonas plecoglossicida* with 99.78% pairwise similarity and 100% query coverage. Phylogenetic analysis also grouped PSB-3 with *P. cyripedii* and PSB-5 with *P. plecoglossicida*. 16S rRNA gene sequences determined in this study was deposited in GenBank of the NCBI under the accession numbers JX556216 and JX556217 for PSB-3 and PSB-5, respectively.

In the present study two efficient phosphate-solubilizing bacteria isolated from rhizospheric soil of *Stevia rebaudiana* were identified as *P. cyripedii* and *P. plecoglossicida*. Although earlier studies on phosphate solubilization and plant growth promotion activities of these genera have reported but they were isolated from the rhizospheric soils of conventional farming system and from other natural habitat not from the organic field and these are the new species of *Pantoea* and *Pseudomonas* for P solubilization. Rodriguez and Fraga (1999) related that strains from *Pseudomonas*, *Bacillus* and *Rhizobium* are among the genera with

the greatest potential for P solubilization. Kucey (1983) reported that mainly the bio-fertilizer bacteria are genus of *Pseudomonas* and *Bacillus*.

Identification of fungal species by classical taxonomy is based mainly on the use of morphological markers. However, number of these markers available is generally low, which makes difficult the classification and/or identification of related species. The development of molecular biology techniques for the genetic differentiation of species has resulted in substantial advances in taxonomy due to their sensitivity and specificity. The amplification of internal transcribed spacer (ITS1-5.8S-ITS2) of ribosomal RNA (rRNA) by the polymerase chain reaction (PCR) (Mullis and Faloona 1987), combined with sequencing of the amplicon and analysis of similarity between the sequences obtained and those already deposited in the Gene Bank, has been frequently employed for identification of fungal species.

In the present study, eight fungal isolates were identified as genus *Aspergillus niger* and one as *Aspergillus tubingensis*. Classical taxonomy has been utilized and it does not allow the discrimination of *A. tubingensis* from *A. niger*. The utilization of molecular methods allowed a better distinction of the *A. niger* group (Kusters-Van Someren et al. 1991; Varga et al. 1993, 1994; Accensi et al. 1999) with *A. tubingensis*. The amplification of the ITS1-5.8S-ITS2 region of rRNA for the nine *Aspergillus* isolates, using the universal primers ITS1 and ITS4 (White et al. 1990) originated a fragment of approximately 600 bp. These result are in accordance to Henry et al. (2000) who found ITS1-5.8S-ITS2 amplicons of sizes varying between 565 and 613 bp. In the present study, the distinction between *A. niger* and *A. tubingensis* was determined by RFLP analysis. This analysis was based on the presence of the restriction site for the endonuclease *RsaI* (GT/AC) in ITS1 sequence from *A. niger* and its absence in *A. tubingensis* (Accensi et al. 1999). Several authors have described *Aspergillus* as major genus of P-solubilizing fungi (Whitelaw et al. 1999; Seshadri et al. 2004; Wang et al. 2005). Nucleotide sequences comparison of fungal isolates was performed using the BLAST

database. Sequence data from BLAST results showed that most efficient fungal isolates PSF-5-, PSF-6 and PSF-7 showed maximum similarity with *Aspergillus niger* and one isolate PSF-4 showed maximum similarity with *Aspergillus tubingensis*.

Effect of carbon and nitrogen sources on P-solubilization by bacterial isolates

Phosphate solubilization by bacterial isolates was evaluated in presence of different carbon and nitrogen sources by replacing the glucose and ammonium sulphate, respectively, from the PKV broth. Maximum P-solubilization was observed in presence of glucose as a carbon source and ammonium sulphate as nitrogen source in culture broth. Nautiyal et al (2000) also showed that in some bacterial isolates, glucose proved to be one of the best carbon sources for insoluble phosphate solubilization. Acid phosphatase, alkaline phosphatase, phytase enzymes and organic acid production was also higher in presence of glucose and ammonium sulphate compared to other carbon and nitrogen sources. Both the strains demonstrated diverse levels of phosphate solubilization activity in presence of various carbon and nitrogen sources. Production of acid phosphatase, alkaline phosphatase, phytase enzymes, pH reduction, P-solubilization and organic acids production was greatly affected by the nature of carbon and nitrogen sources in the media. The effect was more pronounced in presence of different carbon sources as compared to different nitrogen sources. Carbon source is an important parameter for active proliferation of organisms and production of organic acids whereas nitrogen source is important for the production of inorganic acids (Narsian and Patel 2000). In presence of some carbon and nitrogen sources, there was steep decline in pH from 7.0 to 3.7 within three days of incubation and it remains in lower state for many days of incubation, indicating the production of strong acids. Solubilization in presence of other carbon and nitrogen sources was not accompanied with such decline in pH. Glucose, xylose and galactose decreased the pH of the medium to maximum extent and caused higher solubilization of phosphorus. Acid phosphatase, alkaline phosphatase and phytase enzyme

production was found to be higher in carbon sources that showed maximum P solubilization. This showed that organic acid production, acid phosphatase, alkaline phosphatase and phytase enzymes are the mechanism for the P-solubilization. Results showed that nature of carbon sources in growth medium significantly affect the organic acid production, acid phosphatase, alkaline phosphatase and phytase enzymes production that directly affect the P-solubilization. The role of carbon sources is important in P-solubilization, as the production of acids which is common mechanism of P-solubilization (Di Simone 1998), was affected by the carbon sources. The nature of acid produced is more important than the quantity of the acid (Agnihotri 1970). The solubilization activity of microorganism is related to its organic acid production; however the nature of the organic acid produced is also important (Vassileva et al. 1998). The potential mechanism for phosphate solubilization might be acidification either by proton extrusion associated with ammonium assimilation (De Freitas et al. 1997; Reyes et al. 1999) or by organic acid production (Cunningham and Kuyack 1992). Acid phosphatase and phytases secreted by microorganisms also have an important role in phosphate solubilization (Richardson et al. 2000).

Singal et al (1994) found that in case of *Aspergillus japonicus* and *A. fetidus*, phosphate solubilizing activity was highest after 48 hours in presence of glucose and ammonium sulphate as carbon and nitrogen sources. Glucose produced the greatest increase in total soluble phosphate. *Pseudomonas lurida* showed maximum P-solubilization at 10 °C with glucose and ammonium sulphate in TCP containing NBRIP media (Pallavi and Gupta 2013). Glucose was found to be best carbon source followed by sucrose and galactose for phosphate solubilization by *Pseudomonas striata* (Gaur 1990). Narsian and Patel (2000) reported maximum P solubilization by *Aspergillus aculeatus* with arabinose and glucose. The effect of inorganic and organic nitrogen sources on P-solubilization activity of *Schwannomyces occidentalis* with RP was tested and found that ammonium sulphate exhibiting maximum

activity followed by sodium nitrate (Gaur 1990). Ammonium chloride, potassium nitrate, sodium nitrate, and urea were observed to be inferior to the ammonium sulphate when used as nitrogen sources for solubilization of TCP and RP by *Enterobacter aerogenes* (Thakker et al. 1993). A number of bacteria had been reported of being able to solubilize phosphate only in presence of ammonium as the nitrogen source (Illmer and Schinner 1992; Lapeyrie et al. 1991). The nitrogen source in salt form seems to be important, as it was necessary for better solubilization of rock phosphate (Asea 1988).

Effect of carbon and nitrogen sources on P-solubilization by fungal isolates

In the case of fungal isolates (*A. tubingensis* and *A. niger*) glucose as a carbon source and $(\text{NH}_4)_2\text{SO}_4$ as a nitrogen source were found to be the best in solubilization of P and reduction of the medium pH compared to other carbon and nitrogen sources tested. Maximum P solubilization was accompanied by reduction in pH, production of acid, alkaline phosphatase and phytase enzymes and organic acids in growth medium. Oxalic acid, citric acid, malic acid and gluconic acids were significantly produced in growth medium having glucose and ammonium sulphate as carbon and nitrogen source. Results showed that the nature of carbon and nitrogen sources in media significantly affect the fungal P-solubilization, acid phosphatase, alkaline phosphatase, phytase enzymes and organic acid production in growth medium. Previous reports on phosphate-solubilizing microorganisms (Lapeyrie 1991; Carlile and Watkinson 1994) have attributed the differences in phosphate solubilization (when ammonium and nitrate were used) to the use of different mechanisms for the generation of acidity in the culture. The overall results of the study showed that acid production is not only the reason for P solubilization in the medium. Acid phosphatase, alkaline phosphatase and phytase enzyme production was also significantly taking part in P solubilization. This finding was in agreement with data obtained from earlier reports (Abd Alla 1994; Whitelaw 2000). Pardhan and Sukla (2005) also suggested that in case of *Aspergillus* sp. glucose as a carbon

source and ammonium sulphate as a nitrogen source increases the solubilization of phosphorus. The study of phosphate solubilization at different carbon and nitrogen source revealed that all isolates showed maximum phosphate solubilization in presence of glucose and ammonium sulphate (Jadhav 2013).

Four mechanisms of solubilization were reported in fungi: (i) acidolysis, (ii) complexolysis, (iii) redoxolysis, and (iv) the mycelium functioning as a sink (Burgstaller and Schinner 1993). The first two mechanisms occur as a result of the efflux of protons from hyphae, the production of siderophores (for iron) and the production of organic acids (Gadd 2000). Generally, fungi acidify their nutrient medium during growth, although this can depend on the nitrogen source (Illmer and Schinner 1995; Whitelaw et al. 1999). Acidification can result from four main processes: (i) excretion of protons via proton-translocating plasma membrane ATPase; (ii) uptake of nutrients in exchange for protons; (iii) excretion of organic acids; and (iv) acidification through carbon dioxide produced by fungal respiratory activity (Burgstaller and Schinner 1993). The production of organic acids in turn provides a source of protons for solubilization (Gadd 1999). Amongst above mentioned mechanisms for phosphate solubilization, the most recognized one is through the production of organic acids (Reyes et al. 2006). The production and release of organic acids (mainly citric acid, oxalic acid, malic acid and gluconic acid) attribute to ion chelation and solubilization of inorganic P sources (Cunningham and Kuiack 1992; Reyes et al. 2006). The nature and amount of organic acids excreted by fungi are mainly influenced by medium pH and buffering capacity, carbon source and the balance of nitrogen and phosphate (Reyes et al. 1999).

Potential mechanisms for explaining mineral phosphate solubilization (MPS) activity point to acidification either by proton extrusion associated with ammonium assimilation (Roos and Luckner 1984), or by organic acid production (Cunningham and Kuiack 1992). MPS activity is usually measured by using glucose (Asea et al. 1988; Kucey 1983) as the sole carbon

source. Furthermore, in most studies, ammonium was found to be a better N source than nitrate (Asea et al. 1988; Wenzel et al. 1994).

Whitelaw et al (1999), Pradhan and Sukla (2005) and Asea et al (1988) found a higher P solubilization from ammonium assimilation by *Penicillium*. The results show higher organic acid production and higher pH reduction in ammonium sulphate as compared to other N sources. The reduction of pH indicates the possibility of the operation of NH_4^+/H^+ exchange mechanism acidifying the medium, as reported by Roos and Luckner (1984). Hence, acidification due to NH_4^+ is more evident rather than NO_3^- because the acidification of the medium is a result of H^+ efflux from hyphae during NH_4^+ uptake (Jacobs et al. 2002a). Ammonium sulfate or ammonium nitrate has been used as a nitrogen source for organic acid production on large scale. Cerezine et al (1988) reported that ammonical sources increased the solubilization of fluorapatite by *A. niger* more than organic sources of N. Physiologically, ammonium compounds are preferred since their consumption lowers the pH of the medium which is an additional prerequisite of organic acid production. In all the cases, phytase activity was more than phosphatase activity as reported by Aseri et al (2009) that fungi executes extracellular phytase activity many times more than extracellular phosphatase activity.

Plant growth promotion activities of bacterial and fungal isolates

It is desirable that P-solubilizers have additional plant growth promoting properties like IAA and siderophore production ability. Root colonizing bacteria (rhizobacteria) that exert beneficial effect on plant development *via* direct or indirect mechanisms have been defined as plant growth promoting rhizobacteria (PGPR) (Nelson 2004). PGPR enhance plant productivity by a range of direct/indirect mechanisms. In addition to P-solubilization, phosphate-solubilizing microorganisms (PSMs) may also improve the plant productivity by

producing other secondary metabolites like indole acetic acid (IAA) (Nylund et al. 1994) and siderophores (Hariprasad and Niranjana 2009). Direct promotion of growth by plant growth promoting microorganisms occurs when rhizobacteria and rhizofungi produce metabolites that promote plant growth such as auxins as well as through the solubilization of phosphate minerals (De Freitas et al. 1997). Indirect growth promotion occurs through the elimination of pathogens by the production of siderophores. Siderophore and IAA production by *Pseudomonas chlororaphis* and *Bacillus subtilis* play an important role in biocontrol of *Phythium aphanidermatum* inciting damping off in tomato (Kavitha et al. 2003). The present study clearly revealed that both the bacterial and fungal isolates had the ability to produce IAA and consequently, considered as IAA producing bacteria and fungi. Production of IAA, even without addition of precursor indicated that bacteria and fungi were actively involved in the synthesis of IAA in pure culture. *P. cyripedii* showed higher production of IAA as compared to *P. plecoglossicida* both in absence and presence of tryptophan, but production was more comparable when tryptophan was used. Production of IAA by *Pseudomonas fluorescens* RAF15 was observed only in presence of L- tryptophan (Park et al. 2009). Contrary to this, we observed that both the isolates were able to produce IAA without tryptophan also. In case of *P. cyripedii* there was 12-fold increases of IAA production and in case of *P. plecoglossicida*, it was 4-fold when L-tryptophan (TRP) was added to culture medium, in comparison with culture growing without tryptophan. In case of fungal isolates (*A. tubingensis* and *A. niger*) IAA production was observed with and without addition of tryptophan in the growth medium but the amount of IAA produced was higher in presence of tryptophan compared to without tryptophan. PSM cultures release a maximum quantity of IAA in presence of physiological precursor, tryptophan in a culture medium (Ponmurugan and Gopi 2006). *Aspergillus niger* ($85 \mu\text{g ml}^{-1}$) and *Trichoderma harzianum* ($68 \mu\text{g ml}^{-1}$) was showed more significant IAA production at three days of incubation, when these fungi were

grown in synthetic Czapek-Dox broth amended with 1000 $\mu\text{g ml}^{-1}$ L-tryptophan (Yadav et al. 2011). We also recorded similar reports of IAA production in *Aspergillus niger* and were shown by Bilkay et al (2010); Gunasekaran (1978) and Hasan (2002).

Culture supernatant of fungal and bacterial isolates by the addition of 2 % of aqueous FeCl_3 solution showed λ_{max} in between 420-430 nm, indicating the presence of hydroxamate-type of siderophores. Howell et al (1988) and Jagadeesh et al (2001) suggested that some of *Pseudomonas* sp. act as antagonist, inhibiting growth of pathogens through production of siderophores. The deficit of available iron to pathogens might have resulted in death of the pathogenic microorganisms. Jagadeesh et al (2006) suggested that rhizobacteria *Bacillus* sp. suppress the deleterious bacteria and subsequently improve the germination and growth of tomato seedlings. Microbial siderophores are either hydroxamates, catecholates, carboxylates or mixed types. Hydroxamates are produced by bacteria and fungi, catecholates only by bacteria, carboxylates are produced by a few bacteria (*Rhizobium meliloti* and *Staphylococcus hyicus*) (Drechsel et al. 1991) and exclusively by fungi belonging to Mucorales.

Effect of buffering of media on P-solubilization by bacterial and fungal isolates

Soil inoculation with PSMs has been shown to improve solubilization of fixed soil P and applied P resulting in higher crop yields. PSM are found in majority of soils (Subba Rao 1982). However there, performance is severely influence by environmental factors especially under stress condition (Yahya and Al-Azawi 1989; Pal 1998). Microorganisms growing in alkaline soils in India are subjected to high pH steess. These alkaline conditions may results in poor growth and survival of PSM (bacteria and fungi). The available P in these soils is poor, and the most appropriate solution to this situation is to use PSMs as bio-inoculants. However, detailed studies have not been made on PSMs isolated from alkali soils. The decrease in pH clearly indicates the production of acids, which is considered to be

responsible for P-solubilization. It has been suggested that microorganisms which decrease the medium pH during growth are efficient P-solubilizers (Halder et al 1991).

In the present study, P-solubilization by phosphate-solubilizing bacteria (*P. cypridii* and *P. plecoglossicida*) and fungi (*A. tubingensis* and *A. niger*) tested in buffered medium of pH 7.0, 8.0 and 9.0 adjusted with 0.1 M Tris-HCl. Both bacterial and fungal isolates have the ability to solubilize insoluble form of P in presence of such alkaline pH conditions. All selected strains demonstrated a significant increase in P-solubilization with reduction of pH of the medium. All isolates were able to produce acid phosphatase, alkaline phosphatase and phytase enzyme in buffered medium and it was comparable to the enzyme production in normal and un-buffered broth.

P-solubilization ability of microorganisms in soil may be different from that found under laboratory conditions (Gyaneshwar et al 1998). The buffering capacity of soils could limit solubilization of soil phosphates by microorganisms as it has been shown that solubilization of Ca-P complexes are mediated mainly by the lowering the pH of the medium (Maliha et al. 2004). Hence, present study was undertaken to evaluate the effect of buffers in microbial P-solubilization. Our results showed that phosphate-solubilizing microorganisms were able to reduce the pH of the medium in presence of supplemented buffers. The results are in agreement with the earlier observations of Gyaneshwar et al (2002) who suggested that plant growth was limited by availability of P despite the abundance of PSMs in the rhizosphere due to buffering. The reduction of P solubility has been shown to be due to high buffering capacity of soils and reduced or loss of P solubilizing efficiency of bacteria under buffered conditions. Drop in pH in un-buffered conditions is comparable to the drop in pH of media in buffered conditions. The results indicate that buffering of media did not reduce the effectiveness of the PSMs in releasing P from tri-calcium phosphates. In conclusion, the effectiveness of the P-solubilizing bacterial and fungal isolates used in present study were not

affected with buffered conditions and screening of PSMs using buffered media conditions may lead to the selection of more effective PSMs in alkaline soils.

Inoculation of PSMs into soils has been shown to increase the population of PSMs in the rhizosphere but only a few studies show consistent enhancement of phosphorus uptake by plants as well as plant growth (Subba Rao 1982; Kucey et al. 1989). The variations in growth enhancement are attributed to the differences in the composition and properties of soils, the nature and distribution of soil microflora, and the type of crop (Kucey et al. 1989). It has also been suggested that, PSMs present in the rhizosphere of many plants are not effective because their P-solubilization ability could be limited by the availability of carbon and nitrogen sources in the soil conditions. Alternatively, the inconsistency in growth enhancement of inoculated plants could arise due to the inability of some PSMs to release P from soils. Such a view can be considered if P solubilization ability of microorganisms in soils is different from that found under laboratory conditions. This proposition is supported by several observations. Most of the PSMs have been isolated using un-buffered conditions (Kucey et al. 1989) whereas soils rich in Ca-P complexes have a very strong buffering capacity (Ae et al. 1991). The buffering capacity of soils could limit solubilization of soil phosphates by microorganisms as it has been shown that solubilization of Ca-P complexes is mediated mainly by lowering the pH of the medium (Sperber 1957; Kucey et al. 1989; Halder and Chakrabartty 1993).

Field applications of Phosphate-solubilizing microorganisms in organic farming and at multilocal sites

Present study gives evidence that P-solubilizing microorganisms pose wide variety of functions which facilitate their implication in agriculture ecosystem to improve the crop yield and soil health. Soil microorganisms that solubilize mineral phosphates can significantly

affect P cycling in both natural and agricultural ecosystem. P-solubilizers along with RP fertilization seem crucial in improving soil characteristics and crop yield (Reyes et al. 1999).

To increase the availability of phosphorus for plants, large amount of fertilizers are used on regular basis. But after applications, a large proportion of fertilizer phosphorus is quickly transferred to the insoluble form (Omar 1998). Therefore, very little percentage of the applied phosphorus is used, making continuous application necessary (Abd Alla 1994). These beneficial, free-living bacteria enhance emergence, colonize roots, stimulate growth and enhance yield (Rai et al. 2006). Agricultural activities such as tillage (Sharma et al. 2012), intercropping, rotation (Guo et al. 2005) and bio-fertilizers (Himani and Reddy 2012) have significant implications for the microorganisms present in soil. PGPR are bioresources which may be viewed as a novel and potential tool for providing substantial benefits to the agriculture.

- **P-solubilizing microorganisms in organic farming**

The results obtained *in vitro* cannot always be dependably reproduced under field conditions. Further, evaluation of selected isolates on soil-plant system was done to uncover their efficacy as effective plant growth promoting microorganisms. In the present study, two P-solubilizing bacteria *P. cypripedii* and *P. plecoglossicida* and two P-solubilizing fungi *A. tubingensis* and *A. niger* isolated from an organic farm, were used as bio-inoculants in a two year of field study in organic farming. We are reporting for the first time on improvement in growth and yield of maize and wheat crop in an organic farming. There is no report present on characterization of phosphate solubilizing microorganisms from organic farming and their effect as bio-inoculants alone or along with RP fertilization on crop yield and soil fertility of organic farming system.

In the present study a significant increase was found in biometric parameters (shoot height, shoot and root dry biomass) of maize and wheat plants after treatment with PSMs (bacteria and fungi). Inoculation of plant growth promoting microorganisms showed improvement in growth and yield of different crops (Reddy and Rahe 1989; Pandey et al. 1999; Pandey et al. 2006; Kumar et al. 2012; Sharma et al. 2012; Zhang et al. 2004). Seed inoculation with *Bacillus spp.* improves seedling vigour in oil-seed plant *Jatropha curcas* L (Desai et al. 2007). The effect was more pronounced when PSMs inoculation was done along with rock phosphate fertilization. PSMs inoculation along with rock phosphate fertilization showed a stimulatory effect on P uptake of plants. Field study found a significant increase in total P uptake in plant and grain yield after inoculation of bacterial and fungal isolates in respective plots compared to un-inoculated control treatments. Bio-fertilizer inoculation in wheat lead to 15-25 % higher yields when compared to plants grown without the applications of biofertilizers (Sharma and Adholeya 2007).

Bio-inoculations resulted in significantly higher values for phosphorus content of plant components (Pandey et al. 1998). This might be due to better utilization of P from the pool of soil nutrients by the action of P-solubilizing microorganisms (Mamta et al. 2010). Total P level was more pronounced in phosphate-solubilizers inoculation along with rock phosphate fertilization. The highest increase in total P content in plant shoots, roots and grains was observed in the treatments showing highest available P in soil. These results suggested that a subsequent crop will reap the benefits imparted by PSMs to the soil in terms of available P content, physical and biological characteristics of the soil (Mittal et al. 2008). Results showed that, RP amendment along with inoculations had promising positive effects on the yield of maize and wheat crops compared to the control treatments.

Rhizosphere colonization by microbial inoculants has been described as a crucial factor for plant growth promotion (Lugtenberg 2001). Results of the present study are also in agreement

with this statement. Several studies indicated that seed or soil inoculation with PSM improves solubilization of fixed soil phosphorus, and applied phosphates, resulting in higher crop yield (Richardson 2001). Hameeda et al (2008) reported maize growth promotion by inoculating two phosphate-solubilizing bacteria, *Serratia marcescens* and *Pseudomonas* sp. isolated from compost. Khalimi et al (2012) showed that *Pantoea agglomerans* effectively promoted the rice growth and increased the yield. Himani and Reddy (2011) showed that *A. tubingensis* and *A. niger* significantly improved the plant growth, yield and P uptake along with RP fertilization in maize and wheat crop. *Pseudomonas* sp. has a considerable potential in phosphorus uptake efficiency. Due to the ecotype diversity of this species and its tolerance in some environmental stresses, this bacterium is of special importance as a biological fertilizer (Kim et al. 1989; Tilak et al. 1995).

Stimulatory effect of PSMs on growth, yield and nutrient uptake in maize and wheat crop can be correlated with increased population of PSMs in rhizospheric soil by PSMs inoculation along with RP fertilization. This may be subjected to the increased population of PSMs and possibly also the indirect increment in total native microbial populations resulting from altered root exudation. Addition of RP to the inoculated treatment raised the total soil population of PSBs and PSFs in their respective plots, suggesting that RP acts as a substrate for the tested PSMs, and is obviously beneficial for proliferation and survival of these isolates. Similar results were also reported by Yu et al (2011) with TCP amendments.

It was apparent from the results that various treatments had an inherent potential for the improvement of soil properties especially related to organic carbon and available P that directly affect the plant growth, yield and nutrient uptake. Maximum amount of soluble P was observed in the soil with P-solubilizers (bacterial and fungal) along with rock phosphate fertilization treatment. The organic carbon level was significantly increased in all the treatments in comparison to the initial values. Our results are in agreement with Himani and

Reddy (2011) who reported an increase in soil organic carbon level in bio-inoculated and RP fertilized soil compared to control soil. Increased in organic carbon in seed inoculated and RP amended treatments may be due to increase in phosphate-solubilizing bacterial and fungal population in respective plots compared to control soil. Changes in soil organic carbon contents directly associated with changes in microbial biomass and biological activity in soil (Nahro and Dkhar 2010). Little decrease in soil pH was observed in all the treatments compared to control soil. Inorganic P is solubilized by the action of organic and inorganic acids secreted by P-solubilizing microorganisms in which hydroxyl and carboxyl groups of acids chelate cations and decreases the pH in basic soils and increases the concentration of available phosphorus in soil (Stevenson 2005). This may be attributed to the ability of such microorganisms to excrete organic acids, there by decrease the pH, and increase the concentration of soluble phosphorus in soil by mechanisms involving chelation and exchange reactions (Vassilev et al. 1996). The decrease in soil pH in PSM treatments indicated the production of organic acids by selected isolates as also reported for phosphate solubilizing *A. niger* and *A. tubingensis* (Richa et al. 2007). However, less pH reduction in soil during plant growth promotion experiments than P-solubilization in culture medium could be due to the buffering nature of soil (Gyaneshwar et al. 1998).

Soil enzymes have been suggested as potential indicators of soil quality because of their relationship to soil biology, ease of measurement, and rapid response to changes in soil management (Dick et al. 1996). Inorganic P is released from organic matter by hydrolysis of C-O-P ester bonds by phosphatases, which are therefore important in the P nutrition of plants. Dehydrogenases represent a class of enzymes that give us information about the influence of natural environmental conditions on microbial activities of the soil (Schaffer 1993). Activities of enzymes such as dehydrogenase, acid phosphatase, alkaline phosphatase and phytase in all the treatments were higher than the control soil. In all the treatments, phytase activity was

observed to be more than phosphatase activity. This may be due to the higher extracellular phytase enzyme activity of bacterial isolates as compared to extracellular phosphatase enzyme activity. Similar results were reported by Aseri et al (2009) that microbes execute extracellular phytase activity many times more than extracellular phosphatase activity. The potential role of soil microorganisms for increasing the amount of available P from phytase activity has been reported (Richardson 2001). The alkaline phosphatase and phytase activities were slightly decreased after wheat harvest as compared to the initial values. This may be due to the slight reduction in soil pH, a fact which has been also observed by Richardson et al (2005). Higher enzyme activities in soils indicated the potential of soil to affect the biochemical transformations necessary for the maintenance of soil fertility (Rao et al. 1990). The increase of enzymatic activities in soils is involved in an increase in the availability of nutrients to plants, which in turn have a positive influence on soil fertility (Garcia et al. 1997). It is widely accepted that soil enzyme activities are highly sensitive biochemical parameters indicating perturbations caused by soil treatments (Naseby and Lynch 1997). They give an indication of ecosystem function rather than just a measurement of perturbation. Increase in enzyme activities may be related mainly to reactivation of the rhizosphere microbial population due to addition of rock phosphate in combination or not with inoculation treatments.

- **Comparison with DAP treatments**

When we compare the RP fertilization and bio-inoculation treatment with DAP treatments it was observed that bio-inoculation treatments alone or along with RP fertilization have more pronounced effect in improvement of maize and wheat crop yield in two year of field study compared to DAP treatments.

Bio-inoculation and rock phosphate fertilizer applications markedly improve the total biomass, grain yield and total P uptake of maize and wheat crop compared to control soil. Results of bio-inoculation treatment in the presents study are comparable with the earlier findings of inoculation trials (Vasudevan et al. 2002; Pandey et al. 2006; Sharma and Adholeya 2004; Varshney et al. 2002; Klopper et al. 2004) where plant growth and yield was significantly improved and correlated with the stimulation effect of introduced microbes as bio-inoculants in soil compared to control. Results of grain yield of maize and wheat crop showed that all the treatments had significant effect but the inoculation treatments along with RP fertilization showed more pronounced effects than DAP treatments and control treatments. In the field conditions, when PSMs were combined with rock phosphate, yield was more greatly increased. These results are in line with those obtained by Akabari et al (2010) who found that application of RP with PSBs enriched the rhizosphere more than the other treatments. Results showed that highest yield was obtained by bio-inoculation treatments alone or along with RP fertilization. Maize and wheat yield increased by application of PSMs along with RP fertilization compared to single RP fertilization, DAP and control treatments. Pandey et al (1998) suggested that inoculation of *Azotobacter chroococcum* significantly improve the wheat yield up to 1.5 folds over control. Medina and Probanza (2003) also found that PSBs have higher efficiency than chemical phosphate fertilizers in crop production.

Addition of bio-inoculants along with RP fertilization significantly increased P uptake in comparison with RP fertilization alone, chemical fertilizer and inoculation of bio-inoculants alone. Swarnalakshmi et al (2013) also reported that addition of inoculants along with RP significantly increased P uptake in comparison with chemical fertilizers or individual inoculation of bio-inoculants in wheat. Sundara et al (2002) found that application of PSBs combine with RP is more effective than phosphorus fertilizer. Increased yield and total P

uptakes of plants fertilized with bio-fertilizers indicate that this technique has potential as a low- cost alternative to expensive soluble fertilizers.

A variable response in terms of available P content in bio-inoculation treatments alone and along with RP fertilization and DAP treatments was observed. Among the treatments involving PSMs inoculations along with RP fertilization, a marked increase in available P content was observed in both maize and wheat rhizospheric soil. Microbial inoculants are promising components for integrated solution to agro-environmental problems because inoculants possess the capacity to promote plant growth, enhance nutrient availability and uptake, and support the health of plants (Kloepper et al. 2004; Han and Lee 2005; Weller 2007; Adesemoye et al. 2008). Because the fertilizer value of hardly soluble rock phosphate was substantially increased by the exogenous introduction of PSMs replacing the costly chemical fertilizer, the low cost eco-technology engineered through specific P-fertilizers responsible for solubilization of rock phosphate is of considerable economic importance in the developing countries (Sahu and Jana 2000).

The beneficial effect of inoculation of PSMs may be direct, due to an increased supply of available P or indirect through changes in the growth rate and metabolic activities of crop. Results showed that yield, organic carbon level, available P, soil enzyme activities and population density of selected phosphate-solubilizing bacteria and fungi was significantly increased in their respective plots with inoculation alone or along with RP fertilization compared to DAP treatments. Rhizosphere and soil bacteria are important drivers in nearly all biochemical cycles in terrestrial ecosystems and participate in maintaining health and productivity of soil in agriculturally managed systems (Fernando and Ru 2011). DAP treatments showed lowest increase in improvement of soil physiochemical properties like available P, organic carbon and soil enzyme activities. Several plant growth promoting rhizobacteria have been reported as important bio-inoculants due to their multiple bio-

fertilizing activities of improving soil nutrient status, secretion of plant growth regulators, and suppression of soil-borne pathogens (Rodriguez and Fraga 1999; Vyas et al. 2009; kumar et al. 2011b).

Microorganisms can enhance the capacity of plants to acquire P from soil through various mechanisms that can be summarized as: (1) increased root growth through hormonal stimulation of root growth, branching, or root hair development (phytostimulation; e.g. production of indole-3-acetic acid) (Richardson et al. 2009; Hayat et al. 2010); (2) alteration of sorption equilibria that may result in increased net transfer of orthophosphate ions into soil solution or facilitate the mobility of organic P either directly or indirectly through microbial turnover (Seeling and Zasoski 1993); and (3) through induction of metabolic processes that are effective in directly solubilizing and mineralizing P from sparingly available forms of soil inorganic and organic P (Richardson et al. 2009). This includes the efflux of protons and organic anions, production of siderophores, and release of phosphatase enzymes required for the hydrolysis of organic P or mineralization of organic residues and organic matter, respectively. Organic anions and protons are particularly effective in solubilizing precipitated forms of P (e.g. Ca phosphates under alkaline conditions), chelating metal ions that are commonly associated with complex forms of soil P (as is for the role of siderophores in mediating Fe availability), or by facilitating the release of adsorbed orthophosphate or organic P through ligand-exchange reactions (Ryan et al. 2001). Han and Lee (2005) also suggested that inoculation of PSB in conjunction with direct application of rock phosphate into the soil increased P uptake and yield of plants grown on P limited soils.

- **Effect of phosphate solubilizing microorganisms at multilocational sites**

The potential of PSMs isolated from rhizospheric soil of organic farming in contrast to conventional farming, in promoting the crop production and soil fertility at different sites is a

promising area of research. Soil is an unpredictable environment and an intended result is sometimes difficult to obtain (Bashan 1998). The plausible mechanisms adopted by these rhizobacteria in growth promotion, though abundantly documented but still remains to be fully explored. However, their use has not been to the full potential due to inconsistency in their performance and their commercialization limited to few developed countries (Rai et al. 2006). Potential of bacterial inoculums may be determined in a single experiment, but the consistence in performance can only be determined in multiple trials (Kloepper et al. 1989). *Pantoea eucalypti* (Castagno et al. 2011) for *in vitro* plant growth promotion activities, *P. correogata* (Pandey and Palni 1998; Pandey et al. 2004) prevalent in the subtropical and temperate soils and *P. putide* (Pandey et al. 2006) developed as a suitable bio-inoculants for use in higher altitude regions in the mountain, has been examined earlier for plant growth promotion and developed as a carrier based formulation for field application. The isolates in the present study *P. cyripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* isolated from organic farming system seem to be another important isolates that could be developed as suitable bio-inoculants for use in organic farming and at multilocational sites along with RP fertilization.

P. cyripedii, *P. plecoglossicida*, *A. tubingensis* and *A. niger* were first time reported in our present study for their significant effect on improvement of crop productivity and soil fertility of maize and wheat crop fields at multilocational sites of different agroclimatic regions. During the two year of field study of at different sites, it was observed that, the grain yield, total P uptake and rhizospheric soil properties (organic carbon, total P, available P and enzyme activities) in maize and wheat crop was significantly improved in all the treatment. The improvement is more with bio-inoculation along with RP fertilization treatments compared to other treatments. Two year field study of maize and wheat crop showed that considerable crop yield and soil fertility was improved when *P. cyripedii*, *P.*

plecoglossicida, *A. tubingensis* and *A. niger* were applied as bio-inoculants along with RP fertilization at different sites under different agroclimatic regions. This indicated that all the test strains were equally effective in all different sites that are different in their agroclimatic conditions. A very few reports are available on the effect of PSMs as a plant growth promoter and for improvement of soil fertility at different agroclimatic regions. Cakmakci et al (2006) investigated the effectiveness of PGPR in sugar beet at two soils organic matter contents in field and showed that *Bacillus megaterium* and *Pseudomonas putida* has positive effects on growth and sugar yield. The plant growth promotion ability of PGPR inoculations varied with soil organic matter content. Free living microorganisms depends on organic carbon content as a food source, addition of organic matter to the soil will enhance the organic carbon content in soil may be increased the plant growth promoting activity of PGPR (Cakmakci et al. 2006). Chabot et al (1996) reported that, two strains of *Rhizobium leguminosarum* bv. *Phaseoli* solubilizing soil P stimulates the growth of lettuce and maize at three sites having different high to low levels of available P. However, no reports are available on the effect of phosphate-solubilizing bacteria and fungi along with RP fertilization that showed their effect as a plant growth promoter at different sites comes under different agroclimatic regions with different soil physiochemical properties. Results of present study revealed that *P. cyripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* used as bio-inoculants during two years of field study have the ability to adapt to different agroclimatic conditions and equally improved the yield and soil fertility in different sites. An organism with properties of plant growth promotions such as phosphate solubilization (Pandey et al. 2008; Pandey et al. 1999), IAA production, siderophore production (Nylund et al. 1994; Jagadeesh et al. 2006), as well as compatibility with climatic conditions (Desai et al. 2007; Nakkeeran 2005; Kumar et al. 2011a) would seem ideal for selection as suitable bio-inoculant. Fungi have been reported to possess greater ability to solubilize rock phosphate than bacteria (Nahas 1996). The use of

rock phosphate as phosphate fertilizers and its solubilization by bacteria and fungi (Kang et al. 2002), through production of organic acids (Maliha et al. 2004), have become a valid alternative to chemical fertilizers. Rock phosphates are widely distributed throughout the world, both geographically and geologically (Zapata and Roy 2004). In conjugation with PSMs, rock phosphate provides a cheap source of P fertilizer for crop production. In this regard, several studies have conclusively shown that PSM solubilizes the fixed soil P and applied phosphates, resulting in higher crop yields (Zaidi 1999; Gull 2004).

Presently, rock phosphate is being chiefly employed to sustain soil P levels in an available form for plants. In this context, PSM have been reported to solubilize the rock phosphate through the production of organic acids, ion chelation and exchange reaction in the growth environment (Yadav and Dadarwal 1997). As a result of this activity, PSM play an important role in supplementing P to the plants, allowing a sustainable use of phosphatic fertilizers. In the present study, addition of rock phosphate along with microbial cultures greatly enhanced the plant growth and nutrient uptake in maize and wheat crop. It is generally thought that PSM in addition to solubilizing inorganic P also release growth-promoting substances (Kucey et al. 1989), which improve the germination and growth of plants and stimulate microbial activity in the rhizosphere. The present study clearly indicated that when rock phosphate fertilization was done along with bio-inoculation, then insoluble form of RP is transformed into available forms of P. Plant growth promotion activities in field conditions were more pronounce in fungal bio-inoculum compared to bacterial inoculation. That might be due to because P releasing fungi produce more organic acids (Venkateswarlu et al. 1984) than do bacteria, which enhance the solubilization of phosphates.

Present study concluded that *P. cyripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* can be used as bio-inoculants along with RP fertilization at multilocational sites as they showed consistent performance at multilocational sites comes under different agroclimatic

regions. Chemical fertilizers might be therefore, substituted by cheaply available RP and P-solubilizing microorganisms. These results ensure the long term fertility of the soil in relation to P and would provide a cost effective, sustainable and environmental friendly production system for crops.

Development of inoculum formulations of PSMs

The beneficial effect of these rhizobacterial populations can be optimally harvested only when carrier-based formulations that are amenable for field level applications are developed and made available to the clientele (Nakeeran et al. 2005). It is not possible to introduce the bacteria and fungi to the soil directly or as suspension because it has many problems. So, it's basically done by a carrier material. Bacterial and fungal inoculum is applied to solid, semisolid or liquid material that is capable to maintain certain population of bacteria in acceptable number during specified time and be a good means for supplying bacteria for the seed surface or rhizosphere (Shariati et al. 2013). Before recommended an organism as bio-inoculants to crop production, its shelf life in different carrier materials needs to be addressed. The success of these carrier-based formulations largely depends on the bacterial strain used for inoculant production. While *Pseudomonas fluorescens*, *P. putida*, *P. aeruginosa*, *Bacillus subtilis* and *Bacillus spp.* are commonly used for commercial inoculant production, the vast plethora of soil bacteria are yet to be explored for their beneficial interactions with plants (Selvakumar et al. 2009).

Till now, there is no report on survivability of *P. cyripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* in carrier materials. The most important factor which contributes to the high quality inoculant is the presence of viable cells. Therefore, the evaluation of inoculants quality by enumerating the viable cell count is considered as an accurate index of the inoculants potential (Hiltbold et al. 1980).

Inoculum formulations of *P. cypripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* were developed, where in, rock phosphate, fly ash, charcoal and vermiculites were used as a carrier materials. Efforts were made to test compatibility (in context to sustains the viability) of these carrier materials with selected P-solubilizing bacterial and fungal isolates at different temperatures for long storage period. After fifteen days of incubation, the viability of inoculum at 37 °C was slightly increased and after that it was started decreased in both bacterial and fungal isolates in all carrier materials tested. Similar upward trend in viable count of phosphobacterial population in sterilized fly ash formulation was observed by Gaind and Gaur (2004) during initial period of incubation followed by a steady decline in inoculum after long time of incubation. Menaka and Alagawadi (2007) also suggested that in general all the treatments showed increase in population of PSB up to 30 days, latter a downward trend was observed in the survival of PSB with storage period. Similar results on increase in the population upto 30 days and gradual decrease thereafter with increase in incubation period have been reported in case of *Bacillus megaterium* (Dhami et al. 2013) and in *Pseudomonas striata* (Gaur 1990). Mendez and Videira (2005) stated that bacterial maintenance at 28 °C for 41 days caused an increase in number of viable bacterial cells on all carriers so that the population reached nearly 10^9 cfu per gram of carrier. This increase in viability may be due to the presence of small amount of organic carbon and some nutrients in the carrier materials (Gaind and Gaur 2004). All the carrier materials sustain the viability of both bacterial and fungal isolates up to 270 days of incubation at different temperatures. With increase in incubation period, the viability of bacterial and fungal isolates was decreased in all the carrier materials. A decline in population on prolonged incubation may be attributed to the depletion of nutrients, moisture and autolysis of cells (Gaind and Gaur 1990). Results showed that viability of bacterial and fungal isolates was higher at 4 °C compared to 37 °C after 270 days of incubation. The main reason for the population decline can be due to the

decline in organic matter in these materials, because when the bacteria are in growth conditions (15 days incubation), they go to stagnation phase and due to possible limitations of nutrient may encounter with shortage that result in reduced bacterial population. Also, declining in bacterial population could be attributed to drying process and toxins production in this temperature (Dearmon et al. 1962; Cigdem and Merih 2005).

Among the different carrier materials, rock phosphate sustains the maximum viability of bacterial and fungal inoculums compared to other carrier materials at 4 °C and 37 °C. A higher number of viable cells of *Burkholderia* was retained in clay, rice bran and rock phosphate than clay RP pellets (Anandham et al. 2007). Rock phosphate powder is rich in P, it improves the nutritional status of the carrier material and help in growth and multiplication of inoculated PSB. Rock phosphate, which although contains unavailable form of P, is solubilized by the inoculated PSB and utilized phosphorus for its growth and multiplication. Similar results were observed by (Menaka and Alagawadi 2007). P-solubilization activity, plant growth promotion activities, acid phosphatase enzyme, alkaline phosphatase enzyme and phytase enzymes production of 270 days old bacterial and fungal inoculum formulations were comparable to the fresh culture activities. Carrier material increases the survival rate of microbial inoculants by protecting it from desiccation and death of cells (Heijnen et al. 1993). The shelf life of microorganisms (bacteria and fungi) varies depending upon carrier materials and their particle size. The carriers with smaller particle size have increased surface area, which increase resistance to desiccation of bacteria by the increased coverage of microbial cells (Dandurand et al. 1994). The results showed that RP though the basic requirements of a carrier i.e. being cheap, easily available, favorable pH and compatibility with the native soil flora and can be used as carrier for the inoculum formulations of phosphate-solubilizing microorganisms.

Kandasamy and Prasad (1971) recommended the use of lignite as a carrier due to high carbon content, in contrast to vermiculite which contains very low organic matter and N content. The success of microbial inoculants depends on several factors, of which carrier material is the most important. The carrier refers to a solid, semisolid or liquid substance, which can sustain a given number of particular bacteria for a given period of time (Khavazi and Rejali 2000). One of the important properties of a carrier material is its ability to maintain higher population of inoculated organism over longer storage periods. The current tendency for a reduced use of agrochemicals and efficient application of natural materials in agroecosystems, a renewed interest in direct application of rock phosphate (RP) has arisen (Rajan et al. 1996). RP is theoretically the cheapest P fertilizer but most phosphate rock deposits found in the world are classified as low reactive and, therefore, direct application is not always effective without previous treatment.

Phosphorus is one of the most essential macro elements required for the growth and development of plants, deficiency of which restricts crop yields severely. An adequate supply of P is essential for the earliest stages of plant growth. Early season deficiencies of P can lead to restrictions on crop growth from which the plant will not recover, even when the P supply is increased to an adequate level at a later stage (Grant et al. 2005). In the acid-weathered soils of the tropics, subtropics and temperate regions, P is fixed by free oxides and hydroxides of aluminum and iron, while in alkaline soils it is fixed by calcium, causing a low efficiency of soluble P fertilizers and limits crop production in those soils (Rodríguez and Fraga 1999). Therefore phosphate-solubilizing bacteria have been used to enhance the solubilization of fixed P for crop nutrition (Nautiyal et al. 2000). In this powder formulation PSB is supplied along with RP, in which part of the RP is already solubilized. The product works on the principle that in rhizosphere soil, it subsequently disperses around the root zone, so there is a possibility to meet out the crop requirement of P nutrition especially at an early

stage and in later periods of crop growth with this powder inoculant. Also, it can be recommended for various crops grown in P-limited soils. This powder formulation serves a tripartite benefit as an excellent carrier for *P. cyripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger*, enhances the shelf life and also a promising source of soluble P to the crop. However, our study on bio-inoculation along with RRP fertilization in organic farming and at multilocal sites also showed that PSM along with RP fertilization can improve the crop yield and soil fertility hence showed positive response in agronomic performance.

Salient findings

- Efficient phosphate-solubilizing bacteria and fungi were isolated from rhizosphere of *Stevia rebaudiana* grown in organic farming at Pojewal, Punjab, India.
- On the basis of biochemical analysis and 16S rRNA gene sequencing, bacterial isolates were identified as *Pantoea cyripedii* (PSB-3) and *Pseudomonas plecoglossicida* (PSB-5).
- Based on morphological, microscopic and molecular characterization (ITS sequence analysis) fungal isolates were identified as *Aspergillus tubingensis* (PSF-4) and *Aspergillus niger* (PSF-5, PSF-6 and PSF-7).
- Both the bacterial and fungal isolates solubilize insoluble form of P (TCP and RP) into soluble form in PKV broth. All the bacterial and fungal isolates produced significant amount of organic acids such as gluconic acid, citric acid, oxalic acid, malic acid and acetic acid in growth medium. Acid phosphatase, alkaline phosphatase and phytase enzymes production was observed in growth medium by both fungal and bacterial isolates. A significant increase in soluble P was observed with decrease in pH of the growth medium. Production of organic acids and enzymes was higher in fungi compared to bacterial isolates.

- Maximum P-solubilization in both the bacterial isolates was observed with glucose as a carbon source and ammonium sulphate as a nitrogen source. Significant amount of oxalic acid, acetic acid and gluconic acid was produced in presence of glucose as carbon source and ammonium sulphate as a nitrogen source in growth medium.
- In case of fungal isolates, glucose and ammonium sulphate were the best carbon and nitrogen sources for the P-solubilization. Oxalic acid, citric acid, malic acid, succinic acid and gluconic acids were significantly produced in growth medium.
- Nature of carbon and nitrogen sources in growth medium also effects the production of acid phosphatase, alkaline phosphatase, phytase enzymes in case of both bacterial and fungal isolates.
- The mechanism of P-solubilization might be due to pH reduction, organic acid production, acid phosphatase, alkaline phosphatase and by phytase enzymes production in both bacterial and fungal isolates.
- All the selected bacterial and fungal isolates positive for indole acetic acid production and siderophore production.
- Bacterial and fungal isolates showed efficient P-solubilization in buffered media of pH 7, 8 and 9 maintained with Tris-HCl. P-solubilization activity in buffered medium of different pH was comparable to the normal broth without any buffering conditions.
- Two year field study of maize and wheat crop showed that bio-inoculation treatments of bacteria (*P. cyripedii* and *P. Plecoglossicida*) and fungi (*A. tubingensis* and *A. niger*) along with RP fertilization have more significant results in the improvement of crop yield and soil fertility in organic farming compared to other treatments.
- The results of the present study revealed that the use of phosphate-solubilizing bacteria and fungi along with RP fertilization is an efficient approach for the improvement of growth, yield, and nutrient uptake in maize and wheat crops and also

for the improvement in physiochemical properties of soil especially for the maintenance of phosphorus level in an organic farming. The inoculation of P-solubilizing bacteria and fungi along with RP fertilization significantly increase the growth and yield of maize and wheat crops over two years in an organic field. Uniqueness of this study lies in the fact that this is an early report on the RP-solubilization by *P. cyripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* isolated from an organic field and on their significant effect as bio-inoculants along with RP fertilization on improvement of crop yields and soil properties in organic farming.

- To check the consistency in performance of selected phosphate-solubilizing microorganisms (bacteria and fungi), field studies were performed at multilocational sites with and without rock phosphate fertilization for two years under different agroclimatic regions. This is an early report on *P. cyripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* (isolated from an organic field) for their significant effect as inoculant along with RP fertilization on improvement of yield and soil fertility at multilocational sites comes under different agroclimatic regions with different physiochemical properties of soil.
- Application of both bacterial and fungal isolates showed significant effects on maize and wheat grain yield and on soil fertility at all different agro-climatic regions. Grain yield, P uptake, soil organic carbon, available P and enzyme activities in all the experimental crop fields at different sites, significantly increased due to inoculation. Improvement in crop yield and soil fertility at three different sites was more pronounced and significant when RP was supplemented along with inoculation. Present study results suggest that *P. cyripedii*, *P. plecoglossicida*, *A. tubingensis* and

A. niger along with RP fertilization play an important role in plant growth promotion and facilitates improvement in soil fertility in different agro-climatic conditions.

- Present study clearly brings out that rock phosphate that was added once during the maize crop sowing was equally effective on second year wheat crop in which no RP fertilization was done to check the effect of previously (in maize crop) added RP on crop yield and soil fertility. Results showed that RP fertilization done during first year of field study was equally effective in agronomic performance in second year of field study compared to DAP fertilization that was done regularly before sowing of each crop. RP fertilization gave significant and more pronounced results during two year of field study compared to DAP fertilization treatments.
- Rock phosphate can be advantageously utilized in maize-wheat cropping when applied with PSMs inoculation, this practice gives good yields as compared to DAP and also enriches the soil more with available P. Inoculations along with RP fertilization gave better results than inoculation alone and DAP treatments. Therefore, this study concluded that the beneficial PSMs applied in combination with RP fertilization were a better choice for farmers to reduce the use of chemical fertilizers for sustainable crop production. Further, low quantity of RP is sufficient to meet the nutrient requirements of the crop. So the combine use of PSMs and RP fertilization treatments is more economical in terms of crop yield, and it is also a sustainable crop production technology. Thus this approach could be reduce over application of P fertilizer for the profit of farmers and ensure environmental friendly practices.
- It can be concluded from the present study that *P. cyripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* can be used as bio-inoculants along with RP fertilization in organic farming and in multilocational sites as they showed consistent performance at different agroclimatic regions. Chemical fertilizers might be therefore, substituted by

cheaply available RP and P-solubilizing microorganisms. In long term, this approach would ensure cost effective, sustainable and environmental friendly production system for maize and wheat crop at organic farming and at sites comes under different agroclimatic regions.

- Shelf life *P. cyripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* was tested in various carrier materials such as rock phosphate, fly ash, charcoal and vermiculites. Results showed that all the carrier materials tested support the viability of bacterial and fungal inoculum. RP sustains the maximum shelf life of both fungal and bacterial isolates at different temperature (4 °C and 37 °C) up to 270 days of storage compared to other carrier material tested. Potential of these isolates for P-solubilization was also retain in RP as carrier material even after 270 days of storage at different temperatures and was comparable with P-solubilization activities of fresh culture.

Summary

Summary

Phosphorus is an essential mineral fertilizer for plant growth and development. Soluble P is often the limiting mineral nutrient for biomass production in natural ecosystems. Plants utilize only a small fraction of the phosphate fertilizers applied to the soil and the rest is rapidly converted into insoluble complexes in the soil. The nutrient reservoirs in the soil shrink when crops are removed from the field at harvest. This nutrient export creates a P deficit, necessitating regular P addition to replace the harvested P. Several studies investigating whole farm P budgets have found nutrient P deficits in many organic forms and illustrate the need for nutrient additions. This leads to the need of frequent application of phosphate fertilizers. These fertilizers are expensive, and have some harmful impacts on the soil structure, composition, microflora and other specifications of soil. Chemical phosphate fertilizers use on a regular basis has become a costly affair and also environmentally undesirable. Increasingly high cost of chemical fertilizers has been the major stimulus to search for an alternative, naturally-occurring, dependable, biodegradable, phosphate source. Natural rock phosphates have been recognized as a valuable alternative for P fertilizers. In India, it is estimated that there are almost 260 million tons of rock phosphate deposits and this material should provide a cheap source of phosphate fertilizer for crop production. Unfortunately, rock phosphate is not readily available to the plants in soils with a pH > 5.5-6.0. Because of this, extension services are reluctant to be recommended and farmers are hesitant to utilize RP directly. One approach for solubilization of rock phosphate in field conditions is the application of phosphate-solubilizing microorganisms (PSMs). PSMs solubilize insoluble form of phosphates by acidification, chelation and exchange reactions and also by production of organic acids. This process not only compensates for higher cost of manufacturing fertilizers in industry; it also mobilizes the fertilizers applied to the soil. In addition to P-solubilization, phosphate-solubilizing microorganisms (PSMs) may also

improve the plant productivity by producing other secondary metabolites. There are several evidences related to plant growth promotion by PSMs through the production of indole acetic acid (IAA) and siderophores. Organic farming avoids the inputs of synthetic chemicals and their consequences. The build-up of a large and active soil microbial biomass is, therefore, critically important for sustaining the productivity of soils in organic farming systems. The use of PSMs with the aim of improving P nutrient availability for plants is an important practice and necessary for agriculture. However, the potential benefits of these PSMs are not fully realized because of the limitations like inconsistent performance at different sites. There is no doubt that bacterial inocula can increase the yield of various crops significantly, but the performance has generally been inconsistent. A key factor involved in the lack of success has been the rapid decline of the size of populations of active cells, to levels ineffective to achieve the objective, following introduction into soil. Potential of bacterial inoculums may be determined in a single experiment, but the consistent performance can only be determined in multiple trails. The PSMs occur in soil, usually their number are not high enough to compete with other microorganisms commonly established in the rhizosphere. Thus the amount of P liberated by them is generally not sufficient for a substantial increase in plant growth. Therefore, inoculation of plants by a target microorganism at a much higher concentration than the normally found in soil is necessary to take advantage of their phosphate solubilization for enhancement of crop yield and soil fertility. Before recommending an organism as bio-inoculants to crop production, its shelf life in different carrier materials needs to be addressed. Aim of present study was to isolate the phosphate-solubilizing microorganisms (bacteria and fungi) from organic farm and evaluate their effect on maize and wheat crop, as bio-inoculants along with RP fertilization in organic farming and at multilocational sites comes under different agroclimatic regions, and compare bio-inoculation and RP fertilization treatment effects with DAP fertilization for the enhancement

of yield and soil fertility. Inoculum formulations were developed with suitable carriers to sustain the shelf life of these inoculants for long period at different temperatures.

Phosphate-solubilizing microorganisms (bacteria and fungi)

To isolate the phosphate-solubilizing microorganisms, soil samples were collected from different agricultural niches. These different niches have three types of fields, one was conventional agriculture field in which all the chemical fertilizers are used by farmers as per agronomic practices, these conventional agriculture fields were bamboo plants (*Bambusa vulgaris*) (multipurpose agroforestry crop) experimental field of CORE, Thapar University, Patiala (30.30° N and 76.38° E); potato (*Solanum tuberosum*) (tuberous crop) field of CORE, Thapar University, Patiala; mustard (*Brassica campestris*) crop field (oil-seed crop), mature maize (*Zea mays*) crop field (cereal crop), small maize plants crop field, Berseem (*Trifolium alexandrinum*) crop field (fodder crop); sunflower (*Helianthus annuus*) crop (oil-seed crop) grown in agriculture field of Balachaur (31.07° N and 76.32° E), Punjab. Second type of crop field was organic farm of *Stevia rebaudiana* (medicinal plant) at Pojewal (31.65° N and 76.26° E), Punjab. Organic field used in this study is a field where no chemical fertilizers are used since last ten years. Mainly animal manure, vermi-compost and green manure are used to maintain the soil fertility. And third one was the surface soil of CORE, Thapar University, Patiala that is open land area, without any vegetation.

Phosphate-solubilizing bacteria and fungi were isolated by serial dilution method on Pikovskaya's (PKV) agar plates (Pikovskaya's 1948). Quantitative screening of selected bacterial and fungal isolates was done by the method described by Jackson (1973) in tri-calcium phosphate (TCP) and RP supplemented (equivalent to 100 mg P₂O₅ 100 ml⁻¹) PKV broth. Most efficient phosphate-solubilizing bacterial and fungal isolates were come from rhizospheric soil of organic farming. Six bacterial isolates designated as PSB-3, PSB-5, PSB-

6, PSB-7, PSB-12 and PSB-13, isolated from organic farming soil, showed maximum P solubilization up to 429 $\mu\text{g ml}^{-1}$ respectively in TCP amended PKV broth. The main organic acids produced by these selected isolates in TCP solubilization were oxalic acid, citric acid, acetic acid and gluconic acid. These selected bacterial isolates were further screened for solubilization of rock phosphate. Maximum P solubilization of rock phosphate was observed on day five of incubation up to 271 $\mu\text{g ml}^{-1}$ with reduction in pH 3.7 of the medium from its initial value 7.2. The main organic acids produced by these isolates in RP amended PKV broth were oxalic acid and gluconic acid. Production of gluconic acid was higher than that of other organic acids. All the isolates were able to produce significant amounts of acid phosphatase, alkaline phosphatase and phytase enzyme in the TCP and RP amended PKV broth.

Four fungal isolates designated as PSF-4, PSF-5, PSF-6 and PSF-7 showed maximum P solubilization in TCP and RP amended PKV broth were selected for further study. These four fungal isolates produced significant amount of acid phosphatase, alkaline phosphatase, phytase enzymes and organic acid in growth medium supplemented with TCP and RP.

Identification of phosphate-solubilizing bacteria and fungi

For the identification of bacterial isolates, 16S rRNA genes were amplified by 16S rRNA primers. The amplicons were purified with QIA gel extraction kit (Qiagen, USA), and ligated into the pGEM-T easy vector as per the manufacturer's instructions (Promega Inc., USA). Ligated plasmids were transformed into *Escherichia coli* DH5 α cells and recombinant clones with inserts were sequenced. Nucleotide sequences comparison of bacterial isolates was performed using Ez Tcxon-e database. Sequence data from Ez Tcxon-e database showed that two isolates PSB-3 and PSB-6 showed maximum similarity (98.45 %) with *Pantoea cypripedii* and four isolates PSB-5, PSB-7, PSB-12 and PSB-13 showed similarity (99.85 %)

with *Pseudomonas plecoglossicida*. Out of these six isolates, two isolates PSB-3 and PSB-5 were selected for 16S rRNA full length sequence analysis. 16S rRNA full length sequence analysis using the Ez Tcxon-e database revealed that the most closely related type strain for PSB-3 is *Pantoea cypripedii*, which showed 98.67% similarity. For PSB-5, the most closely related sequence is *Pseudomonas plecoglossicida*, with 99.78% similarity. Phylogenetic analysis (Neighbor-Joining Tree) also grouped PSB-3 with *P. cypripedii* and PSB-5 with *P. plecoglossicida*.

The morphological and microscopic identification of fungal isolates revealed that all the nine isolates belong to the genera *Aspergillus*. For molecular identification of fungal isolates the ITS region of the genomic DNA of fungal isolates was amplified by using ITS 1 and ITS 4 primers. Results of RFLP analysis of ITS amplified products with restriction enzyme *RsaI* showed that one isolate PSF-4 does not show any restriction digestion with *RsaI* and other eight isolates showed restriction digestion with *RsaI*. So on the basis of RFLP analysis PSF-4 was grouped under *Aspergillus tubingensis* and other isolates were grouped under *Aspergillus niger*. Further PSF-4, PSF-5, PSF-6 and PSF-7, which showed maximum P-solubilization, were selected for nucleotide sequencing of the ITS region. Nucleotide sequence comparison of fungal isolates was performed using the BLAST database. Sequence data from BLAST results showed that out of four, three fungal isolates (PSF-5 (99%), PSF-6 (100%) and PSF-7 (99%)) showed maximum similarity with *Aspergillus niger* and one isolate PSF-4 showed maximum similarity (99%) with *Aspergillus tubingensis*.

Effect of different carbon and nitrogen sources on P-solubilization of selected bacterial and fungal isolates

To test the effect of different carbon and nitrogen sources on P-solubilization by bacterial (*Pantoea cypripedii* and *Pseudomonas plecoglossicida*) and fungal isolates (*Aspergillus*

tubingensis and *Aspergillus niger*), PKV broth containing different carbon (fructose, arabinose, galactose, mannitol, maltose, lactose, sucrose, xylose and glucose) and nitrogen sources (NaNO_3 , NH_4NO_3 , NaNO_2 , KNO_3 , tryptophan, NH_4Cl , urea and $(\text{NH}_4)_2\text{SO}_4$) with tri-calcium phosphate ($100 \text{ mg P}_2\text{O}_5 \text{ 100 ml}^{-1}$) were prepared and inoculated with these isolates. The pattern of P solubilization by *P. cyripedii* in different carbon sources was glucose > xylose > galactose > fructose > arabinose > lactose = sucrose > mannitol > maltose while for *P. plecoglossicida* glucose > xylose > galactose > sucrose > maltose > lactose > fructose > mannitol > arabinose. Main organic acids, produced by *P. cyripedii*, in different carbon sources were oxalic acid, acetic acid and gluconic acid, where as *P. plecoglossicida* produced oxalic acid, citric acid and gluconic acid. *Aspergillus tubingensis*, in presence of different carbon sources, showed pattern of P solubilization as glucose > mannitol > sucrose > xylose > maltose > fructose > galactose > arabinose > lactose. Organic acids produced by *A. tubingensis* in presence of different carbon sources were oxalic acid, succinic acid and gluconic acid. In case of *A. niger* pattern of P solubilization in presence of different carbon sources was glucose > mannitol > maltose > xylose > sucrose > fructose > galactose > arabinose > lactose. Organic acids produced by *A. niger* were oxalic acid, citric acid, malic acid and gluconic acid and maximum acid production was observed in presence of glucose compared to other carbon sources. In both bacterial and fungal isolates, it was observed that acid phosphatase, alkaline phosphatase and phytase enzymes production was higher in presence of glucose compared to other carbon sources in growth medium.

Among the nitrogen sources, ammonium sulphate showed maximum P-solubilization in all the selected bacterial and fungal isolates. In case of *P. cyripedii* pattern of P solubilization was $(\text{NH}_4)_2\text{SO}_4 > \text{KNO}_3 > \text{NH}_4\text{NO}_3 > \text{NaNO}_2 > \text{tryptophan} > \text{NaNO}_3 > \text{NH}_4\text{Cl} > \text{urea}$, while in *P. plecoglossicida* the pattern was $(\text{NH}_4)_2\text{SO}_4 > \text{urea} > \text{tryptophan} > \text{KNO}_3 > \text{NH}_4\text{Cl} > \text{NaNO}_2 > \text{NaNO}_3 > \text{NH}_4\text{NO}_3$. In *A. tubingensis* P-solubilization pattern was $(\text{NH}_4)_2\text{SO}_4 >$

$\text{KNO}_3 > \text{NH}_4\text{NO}_3 > \text{NH}_4\text{Cl} > \text{urea} > \text{tryptophan} > \text{NaNO}_3 > \text{NaNO}_2$. While in *A. niger* it was $(\text{NH}_4)_2\text{SO}_4 > \text{KNO}_3 > \text{NaNO}_2 > \text{NaNO}_3 > \text{tryptophan} > \text{NH}_4\text{Cl} > \text{urea} > \text{NH}_4\text{NO}_3$. *Pantoea cypripedii* produce oxalic acid and gluconic acid as main organic acids in all the nitrogen sources. Organic acids produced by *P. plecoglossicida* in different nitrogen sources were oxalic acid, citric acid, malic acid and gluconic acid. *Aspergillus tubingensis* produced oxalic acid, citric acid, malic acid, succinic acid and gluconic acid, while *A. niger* produced oxalic acid, malic acid and gluconic acid. Acid phosphatase, alkaline phosphatase and phytase enzymes production was higher in presence of ammonium sulphate compared to other nitrogen sources both in bacterial and fungal isolates. It was observed that in both bacterial and fungal isolates, nature of carbon and nitrogen sources affect the P-solubilization. The mechanism of P-solubilization might be due to pH reduction, organic acid production, acid phosphatase, alkaline phosphatase and by phytase enzymes production in both bacterial and fungal isolates.

Plant growth promotion activities of bacterial and fungal isolates

Indole acetic acid (IAA) production was detected in bacterial and fungal isolates with (0.1% L-tryptophan in growth medium) and without addition of L-tryptophan, but the rate of IAA production was higher in presence of L- tryptophan. IAA production in presence of L-tryptophan was found to be higher in case of *P. cypripedii* ($92.9 \mu\text{g ml}^{-1}$) compared to *P. plecoglossicida* ($26.9 \mu\text{g ml}^{-1}$). IAA production in presence of L-tryptophan in case of *A. tubingensis* was $50 \mu\text{g ml}^{-1}$ and in case of *A. niger*, it was $57 \mu\text{g ml}^{-1}$. *P. cypripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* were observed to be positive for siderophore production and were negative for hydrogen cyanide production. Both the bacterial and fungal isolates produced hydroxamate type of siderophore.

Effect of buffering on P-solubilization of selected bacterial and fungal isolates

Phosphate solubilization efficiency of selected bacterial and fungal isolates was tested in buffered medium. For this selected isolates were inoculated into Pikovskaya's broth having TCP (equivalent to 100 mg P₂O₅ 100 ml⁻¹) and the pH of the growth medium was maintained 7.0, 8.0 and 9.0 with 0.1 M Tris-HCl buffer. P solubilization by selected isolates was compared with un-buffered medium of pH 7.0, 8.0 and 9.0 adjusted with 0.1 M NaOH. Normal PKV broth having TCP (equivalent to 100 mg P₂O₅ 100 ml⁻¹) inoculated with *P. cypripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* were kept as control. It was observed that in case of all the isolates, there were no negative effect of buffering of media on P solubilization when compared with control medium and un-buffered medium of different pH ranges. Reduction in pH was observed with increase in soluble P concentration in buffered and un-buffered PKV broth. All the bacterial and fungal isolates were able to produce acid phosphatase, alkaline phosphatase and phytase enzymes in buffered and un-buffered conditions and these enzymes production were comparable with normal broth (control).

Role of phosphate-solubilizing bacteria and fungi in organic farming

A two year field experiment of wheat and maize crop was conducted in organic farming to test the effect of selected bacterial and fungal isolates as bio-inoculants in organic farming alone or along with RP fertilization. All the bacterial and fungal bio-inoculation and RP fertilization treatments were compared with chemical phosphate fertilizer (DAP) treatments and with control treatments. It was observed that there was a significant improvement in plant growth parameters (shoot height, shoot and root dry weight), yield and in total P uptake in both the maize and wheat crop field experiments in all the treatments compared to control soil. Results were more significant and pronounced in inoculation along with RP fertilization

treatments compared to bio-inoculation and DAP treatments. Similarly during the two year of field study, soil fertility was significantly improved in context to organic carbon, total P, available P, soil enzyme activities (acid phosphatase, alkaline phosphatase, phytase enzymes and dehydrogenase enzymes) and phosphate-solubilizing bacterial and fungal population in respective plots compared to control. It was observed that soil total P level was significantly increased with DAP and RP fertilization but available P level was significantly increased only in inoculation treatments along with RP fertilization treatments. After harvesting of maize and wheat crop, reduction in soil pH was observed in bio-inoculation treatments alone or along with RP fertilization compared to control. This may be attributed to ability of phosphate-solubilizing microorganisms to produce organic acids, thereby decreasing the pH and increasing the concentration of phosphorus in soil by mechanism involving chelation and exchange reactions. Results showed that inoculation treatments along with RP fertilization significantly improve the yield and soil fertility in organic farming compared to inoculation alone, RP fertilization alone and DAP treatments. Present study suggested that these phosphate-solubilizing bacterial and fungal strains along with RP fertilization can substitute the chemical fertilizers, might be used to reduce the alkalinity of soil by neutralization phenomenon through organic acid exudation and can survive in the soil system to retain the phosphate solubilizing potential for long time.

P-solubilizing bacteria and fungi at multilocal sites

A two year field experiment was conducted at multilocal sites comes under different agroclimatic regions, to test the consistency in performance of selected phosphate-solubilizing microorganisms (bacteria and fungi), with and without rock phosphate fertilization. Maize and wheat were selected as test crops. Application of both bacterial and fungal isolates showed significant effect on maize and wheat grain yield and on soil fertility at all different agro-climatic regions. Grain yield, P uptake, soil organic carbon, available P

and enzyme activities in all the experimental crop fields at different sites, significantly increased due to inoculation. Improvement in crop yield and soil fertility at three different sites was more pronounced and significantly higher when RP was supplemented along with bio-inoculation compared to inoculation, RP fertilization, DAP treatments. Results of the present study suggest that *P. cyripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* along with RP fertilization play an important role in plant growth promotion and facilitates improvement in soil fertility in different agro-climatic conditions.

Development of inoculum formulations

Inoculum formulations of *P. cyripedii*, *P. plecoglossicida*, *A. tubingensis* and *A. niger* were developed, where in, rock phosphate, fly ash, charcoal and vermiculite were used as a carrier materials. The final moisture content of the carrier materials were adjusted to 30 % before packing separate bags. Bacterial and fungal population in all carrier materials at 37 °C showed an upward trend up to 15 days of incubation after that it was started decreased in all the carrier materials, while there was no increase in population at 4 °C. Results showed that viability of bacterial and fungal isolates was higher at 4 °C compared to 37 °C. Among the different carriers tested, rock phosphate formulations supported the maximum viability of fugal and bacterial inoculum up to 270 days of storage period at 4 °C and 37 °C compared to other carriers tested. Both bacterial and fungal P-solubilization, enzyme activities and plant growth promotion activities were not affected even after a long storage period of 270 days at different temperatures and were comparable to fresh culture activities. Results showed that RP can be used as a carrier material for the development of inoculum formulations of selected bacterial and fungal isolates that maintain the viability and P solubilization efficiency of selected isolates up to 270 days of storage at 4 °C and 37 °C. Rock phosphate may be the best carrier material for PSMs due to its low cost, positive effects on crop yield and on improvement of soil fertility on its amendments in soil.

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Appendix

APPENDIX-I

Ampicillin stock solution (50 mg/ml)

Dissolved 2.5 g of ampicillin sodium salt in 50 ml of deionized water. Filter sterilized and stored in aliquots at -20 °C.

X-Gal stock solution (20 mg/ml)

200 mg X-Gal (5-bromo-4-chloro-3-indolyl- β -Dgalactopyranoside) dissolve in 10 ml N,N'-dimethylformamide. Cover with aluminum foil and store at -20 °C in a dark..

IPTG stock solution (100 mM)

1.2 g IPTG

Add deionized water to 50 ml final volume. Filter sterilize and store at 4°C.

LB-ampicillin X-Gal/IPTG plates

- Prepare LB-agar medium (1 liter), weigh out:

Bacto-tryptone	10 g
Bacto-yeast extract	5 g
NaCl	5 g

Dissolved in 800 ml water, adjust pH to 7.0 with NaOH and adjust the volume with water to 1000 ml. Add 15 g of agar and autoclave at 121 °C.

- Before pouring the plates, allow the medium to cool to 55 °C. Then, add 1 ml of ampicillin stock solution (50 mg/ml) to a final concentration of 50 μ g/ml. Mixed gently and pour the plates. Allowed the LB-ampicillin agar medium to solidify. Dry plates opened at room temperature under UV light for 30 min.
- Add 40 μ l of X-Gal stock solution (20 mg/ml) and 40 μ l of IPTG 100 Mm, spread evenly with a sterile spatula.

Genomic DNA Extraction buffer

Sodium acetate	100 mM
Na ₂ EDTA	50 mM
NaCl	500 mM
SDS	1%

TBE Buffer (5x)

Tris base	54 g/l
Boric acid	27.5 g/l
0.5 M EDTA (pH 8)	20 ml/l

Plasmid extraction solution I (10X)

Tris-HCl	25 mM (pH 8.0)
Glucose	50 mM
Na ₂ EDTA	10mM

Plasmid extraction solution II

NaOH	5M
SDS	10%

Plasmid extraction solution III

5.0 M Potassium acetate (pH 4.5)

Agarose gel loading dye (6X)

Bromophenol blue	0.25%
Xylene cyanol FF	0.25%
Sucrose in water	40.0%

TE buffer 10X

Tris-HCl	0.1 M (pH 8)
Na ₂ EDTA	10 M (pH 8)

APPENDIX-II

Pantoea cyripedii strain PSB-3 16S ribosomal RNA gene, partial sequence

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AUTHORS Reddy,S.M. and Kaur,G.
TITLE Phosphate solubilizing rhizobacteria from organic farm and their influence on the growth and yield of maize (*Zea mays* L.)
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1399)
AUTHORS Reddy,S.M. and Kaur,G.
TITLE Direct Submission
JOURNAL Submitted (29-AUG-2012) Department of Biotechnology, Thapar University, Bhadson Road, Patiala, Punjab 147004, India
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Pseudomonas putida strain PSB-5 16S ribosomal RNA gene, partial sequence

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AUTHORS Reddy,S.M. and Kaur,G.
TITLE Phosphate solubilizing rhizobacteria from organic farm and their influence on the growth and yield of maize (*Zea mays* L.)
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1394)
AUTHORS Reddy,S.M. and Kaur,G.
TITLE Direct Submission
JOURNAL Submitted (29-AUG-2012) Department of Biotechnology, Thapar University, Bhadson Road, Patiala, Punjab 147004, India
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Aspergillus tubingensis strain PSF-4 18S ribosomal RNA gene, partial sequence

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AUTHORS Kaur,G. and Reddy,M.S.
TITLE Phosphate solubilizing fungi isolated from organic farming
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 599)
AUTHORS Kaur,G. and Reddy,M.S.
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TITLE Phosphate solubilizing fungi isolated from organic farming
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 600)
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TITLE Direct Submission
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Aspergillus niger strain PSF-6 18S ribosomal RNA gene, partial sequence

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Aspergillus.
REFERENCE 1 (bases 1 to 601)
AUTHORS Kaur,G. and Reddy,M.S.
TITLE Phosphate solubilizing fungi isolated from organic farming
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 601)
AUTHORS Kaur,G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (04-FEB-2014) Department of Biotechnology, Thapar
University, Bhadson Road, Patiala, Punjab 147004, India
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Bankit Comment: BankIt1697000.

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***Aspergillus niger* strain PSF-7 18S ribosomal RNA gene, partial sequence**

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Aspergillus.
REFERENCE 1 (bases 1 to 600)
AUTHORS Kaur,G. and Reddy,M.S.
TITLE Phosphate solubilizing fungi isolated from organic farming
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 600)
AUTHORS Kaur,G. and Reddy,M.S.
TITLE Direct Submission
JOURNAL Submitted (04-FEB-2014) Department of Biotechnology, Thapar
University, Bhadson Road, Patiala, Punjab 147004, India
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Publications

Full Paper

Phosphate solubilizing rhizobacteria from an organic farm and their influence on the growth and yield of maize (*Zea mays* L.)

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(Received March 28, 2013; Accepted April 25, 2013)

Organic farming is gaining popularity all over the world as it avoids the use of synthetic chemicals. Plant production in organic farming mainly depends on nutrient release as a function of mineralization processes in soils. In the present study, efficient phosphate mineralizing bacteria were isolated and their efficacy tested in plant mineral uptake and soil fertility of an organic field. Amongst 12 P-solubilizing bacteria (PSB) isolated from an organic field, two isolates were selected for field inoculation based on their rock phosphate (RP) solubilizing ability, exudation of organic acids, phosphatase and phytase activity and production of indole acetic acid and siderophores. On the basis of biochemical characterization and 16S rRNA sequence analysis, these isolates were identified as *Pantoea cyripedii* (PSB-3) and *Pseudomonas plecoglossicida* (PSB-5). These isolates significantly increased yield and total P uptake in maize. Soil analysis showed that available P, organic carbon and soil enzyme activities were significantly increased. Present study results suggested that inoculation of these bacteria has great application potential in improving the crop yield and soil fertility in organic farming.

Key Words—indole acetic acid; organic farming; *Pantoea cyripedii*; phosphatase; phytase; *Pseudomonas plecoglossicida*; siderophores

Introduction

Organic farming is a method of farming which primarily aims at cultivating the land and raising crops in such a way as to keep the soil alive and in good health by use of organic wastes (crop, animal and aquatic wastes) and other biological materials along with beneficial microorganisms (biofertilizers) to release nutrients to crops for increased sustainable production in an eco-friendly pollution-free environment. Organic farming avoids or largely excludes the use of synthetic inputs such as fertilizers, pesticides, hormones, and

feed additives (Hajra, 2001). To the maximum extent feasible, it relies upon crop rotations, crop residues, animal manures, off-farm organic waste, and mineral-grade rock additives (Lampkin, 1990). Phosphorus (P) is an important component of macromolecules, present in living systems. It is essential for the fertility of soils and plant growth. Despite high total soil concentrations of P, its soluble concentration in the soil is very low (Barber, 1995). A large proportion of the supplied P gets fixed in the soil as phosphates of iron, aluminium and calcium (Altomare et al., 1999). This leads to the need of frequent application of phosphate fertilizers, but its use on a regular basis has become a costly affair and also environmentally undesirable, and there is a need for alternative sources (Reddy et al., 2002). Organic applications increases the nutrient status, microbial activity and productive potential of soil while the use of only chemical fertilizers in the cropping sys-

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tem results in a poor microbial activity and productive potential of soil (Kang et al., 2005). Phosphate-solubilizing bacteria (PSB) are well known to promote plant growth because of their ability to convert an insoluble form of P to a soluble form by the process of acidification, chelation and exchange reactions that can be readily taken up by the plant roots. Acid phosphatases and phytases secreted by P-solubilizing microorganisms also have an important role in phosphate solubilization (Relwani et al., 2008). In addition to P-solubilization, PSBs may also improve the plant productivity by producing other secondary metabolites. Several evidences related to plant growth promotion by PSBs through the production of indole acetic acid (IAA) and siderophore make the PSBs more suitable as biofertilizers (Hariprasad and Niranjana, 2009). Inoculation of P-solubilizing microorganisms showed improvement in growth and yield of different crops (Omar, 1998). Keeping in mind the effect of conventional (chemical fertilization) methods and organic farming practices on diversity and productivity of microorganisms, in the present investigation we have isolated P-solubilizing bacteria from the rhizospheric soil of an organic farm and studied their impact on the yield of maize and on soil fertility in an organic field.

Materials and Methods

Isolation and screening of PSBs. Rhizospheric soil samples were in sterile plastic bags collected from the organic farm at Pojewal, Punjab, India under *Stevia rebaudiana* plants. Samples were collected at 0–10 cm depth, brought to the lab and stored at 4°C. Soil samples were analyzed for their physiochemical properties. Soil was loamy sand in texture, with pH: 8.4, electric conductivity: 0.14 mScm⁻¹, organic carbon: 0.4%, organic matter: 0.7 mg, available P: 4.0 mg kg⁻¹, total P: 219 mg kg⁻¹ and total nitrogen: 0.03%. For isolation of P-solubilizing bacteria, soil samples were serially diluted in sterile physiological saline by the serial dilution method and spread plating was done on Pikovskaya's agar plates (Pikovskaya, 1948) supplemented with 0.5% tri-calcium phosphate as the sole P source and incubated at 30°C. The composition of PKV medium was (g L⁻¹): glucose: 10.0; (NH₄)₂SO₄: 0.50; KCl: 0.20; Mg₂SO₄·7H₂O: 0.010; Mn₂SO₄·H₂O: 0.0001; Fe₂SO₄·7H₂O: 0.0001, yeast extract: 0.50; pH was adjusted to 7.0. Colonies showing the zone of solubilization were selected and maintained in pure culture for

further studies. Quantitative analysis of soluble P was determined by growing these isolates in PKV medium supplemented with tri-calcium phosphate (TCP) (equivalent to 100 mg P₂O₅ per 100 ml medium). Soluble P content in the supernatant was spectrophotometrically estimated by the chlorostannous reduced molybdophosphoric acid blue method (Jackson, 1973) and was expressed in terms of µg ml⁻¹ phosphorus released in culture medium. Isolates showing maximum P-solubilization in TCP were further screened for rock phosphate (RP) solubilization by supplementing RP equivalent to 100 mg P₂O₅/100 ml. Rock phosphate used in this study was obtained from Rajasthan State Mines and Minerals, Ltd., Udaipur, India. The chemical constituents of the RP were: 31.5% P₂O₅, 45.4% CaO, 3.4% MgO, 8.4% Al₂O₃, 3.1% fluoride, 0.044% organic carbon and 0.003% available P. Acid and alkaline phosphatase activities were estimated using a method described by Tabatabai and Bremner (1969) and phytase activity by Heinonen and Lahti (1981). Organic acids exudated by bacterial isolates during P-solubilization were determined using an HPLC method as described in Relwani et al. (2008). Two isolates (PSB-3 and PSB-5) showing maximum P solubilizing activity were selected for further studies.

Biochemical and 16S rDNA sequence analysis. Biochemical characteristics of the selected bacteria (PSB-3 and PSB-5) were studied by performing Gram staining, a motility test, the Methyl red-Voges-Proskauer (MR-VP) test, indole production, citrate utilization, oxidase, catalase, nitrate reduction, urease, cellulose utilization, starch utilization, and an acid-gas production test by utilization of different carbon sources (glucose, lactose, fructose, and sucrose) as per standard methods described in Bergey's Manual of Systematic Bacteriology (Holt et al., 2000). Susceptibility towards different antibiotics was observed by using a Himedia kit- HK001-1PK (Himedia, Mumbai, India) as per the manufacturer's instructions.

Genomic DNA was extracted from cultures grown overnight and 16S rRNA genes were amplified as described in Karn et al. (2010). The amplicons were purified with a QIA gel extraction kit (Qiagen, Germantown, MD), and ligated into the pGEM-T easy vector as per the manufacturer's instructions (Promega, Inc., Madison, WI). Ligated plasmids were transformed into *Escherichia coli* DH5a cells and recombinant clones with inserts were sequenced (DNA Sequencing Facility, Delhi University, India). The sequences were compared

against the available DNA sequences in Ez Taxon-e database (<http://ez-taxon-e-ezbiocloud.net>) (Kim et al., 2012). The phylogenetic tree (Neighbor Joining) was constructed using the MEGA 5.1 (Tamura et al., 2011) software and the bootstrap values were inferred from 1,000 replicates.

Indole acetic acid (IAA) production. Indole acetic acid (IAA) production was analyzed in nutrient broth with supplementation of 0.1% L-tryptophan and without addition of L-tryptophan. IAA was assayed by the colorimetric method using ferric chloride-perchloric acid reagent ($\text{FeCl}_3\text{-HClO}_4$) (Gordon and Paleg, 1957).

Hydrogen cyanide (HCN) production. HCN production was tested by the method of Bakker and Schippers (1987). Briefly, the bacterial isolates were inoculated on Petri dishes containing nutrient agar supplemented with glycine (4.4 g L^{-1}). A Whatman No. 1 filter paper soaked in 2% (w/v) sodium carbonate in 0.5% (w/v) picric acid solution was placed inside the lid of a Petri dish. The plate was then sealed with parafilm and incubated at 30°C for 2 days. A change in filter paper color from yellow to reddish brown was considered to be an indication of HCN production.

Siderophore production. Bacteria were tested for production of siderophores by the method of Schwyn and Neilands (1987) using chrome azurol S (CAS) agar plates. The siderophore levels produced by the isolates were recorded as the diameter of the orange halo produced by the colony. The presence of catechol and/or hydroxamate siderophores in culture supernatants obtained from bacteria grown in CAS broth (succinic acid: 4.0 g, K_2HPO_4 : 3.0 g, $(\text{NH}_4)_2\text{SO}_4 \cdot \text{H}_2\text{O}$: 0.2 g, and distilled water: 1 L, at pH: 7.0) was determined by the method described by Neilands (1981). For this 1 ml of culture supernatant (centrifuged at 10,000 for 10 min) and 3 ml of freshly prepared 2% aqueous FeCl_3 solution were added and the absorbance between 200 and 600 nm was recorded. A peak at 420–450 nm indicated the presence of ferrate hydroxamate siderophore.

Field experiment. To test the efficacy of these two bacterial isolates, a field experiment was conducted in an organic field at Pojewal, Punjab. The site is situated at 31.65° N latitude and 76.26° E longitude and falls under the sub-mountain undulating agroclimatic region of Punjab State. The region possesses a dry sub-humid type of climate. The experiment was designed with a complete randomized block design with three replications. The treatments consisted of: soil; soil +

PSB-3; and soil + PSB-5. Bacterial strains were inoculated as a seed treatment. For the seed coating of bacterial strains, surface sterilization of seeds was performed by dipping them in 95% ethanol for 3 min and 3% sodium hypochlorite for 5 min, and subsequently washing them with sterile distilled water followed by treatment with slurry containing 40% gum arabic and 10% sugar solution and bacterial suspension. Seeds treated with 40% gum arabic and 10% sugar solution that did not contain bacterial suspension served as a control. Seeding of maize variety DKC-9106 was done in July, 2011. At the time of seeding the size of inoculum per maize seed was $2.5\text{--}3.0 \times 10^5 \text{ cfu}$. All the plots were irrigated once before the sowing to ensure proper germination of seeds and then regularly during crop growth. Nitrogen fertilizer at 272 kg ha^{-1} was used in the form of urea as per agronomic practices. No other conventional fertilizers were used during the experiment. The crop was harvested in October, 2011. From each plot, 10 randomly selected plants were uprooted and shoot height, shoot and root dry biomass were measured. Grain yield was recorded for the entire plot. Total phosphorus content in plant samples were determined by the Vanado-molybdo-phosphoric yellow color method described by Kitson and Mellon (1944). For soil analysis, rhizospheric soil was collected (5–10 cm depth) from 10 randomly selected plants and a composite sample was prepared for each plot. Soil samples of each plot were analyzed for their organic carbon (Walkley and Black, 1934), available P (Olsen et al., 1954), acid and alkaline phosphatase activity, phytase activity and dehydrogenase activity (Casida, 1977).

The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey's test at $p < 0.05$. All the analyses were performed using GraphPad Prism (5) software.

Results

Isolation and screening of PSBs

A total of 12 PSBs were isolated from the rhizospheric soil of *Stevia rebaudiana* plants grown on an organic farm, showing more than 5 mm zone of solubilization on PKV agar plates. These were further screened for TCP solubilization and two isolates, PSB-3 and PSB-5, showed maximum P solubilization (Table 1).

Rock phosphate (RP) solubilization

Both the isolates were able to solubilize insoluble

Table 1. Release of soluble P and pH reduction by bacterial isolates in Pikovskayas broth supplemented with tri-calcium phosphate (equivalent to 100 mg P₂O₅/100 ml).

Isolates	pH	Soluble P ($\mu\text{g ml}^{-1}$)	Isolates	pH	Soluble P ($\mu\text{g ml}^{-1}$)
PSB-1	4.12 \pm 0.05 ef	365 \pm 11 c	PSB-8	4.72 \pm 0.07 d	398 \pm 6 b
PSB-3	3.89 \pm 0.04 g	422 \pm 4 a	PSB-9	5.13 \pm 0.03 c	330 \pm 9 d
PSB-4	5.38 \pm 0.07 b	296 \pm 11 e	PSB-10	5.79 \pm 0.05 a	113 \pm 3 g
PSB-5	3.61 \pm 0.06 h	429 \pm 4 a	PSB-11	5.39 \pm 0.08 b	199 \pm 2 f
PSB-6	4.18 \pm 0.06 e	401 \pm 5 b	PSB-12	3.94 \pm 0.01 g	415 \pm 6 ab
PSB-7	4.21 \pm 0.06 e	410 \pm 11 ab	PSB-13	3.97 \pm 0.03 fg	413 \pm 3 ab

Values sharing a common letter within a column are not significant at $p < 0.05$; values are mean \pm SD ($n=3$).

RP and the maximum amount of P was recorded on day 5. The pH reduction which was observed corresponds to the respective increase in soluble P concentration (Fig. 1). PSB-3 solubilized 253 $\mu\text{g P ml}^{-1}$ and PSB-5 271 $\mu\text{g P ml}^{-1}$. P-solubilization was accompanied by a decrease in the pH of the culture filtrate up to 3.7 in both cases from the initial pH of 7.2. Both the isolates were able to produce significant amounts of acid phosphatase, alkaline phosphatase and phytase enzymes in the RP-amended medium. The main organic acids produced by these isolates were oxalic acid, gluconic acid and succinic acid. Production of gluconic acid was higher than that of other organic acids (Table 2).

Identification of selected PSBs

Biochemical characterization of both the isolates is presented in Table 3. 16S rRNA sequence analysis using the Ez Txzon-e database revealed that the most closely related type strain for PSB-3 is *Pantoea cyripedii*, which showed 98.67% similarity. For PSB-5, the most closely related sequence is *Pseudomonas plecoglossicida*, with 99.78% similarity. Phylogenetic analysis also grouped PSB-3 with *P. cyripedii* and PSB-5 with *P. plecoglossicida* (Fig. 2). 16S rRNA gene sequences determined in this study were deposited in GenBank of the NCBI under the accession numbers JX556216 and JX556217 for PSB-3 and PSB-5, respectively.

Indole acetic acid, HCN and siderophore production

Indole acetic acid (IAA) production was detected in both isolates with and without addition of L-tryptophan but the rate of IAA production was higher in presence of L-tryptophan. Maximum production was observed after 5 days of incubation in nutrient broth supplemented with 0.1% L-tryptophan. IAA production in the presence of L-tryptophan was found to be greater in

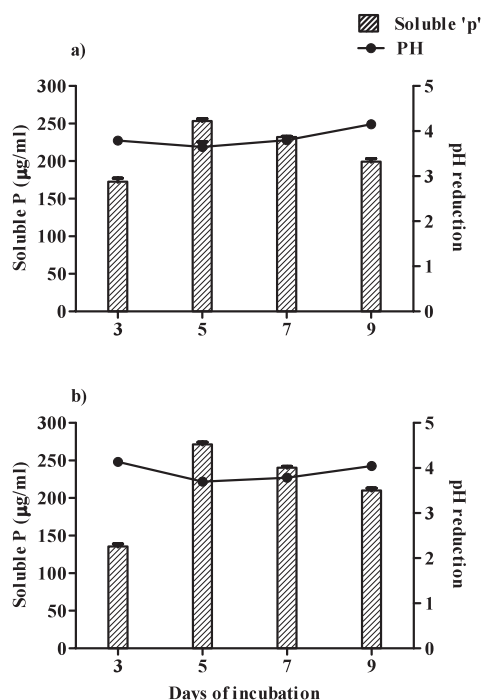


Fig. 1. Phosphorus solubilization and pH reduction by (a) *Pantoea cyripedii* (PSB-3) and (b) *Pseudomonas plecoglossicida* (PSB-5) in Pikovskayas broth having rock phosphate (equivalent to 100 mg P₂O₅/100 ml) as the sole phosphate source during 9 days of incubation.

the case of *P. cyripedii* (92.9 $\mu\text{g ml}^{-1}$) as compared to *P. plecoglossicida* (26.9 $\mu\text{g ml}^{-1}$) (Table 2). Both isolates were negative for HCN production, as no change in color of filter paper from yellow to reddish brown was observed. Siderophore production was confirmed by the development of an orange halo zone around the bacterial colonies on the CAS agar plate. Siderophore production activity of both the isolates was similar (1.5 and 1.6) as calculated by the z/c ratio where z is the colony diameter plus orange halo zone diameter and c is the dia. of the colony (Table 2). The supernatant was further tested for the detection of the catechol and hydroxamate type of siderophores, because CAS

Table 2. Enzyme activities, organic acid exudation, indole acetic acid and siderophore production by *Pantoea cyripedii* (PSB-3) and *Pseudomonas plecoglossicida* (PSB-5).

Activities	<i>P. cyripedii</i>	<i>P. plecoglossicida</i>
Enzyme activities		
Acid phosphatase ($\mu\text{M p.NPP/ml/h}$)	66.2 \pm 3.3	89.6 \pm 16
Alkaline phosphatase ($\mu\text{M p.NPP/ml/h}$)	20.6 \pm 3.0	17.7 \pm 3.2
Phytase ($\mu\text{M pi/ml/h}$)	3,446 \pm 33	3,602 \pm 281
Organic acids (nmol ml^{-1})		
Oxalate	33 \pm 0.9	84 \pm 1.3
Gluconate	4,222 \pm 53	3,816 \pm 49
Acetate	ND	ND
Citrate	ND	ND
Succinate	ND	61 \pm 0.4
Indole acetic acid ($\mu\text{g ml}^{-1}$)		
Without tryptophan	7.6 \pm 0.42	6.8 \pm 0.19
With tryptophan	93.0 \pm 0.44	27.0 \pm 0.04
Siderophore (zone size in mm)	1.5 \pm 0.03	1.6 \pm 0.01

ND, Not detected. Values sharing a common letter within a column are not significant at $p < 0.05$; values are mean \pm SD ($n=3$).

Table 3. Biochemical characterization of *Pantoea cyripedii* (PSB-3) and *Pseudomonas plecoglossicida* (PSB-5) isolates isolated from an organic farm.

Biochemical reactions	<i>P. cyripedii</i>	<i>P. plecoglossicida</i>
Gram staining	—	—
Morphology	Rod	Rod
Motility	+	+
Oxidase	—	+
Catalase	+	+
IMViC test		
Indole production	—	—
Methyl red	—	—
Voges-Proskauer	+	—
Citrate (Simmons)	+	+
Urease	—	—
Nitrate reduction	+	+
Starch utilization	+	+
Cellulose utilization	—	—
Sugar fermentation		
Glucose	+	+
Fructose	+	—
Sucrose	+	—
Lactose	—	—
Gas production during sugar fermentation		
Glucose	+	—
Fructose	+	—
Sucrose	+	—
Lactose	—	—
Antibiotic profile		
Penicillin G (10 U), Cephalothin (30 μg), Clindamycin (2 μg), Erythromycin (15 μg), Amoxycylav (30 μg), Vancomycin (30 μg), Ofloxacin (5 μg), Teicoplanin (30 μg), Ceftazidime (30 μg), Gentamicin (10 μg), Cephoxithin (30 μg)	+	+
Oxacillin (1 μg)	+	—

+: positive; —: negative.

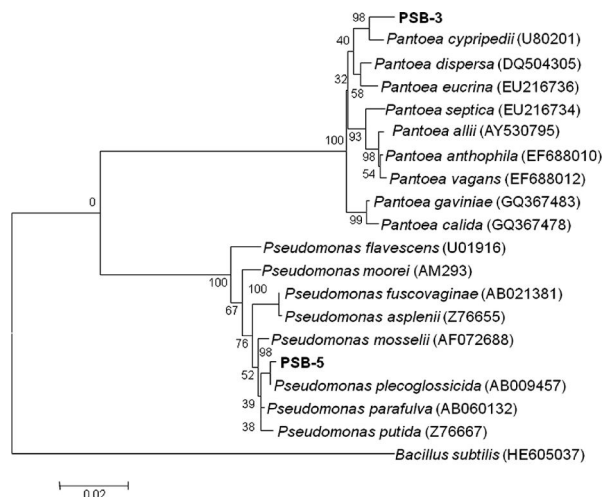


Fig. 2. Phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between the present study isolates and representatives of other related taxa.

Bacillus subtilis was used as the outgroup taxon.

assay does not indicate the type of siderophores being produced. When 3 ml of freshly prepared 2% of aqueous FeCl_3 solution was added to 1 ml of culture supernatant of both the isolates, and the absorbance between 200 and 600 nm was recorded, maximum absorbance was obtained at 425 nm. This indicates the hydroxamate nature of the siderophores produced by both the isolates.

Field experiment

A field experiment was undertaken to evaluate the effectiveness of these bacteria on improvement in crop productivity and soil fertility in a maize crop on an organic farm. Inoculation of these strains significantly increased the grain yield up to 18% and total P uptake in maize grains, shoot and root up to 26%, 22% and 42% respectively in all treatments compared to the uninoculated control soil (Table 4). The available P level and organic carbon level were significantly improved up to 40% and 45% in all treatments compared to uninoculated soil. Dehydrogenase, acid phosphatase, alkaline phosphatase and phytase enzyme activities were significantly higher in all the treatments than in uninoculated soil. Phytase activities were significantly higher in all the treatments compared to acid and alkaline phosphatase activities (Table 5).

Discussion

In the present study two efficient phosphate-solubilizing bacteria isolated from rhizospheric soil of *Stevia rebaeodiana* were identified as *P. cyripedii* and *P. plecoglossicida*. Although earlier studies on phosphate solubilization and plant growth promotion activities of these genera have been reported, they were isolated from the rhizospheric soils of conventional farming systems and from other natural habitats, not from organic fields.

Table 4. Effect of *P. cyripedii* (*Pc*) and *P. plecoglossicida* (*Pp*) on the growth parameters and P uptake of maize plants grown in an organic field.

Treatments	Shoot length (cm)	Shoot dry weight (g)	Root dry weight (g)	Grain yield (ton/ha)	Total P (mg kg ⁻¹) in tissue		
					Grains	Shoot	Root
Soil	225 ± 3.3 a	36.4 ± 1.9 a	9.7 ± 1.0 b	5.66 ± 0.14 b	143 ± 10.5 b	135 ± 8.6 b	127 ± 10.5 b
Soil + <i>Pc</i>	236 ± 4.7 a	47.0 ± 1.3 a	11.3 ± 0.3 a	6.61 ± 0.05 a	177 ± 8.6 a	162 ± 4.2 a	165 ± 9.6 a
Soil + <i>Pp</i>	238 ± 3.1 a	47.4 ± 10.1 a	11.3 ± 0.3 a	6.66 ± 0.03 a	180 ± 16.8 a	165 ± 10.5 a	180 ± 12.7 a

Values sharing a common letter within a column are not significant at $p < 0.05$; values are mean ± SD ($n=3$).

Table 5. Effect of *P. cyripedii* (*Pc*) and *P. plecoglossicida* (*Pp*) on rhizosphere soil characteristics of maize plants grown in an organic field.

Treatments	pH	Organic carbon (%)	Available P (mg kg ⁻¹)	Acid phosphatase ($\mu\text{M g}^{-1} \text{h}^{-1}$)	Alkaline phosphatase ($\mu\text{M g}^{-1} \text{h}^{-1}$)	Phytase activity ($\mu\text{M g}^{-1} \text{h}^{-1}$)	Dehydrogenase activity (ppm)
Soil	8.33 ± 0.03 a	0.38 ± 0.05 b	4.02 ± 0.17 b	370 ± 1.2 c	530 ± 1.8 b	6,237 ± 500 b	8.6 ± 0.9 b
Soil + <i>Pc</i>	7.84 ± 0.02 b	0.52 ± 0.02 a	5.61 ± 0.16 a	471 ± 2.8 b	712 ± 13.1 a	11,904 ± 1,703 a	10.4 ± 0.1 a
Soil + <i>Pp</i>	7.85 ± 0.01 b	0.55 ± 0.05 a	5.63 ± 0.05 a	479 ± 3.6 a	699 ± 61.7 a	11,665 ± 1,663 a	9.8 ± 0.1 ab

Values sharing a common letter within a column are not significant at $p < 0.05$; values are mean ± SD ($n=3$).

Both the strains solubilized RP in culture medium and there was a significant increase in P solubilization with a reduction in the pH of the culture supernatant. Himani and Reddy (2012) showed a significant relationship between quantities of phosphate solubilized and drop in pH of culture filtrate. The P-solubilizing activity is also determined by the biochemical ability of the bacteria to produce and release organic acids. Both the isolates used in this study produced significant amounts of organic acids in RP-amended medium. Gluconic acid was found to be predominant in both the isolates. Gluconic acid seems to be the most frequent agent of mineral phosphate solubilization (Illmer and Schinner, 1992). The major microbiological means by which the insoluble P compounds are mobilized is by the production of organic acids, accompanied by acidification of the medium. Both isolates produced acid phosphatase, alkaline phosphatase, and phytase enzymes in the RP-amended medium. Positive correlation between phosphate solubilization capacity and phosphatase activity was observed. Relwani et al. (2008) suggested that both acid phosphatase and phytase play a major role in P solubilization, apart from other phosphate solubilization mechanisms.

It is desirable that P-solubilizers have additional plant growth-promoting properties like IAA- and siderophore-production ability. Root-colonizing bacteria (rhizobacteria) that exert beneficial effects on plant development via direct or indirect mechanisms have been defined as plant growth-promoting rhizobacteria (PGPR) (Nelson, 2004). PGPR enhance plant productivity by a range of direct/indirect mechanisms. Direct promotion of growth by PGPR occurs when rhizobacteria produce metabolites that promote plant growth such as auxins as well as through the solubilization of phosphate minerals (De Freitas et al., 1997). Indirect growth promotion occurs through the elimination of pathogens by the production of siderophores. The present study clearly revealed that both the isolates used in this study had the ability to produce IAA and consequently, should be considered as IAA-producing rhizobacteria. Production of IAA, even without addition of a precursor, indicated that the bacteria were actively involved in the synthesis of IAA in pure culture. *Pantoea cyripedii* showed higher production of IAA as compared to *P. plecoglossicida* both in the absence and presence of tryptophan, but production was more comparable when tryptophan was used. Production of IAA by *Pseudomonas fluorescens* RAF15 was ob-

served only in the presence of L-tryptophan (Park et al., 2009). Contrary to this, we observed that both the isolates were able to produce IAA without tryptophan as well. In the case of *P. cyripedii* there were 12-fold increases of IAA production and for *P. plecoglossicida* a 4-fold increase was recorded when L-tryptophan was added to culture medium, in comparison with culture growing without tryptophan. This shows that these strains probably synthesize IAA through tryptophan pathways. The culture supernatant of both isolates by the addition of 2% of aqueous FeCl₃ solution showed λ_{\max} in at 420–430 nm, indicating the presence of the hydroxamate type of siderophores. Howell et al. (1988) indicated that some bacterial species such as *Pseudomonas putida* act as antagonists, inhibiting the growth of pathogens through production of siderophores. The deficit of available iron to pathogens might have resulted in the death of the pathogenic microorganisms.

The results obtained in vitro cannot always be dependably reproduced under field conditions. Further evaluation of both the isolates on a soil-plant system was done to uncover their efficacy as plant growth-promoting bacteria. The field study found a significant increase in total P uptake in plants and grain yield after inoculation of these bacteria. Hameeda et al., (2008) reported maize growth promotion by inoculating two phosphate-solubilizing bacteria, *Serratia marcescens* and *Pseudomonas* sp., isolated from compost. Khalimi et al. (2012) showed that *Pantoea agglomerans* effectively promoted rice growth and increased the yield. The available P levels and organic carbon were significantly improved in all treatments compared to the initial values. The changes in soil organic carbon contents were directly associated with changes in microbial biomass carbon and biological activity in soil (Nakhro and Dkhar, 2010). The significant increase in available P might be due to better utilization of P from the pool of soil nutrients by the action of PSB (Mamta et al., 2010). All the treatments showed a drop in pH values of the soil compared to the control. This may be attributable to the ability of such microorganisms to excrete organic acids, thereby decreasing the pH and increasing the concentration of soluble phosphorus in the soil by mechanisms involving chelation and exchange reactions (Vasilev et al., 1996). The dehydrogenase, acid phosphatase, alkaline phosphatases and phytases enzyme activities of all the treatment soils were higher as compared to control soil. The increase of soil enzyme activities upon inoculation observed in this study is sup-

ported microbial build-up in the rhizosphere (Tarafdar and Rao, 1996). In all the treatments phytase enzyme activities were more pronounced than phosphatase enzyme activities, as reported by Aseri et al. (2009) that microbes execute extracellular phytase activity many times more than extracellular phosphatase activity. Simultaneous exudation of phosphatases could increase P solubility, by releasing bound organic phosphates and its mineralization by increasing the rate of hydrolytic cleavage (George et al., 2002).

Conclusion

From the present study it was concluded that *P. cypripedii* and *P. plecoglossicida* isolated from an organic field are efficient phosphate solubilizers. Inoculation of these bacteria significantly increased the plant growth, organic carbon and available P level in soil as well as P uptake in plants and also the crop yield. This is the first report on phosphate solubilization and plant growth promotion activity by *P. cypripedii* and *P. plecoglossicida* strains isolated from rhizospheric soil of an organic field and their effect on the yield of a maize crop grown in an organic field. The results of the present study suggest that inoculation of plant growth-promoting rhizobacteria isolates from an organic field is a good tool to enhance the yield and to improve the soil's available P level in organic farming without the use of chemical phosphorous fertilizers for sustainable cultivation.

Acknowledgments

The authors are thankful to UGC [F. No.-34.67/2008 (SR)] for financial assistance and TIFAC-CORE for facilities.

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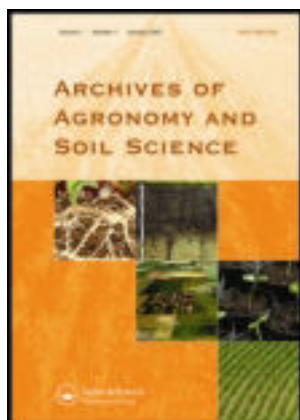
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Archives of Agronomy and Soil Science

Publication details, including instructions for authors and subscription information:

<http://www.tandfonline.com/loi/gags20>

Role of phosphate-solubilizing bacteria in improving the soil fertility and crop productivity in organic farming

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Accepted author version posted online: 25 Jun 2013. Published online: 16 Jul 2013.

To cite this article: Gurdeep Kaur & M. Sudhakara Reddy (2014) Role of phosphate-solubilizing bacteria in improving the soil fertility and crop productivity in organic farming, Archives of Agronomy and Soil Science, 60:4, 549-564, DOI: [10.1080/03650340.2013.817667](https://doi.org/10.1080/03650340.2013.817667)

To link to this article: <http://dx.doi.org/10.1080/03650340.2013.817667>

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Role of phosphate-solubilizing bacteria in improving the soil fertility and crop productivity in organic farming

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(Received 11 March 2013; final version received 16 June 2013)

In the present study, two phosphate-solubilizing bacteria *Pantoea cyripedii* and *Pseudomonas plecoglossicida* isolated from an organic field were tested for their efficacy to solubilize rock phosphate (RP) and other plant growth-promoting activities such as nitrogen fixation and the production of indole acetic acid (IAA) and siderophores. Both of the bacteria effectively solubilized RP and released significant amount of phosphorous (up to 271 $\mu\text{g P ml}^{-1}$) in the culture medium. These isolates produced IAA and siderophores and failed to fix nitrogen as determined by acetylene reduction. Two-year field study was conducted to test the efficacy of these bacteria on the growth and yield of maize and wheat crops grown in organic farm with and without RP. Field trials for both crops were conducted in a completely randomized block design consisting of six treatments and each treatment with triplicates. Significant increase in growth parameters, grain yield, total P uptake, and soil fertility in context of organic carbon, available P, total P, and enzyme activities were observed in inoculation treatments along with RP fertilization compared with control. This study suggested that *P. cyripedii* and *P. plecoglossicida* along with RP play an important role in improving crop productivity in organic farming.

Keywords: phosphate-solubilizing bacteria; *Pantoea cyripedii*; *Pseudomonas plecoglossicida*; organic farming

Introduction

Organic farming is an eco-friendly system of farming which helps to maintain health of soil in terms of soil's biological fertility and productivity. Favorable effects of various components of organic farming over conventional farming systems or integrated nutrient management practices are to be considered holistically rather than looking for short-term benefits (Krishnakumar et al. 2005). Organic farming has been expanding at an annual rate of approximately 20% in the last decade and has become a mainstream practice for some crops (Organic farming... 2004). Organic applications increase the nutrient status, microbial activity, and productive potential of soil. In comparison with conventional farming, organic farming helps to alleviate environmental stress and improves food quality and safety (Giles 2004). Crop production in organic farming mainly depends on nutrient release as a result of mineralization processes in soils. An active soil microflora and a considerable pool of accessible nutrients are, therefore, important priorities in organic farming (Fließbach & Mader 2000). Phosphorus is an essential mineral fertilizer for plant growth and development. Soluble P is often the limiting mineral nutrient for biomass production in natural ecosystems. Plants utilize only a small fraction of the phosphate

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fertilizers applied to the soil and the rest is rapidly converted into insoluble complexes in the soil (Yu et al. 2011). The nutrient reservoirs in the soil shrink when crops are removed from the field at harvest. This nutrient export creates a P deficit, necessitating regular P addition to replace the harvested P. Several studies investigating whole-form P budgets have found nutrient P deficits in many organic farms and illustrate the need for nutrient additions (Nelson & Mikkelsen 2008). This leads to the need of frequent application of phosphate fertilizers, but its use on a regular basis has become a costly affair and also environmentally undesirable (Reddy et al. 2002). Natural phosphate rocks have been recognized as a valuable alternative for P fertilizers. In India, it is estimated that there are almost 260 million tons of phosphate rock deposits and this material should provide a cheap source of phosphate fertilizer for crop production (FAI 2002). Unfortunately, rock phosphate (RP) is not readily available to the plants in soils with a pH > 5.5–6.0. Because of this, extension services are reluctant to be recommended and farmers are hesitant to utilize RP directly. One approach for solubilization of RP in field conditions is the application of phosphate-solubilizing microorganisms (PSMs). PSMs solubilize insoluble form of phosphates by acidification, chelation, and exchange reactions and also by production of organic acids (Chung et al. 2005). This process not only compensates for higher cost of manufacturing fertilizers in industry, but it also mobilizes the fertilizers applied to the soil. In addition to P-solubilization, phosphate-solubilizing bacteria (PSBs) may also improve the plant productivity by producing other secondary metabolites. There are several evidences related to plant growth promotion by PSBs through the production of indole acetic acid (IAA) and siderophores (Hariprasad & Niranjana 2009). Organic farming avoids the inputs of synthetic chemicals and their consequences. The build-up of a large and active soil microbial biomass is, therefore, critically important for sustaining the productivity of soils in organic farming systems (Tu et al. 2006). The present study was conducted to assess the impact of PSBs, *Pantoea cyripedii*, and *Pseudomonas plecoglossicida* isolated from organic field as P-solubilizers for the improvement in growth and yield of maize and wheat crops during 2-year field experiment in organic field with and without RP fertilization. This study also reports the effect of RP fertilization and phosphate-solubilizing bacterial seed inoculation on the physiochemical and enzymatic properties of organic farm soil as compared with non-fertilized and control soil.

Materials and methods

Isolation and plant growth-promoting activities of bacteria

Soil samples to the depth of 5–10 cm were drawn carefully from rhizosphere of six *Stevia rebaudiana* plants growing in an organic farm at Pojewal, Punjab, India, and were collected in sterile plastic bags and brought to the laboratory. From these rhizospheric soil samples, a homogeneous composite sample was prepared and stored at 4°C until used for the isolation of PSBs. For physiochemical analysis, soil samples were air dried and then passed through 2.0 mm sieve and stored in labeled polythene bag before analysis. Soil samples were analyzed for their physiochemical properties. Soil was loamy in texture, with pH, 8.4; electric conductivity, 0.14 mS cm⁻¹; organic carbon, 0.4%; organic matter, 0.7 mg kg⁻¹; available P (Olsen P), 4.0 mg kg⁻¹; total P, 219 mg kg⁻¹; and total nitrogen 0.03%. For isolation of PSBs, soil samples were serially diluted in sterile physiological saline solution (0.85% NaCl in distilled water) by serial dilution method and spread plating was done on Pikovskaya (PKV) agar plates (Pikovskaya 1948) supplemented with 0.5% tri-calcium phosphate (TCP) and incubated at 30°C. Colonies

showing the zone of solubilization were streaked on PKV agar plates to check their purity and stored for further studies.

The spot inoculation was carried out using a sterile needle, and bacterial colonies showing more than 5 mm zone of solubilization on PKV agar plates were further selected for liquid assay. Phosphate-solubilization was estimated in PKV broth supplemented with TCP (equivalent to 22 mg P per 100 ml medium). The selected isolates were grown in a 100 ml PKV broth at 30°C on a rotary shaker (130 rpm). Five milliliters of culture suspension was taken out in sterile conditions at regular interval of 2 days from third day onwards and centrifuged at 10,000 rpm for 10 min, and the pH of the supernatant was determined. Soluble P content was estimated in 500 µl aliquot of supernatant by the chlorostannous reduced molybdo-phosphoric acid blue method (Jackson 1973) and was expressed in terms of micrograms per liter phosphorous released in culture medium. Isolates showing maximum P-solubilization in TCP amended PKV broth were further selected for RP-solubilization by supplementing RP equivalent to 22 mg P (100 ml)⁻¹ medium. RP used in this study was obtained from Rajasthan state mines and minerals limited, Udaipur, India. The chemical constituents of the RP were as follows: 31.5%, P; 45.4%, Ca; 3.4%, Mg; 4.3%, Al; 3.1%, fluoride; 0.044%, organic carbon; and 0.003% available P (Olsen et al. 1954). Acid phosphatase activities by bacterial isolates during RP-solubilization were estimated using a method described by Tabatabai and Bremner (1969). Organic acids produced by bacterial isolates during RP-solubilization were determined using high pressure liquid chromatography (HPLC) method as described by Relwani et al. (2008). Un-inoculated PKV broth supplemented with RP equivalent to 22 mg P (100 ml⁻¹) was kept as control in organic acid estimation. The strains were selected for further studies on the basis of RP-solubilization.

The ability of the bacteria to fix di-nitrogen was measured based on acetylene reduction activity as described in Hardy et al. (1973). The bacterial culture (100 µl of OD₆₀₀ = 1.0) was inoculated in 10 ml of Jensen broth (Jensen 1942) in test tubes. After 3 days of incubation at 30°C, the cotton plugs were replaced with a rubber stopper, and 1 ml of air was removed from the tube with a syringe and 1 ml of acetylene (0.1 atm) was injected into the tubes which were then incubated for 24 h. One milliliter of the gas was sampled and the ethylene concentration was measured by gas chromatography.

Bacteria were further tested for the production of siderophores by the method of Schwyn and Neilands (1987) using chrome azurol S (CAS) agar plates. IAA production was analyzed in nutrient broth with supplementation of 0.1% L-Tryptophan and without L-Tryptophan. IAA was assayed by the colorimetric method using ferric chloride-perchloric acid reagent (FeCl₃-HClO₄) as described by Gordon and Paleg (1957).

Selected strains were identified as *P. cyripedii* (PSB-3) and *P. plecoglossicida* (PSB-5) based on 16S rRNA gene sequence analysis using Ez Taxon-e database (<http://eztaxon-e-zbiocloud.net>) (Kim et al. 2012). The 16S rRNA gene sequences were submitted to the National Center for Biotechnology Information (NCBI) under the accession number JX556216 and JX556217 for *P. cyripedii* (PSB-3) and *P. plecoglossicida* (PSB-5), respectively. The phylogenetic tree (neighbor joining) was constructed using the MEGA 5.1 (Molecular Evolutionary Genetics Analysis; <http://www.megasoftware.net/>) (Tamura et al. 2011) software and the bootstrap values were inferred from 1000 replicates.

Field experiment

To test the efficacy of bacterial isolates as bio-inoculants, a field experiment was conducted for 2 years in the organic field at Pojewal, Punjab, India. In the first year, under

maize and wheat cropping system, maize variety DKC-9106 (20 kg ha⁻¹) was first cultivated in the rainy season (sowing 6 July 2011); after maize harvest (21 October 2011) in the same field, wheat variety PBW-621 (99 kg ha⁻¹) was grown in winter season (sowing 9 November 2011). Field trials for both crops were conducted in a completely randomized block design. Each plot size was 4 m × 4 m (16 m²), which included six treatments: soil; soil + *P. cyripedii*; soil + *P. plecoglossicida*; soil + RP; soil + RP + *P. cyripedii*; and soil + RP + *P. plecoglossicida*, each with three replicates. RP was amended in respective plots at the rate of 13 kg P ha⁻¹ before seeding only once during maize cropping. Bacterial strains were inoculated as seed treatment. For seed inoculation, surface sterilization of seeds was performed by dipping them in 95% ethanol for 3 min followed by 3% sodium hypochlorite for 5 min and subsequently washing with sterile distilled water followed by treatment with slurry containing 40% gum arabic and 10% sugar solution and bacterial suspension. At the time of seeding, size of inoculum per maize seed was 2.5–3.0 × 10⁵ cfu per seed, and per wheat seed was 1.7–2.0 × 10⁵ cfu per seed. All the plots were irrigated once before sowing to ensure proper germination of seeds and then regularly during crop growth as per agronomic practices. Nitrogen fertilizer in the form of urea was used at the rate of 125 kg N ha⁻¹ as per agronomic practices. No other chemical fertilizer was applied to any of the two crops. After wheat harvesting (7 April 2012), the field was left empty for 6 months and wheat variety PBW-621 (99 kg ha⁻¹) was again grown in winter season (sowing 2 November 2012) with booster dose of bio-inoculants 0.6–1.0 × 10⁵ cfu per wheat seed without addition of any phosphate fertilizer to check the effects of previously added (in maize field) RP on wheat crop growth in the second year and was harvested at 5 April 2013. Crops were harvested and data were recorded on 10 randomly selected plants of each plot for growth parameters and yield for the entire plot. At crop maturity, root, shoot, and seed samples of 10 randomly selected plants of each plot were oven dried at 65°C for 72 h and were ground to pass through a 0.5 mm sieve and analyzed for total P content. Total phosphorus content in plant roots, shoot, and seed were determined by vanadomolybdophosphoric yellow color method described by Kitson and Mellon (1944). After harvesting, in order to assess the effect of different treatments on physiochemical properties of rhizospheric soil, soil samples to the depth of 5–10 cm were drawn carefully from rhizosphere of 10 randomly selected plants from each plot and homogeneous composite sample was prepared. Rhizospheric soil samples were stored at 4°C and analyzed within week for soil enzyme activities. For physiochemical analysis soil samples were air dried and then passed through 2.0 mm sieve and stored in labeled polythene bag before analysis. Soil samples of each plot were analyzed for pH, organic carbon (OC) (Walkley & Black 1934), available P (Olsen et al. 1954), total P (Kitson & Mellon 1944), total nitrogen (Piper 1966), acid and alkaline phosphatase activities (Tabatabai & Bremner 1969), phytase activity (Heinonen & Lathi 1981), and dehydrogenase activity (Cassida 1977).

Population density of PSBs

At the end of each field trail, rhizospheric soil samples were tested for phosphate-solubilizing bacterial population. The rhizosphere soil adhered to the roots of harvested plants was separated by gentle tapping and stored in sterilized Petri plates at 4°C. One gram soil of each replicate soil samples was serially diluted and plated on PKV agar plates. The plates were incubated at 30°C, and the colonies showing a clear zone of phosphate-solubilization were counted at the end of incubation.

Statistical analysis

All experiments were performed in triplicates. The data were analyzed by analysis of variance and the means were compared with Tukey's test at $P < 0.05$. All the analyses were performed by using Graph Pad Prism 5.1 software (Dennis Radushev, San Diego, CA, USA).

Results

Isolation and plant growth-promoting activities of bacteria

A total of 12 PSBs, showing more than 5 mm zone of solubilization on PKV agar medium, were isolated from rhizosphere of *Stevia rebaudiana* plants growing in organic farm. P-solubilization activity of these isolates varied when grown in PKV medium supplemented with TCP (equivalent to 22 mg P per 100 ml medium). The two isolates PSB-3 and PSB-5 showed maximum P-solubilization of 422 and 427 $\mu\text{g ml}^{-1}$ from TCP, respectively, on day five with reducing pH up to 3.6 from the initial pH of 7.0. These two isolates were further selected to determine their RP-solubilization. Both isolates were able to solubilize insoluble RP, and the maximum amount of P was recorded on day five. PSB-3 solubilized 253 $\mu\text{g P ml}^{-1}$ and PSB-5 271 $\mu\text{g P ml}^{-1}$ from RP with reduction in pH up to 3.7 from the initial pH of 7.0. These bacteria were identified as *P. cyripedii* (PSB-3) and *P. plecoglossicida* (PSB-5) based on 16S rRNA sequence analysis. Phylogenetic analysis grouped PSB-3 with *P. cyripedii* and PSB-5 with *P. plecoglossicida* (Figure 1).

Both isolates were able to produce significant amount of acid phosphatase enzymes in the RP-amended medium. Acid phosphatase produced by PSB-3 was 66 μM

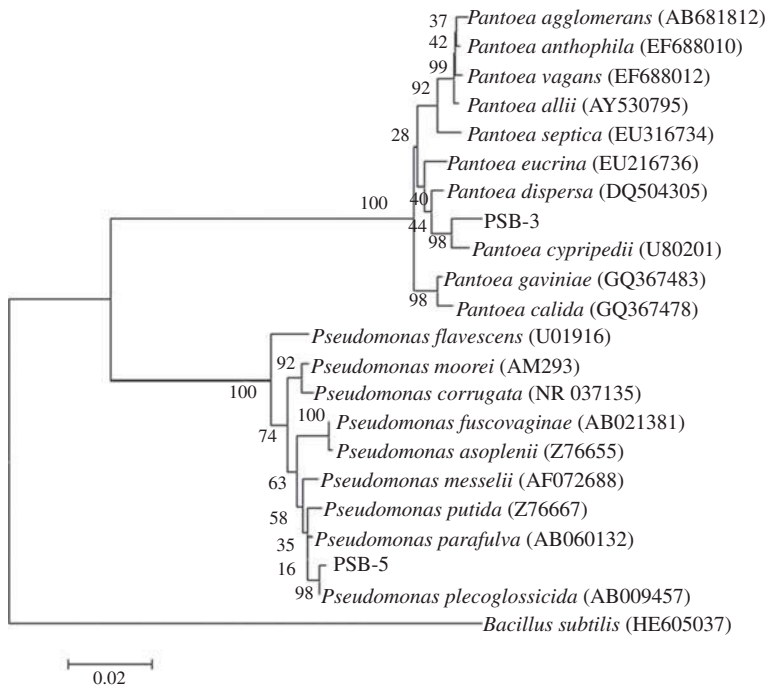


Figure 1. Phylogenetic tree based on 16S rRNA gene sequences, showing the relationship between present study isolates and the representatives of other related taxa with validly published names. *Bacillus subtilis* (HE605037) was used as out group.

p-nitrophenol (PNP) $\text{ml}^{-1} \text{h}^{-1}$ and by PSB-5 was $90 \mu\text{M PNP ml}^{-1} \text{h}^{-1}$ on fifth day of incubation in RP-amended PKV broth. A positive correlation was observed between acid phosphatase enzyme production and release of soluble P by *P. cyripedii* ($r = 0.968$) and *P. plecoglossicida* ($r = 0.896$). Phosphate-solubilization activity was accompanied by the production of organic acids in the culture medium. The main organic acid produced by PSB-3 and PSB-5 was gluconic acid ranged up to $4222 \text{ nmol ml}^{-1}$ and $3816 \text{ nmol ml}^{-1}$, respectively, followed by oxalic acid and succinic acid. No acetylene to ethylene reduction was detected in gas chromatography indicating both of the isolates were not able to fix nitrogen. IAA production was detected in both isolates with and without addition of L-tryptophan but the rate of IAA production was higher in presence of L-tryptophan. *P. cyripedii* showed $7.6 \mu\text{g ml}^{-1}$ and *P. plecoglossicida* showed $6.8 \mu\text{g ml}^{-1}$ of IAA productions in nutrient broth without any addition of tryptophane. IAA production in presence of L-tryptophan was found to be more in case of *P. cyripedii* ($92.9 \mu\text{g ml}^{-1}$) as compared with *P. plecoglossicida* ($26.9 \mu\text{g ml}^{-1}$). Siderophore production was confirmed by the development of an orange halo zone around the bacterial colonies on CAS agar plate. Siderophore production activity of both isolates was almost similar (1.5 and 1.6) as calculated by z/c ratio, where z is the colony diameter plus orange halo zone diameter and c is the diameter of colony.

Effect of PSBs and RP fertilization on maize and wheat crops

Both isolates showed a stimulatory effect on growth parameters of maize and wheat crops (Table 1). Seed inoculation of bacterial strains improved the yield of maize and wheat crops (Figure 2). The stimulatory effect of PSBs on plant growth was found to be more pronounced when inoculated along with RP fertilization. Total P in plant tissues was significantly increased when inoculated seeds were grown in RP-amended soil compared with non-amended soil (Figure 3). RP fertilization with seed inoculations increased the yield of maize grain up to 20% compared with control treatments. Results of wheat crop field trial showed that during the first year of field experiment, grain yield was increased significantly up to 16% and in the second year, grain yield was increased up to 26% in RP fertilization along with bacterial inoculation treatment compared with control.

Effect of PSBs and RP fertilization in rhizospheric soil of maize and wheat crop field

There was a 113% increase in total P level in RP-amended soil in comparison with unfertilized control soil in the maize experimental field (Table 2). Organic carbon was significantly increased (45%) in bacterial inoculated and RP-amended soil, respectively, as compared with control soils (Table 2). Available P increased significantly (40%) due to bacterial inoculation, and with RP amendment, it rose up to 113% in comparison with control soil (Table 2). The pH of soil was decreased in all treatments as compared with control soil (Table 2). Dehydrogenase, acid phosphatase, alkaline phosphatase, and phytase enzyme activities were significantly improved in case of all the treatments when compared with the control treatments. Phytase activity in soil was significantly higher compared with acid and alkaline phosphatase activity (Table 2). All the tested properties of soil were significantly increased in all the treatments compared with controls treatment, but the effects were more pronounced in seed inoculation with RP-fertilization treatment.

Similar to the maize field experiment, field study of wheat crop showed that rhizospheric soil properties such as total and available P, organic carbon, and soil enzyme

Table 1. Effect of *Pantoea cypripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) on growth parameters of maize and wheat crops grown during the first and second year of field trials in organic field.

Treatments	First year						Second year					
	Maize			Wheat			Maize			Wheat		
	Shoot height (cm)	Shoot dry weight (g)	Root dry weight (g)	Shoot height (cm)	Shoot dry weight (g)	Root dry weight (g)	Shoot height (cm)	Shoot dry weight (g)	Root dry weight (g)	Shoot height (cm)	Shoot dry weight (g)	Root dry weight (g)
Soil	225 ± 3.3b	36.4 ± 1.9c	9.70 ± 1.0b	99 ± 8.1b	1.44 ± 0.10c	0.66 ± 0.07b	100 ± 2.4d	1.47 ± 0.08d	0.69 ± 0.04c			
Soil + Pc	236 ± 4.7b	47.0 ± 1.3c	11.3 ± 0.3b	104 ± 5.8b	1.82 ± 0.03b	0.79 ± 0.07ab	112 ± 2.3c	1.95 ± 0.01c	0.82 ± 0.03bc			
Soil + Pp	238 ± 3.1b	47.4 ± 10c	11.3 ± 0.3b	104 ± 2.0b	1.86 ± 0.01b	0.84 ± 0.08ab	115 ± 2.0bc	2.06 ± 0.13bc	0.85 ± 0.08b			
Soil + RP	237 ± 3.0b	51.2 ± 5.1bc	9.90 ± 0.5b	109 ± 3.8ab	1.70 ± 0.05bc	0.79 ± 0.16ab	119 ± 2.1b	2.24 ± 0.05b	0.87 ± 0.08b			
Soil + RP + Pc	257 ± 4.7a	63.2 ± 6.6ab	13.7 ± 1.0a	119 ± 0.9a	2.56 ± 0.16a	0.93 ± 0.05a	127 ± 1.1a	2.69 ± 0.01a	1.24 ± 0.01a			
Soil + RP + Pp	265 ± 6.1a	69.4 ± 2.1a	14.2 ± 0.7a	120 ± 1.4a	2.54 ± 0.16a	1.03 ± 0.06a	128 ± 1.2a	2.75 ± 0.04a	1.29 ± 0.03a			
LSD ($P < 0.05$)	10.7	9.7	1.21	8.4	0.18	0.16	3.48	0.12	0.09			

Notes: Means sharing a common letter within the column are not significant at $P < 0.05$. Values are mean ± SD ($n = 10$).

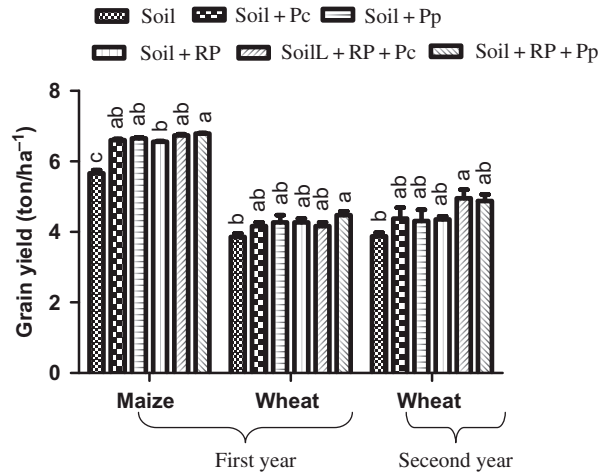


Figure 2. Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) on the yield of maize and wheat crops grown in an organic field with and without supplementation of RP. Bars sharing a common letter within the crop are not significant at $P < 0.05$.

activities were significantly improved in all the treatments, but results were more pronounced in the bacterial inoculation along with RP-amended soil treatments as compared with control treatments (Tables 3 and 4). In the first year and second year of trial, there was 28% and 48% of increase, respectively, in organic carbon in seed inoculation along with RP-amended soil compared with the control treatments. Similarly seed inoculation increased 41% and 77% of available P and seed inoculation along with RP amendment increased available P level up to 86% and 147% than control treatment soil in the first and second year of field trial, respectively (Tables 3 and 4). The pH of soil was decreased in all treatments as compared with control soil (Tables 3 and 4). Two-year wheat study showed that soil enzyme activities such as dehydrogenase, acid phosphatase, alkaline phosphatase, and phytase enzyme activities were significantly improved in case of all the treatments when compared with the uninoculated seed treatments. Phytase activity in soil was significantly higher compared with acid and alkaline phosphatase activities (Tables 3 and 4). The PSBs population in rhizosphere soil of maize and wheat crops was increased significantly by seed inoculation treatments compared with control. Addition of RP along with seed inoculants further increased the PSBs population (Tables 2–4).

Discussion

In the present study, two PSBs, *P. cyripedii* and *P. plecoglossicida*, isolated from an organic farm were used as bio-inoculants in a field study. We are reporting for the first time on improvement in growth and yield of maize and wheat crops in an organic farming.

Both strains solubilized RP in the culture medium and there was a significant increase in P-solubilization with reduction in pH of the culture supernatant. Himani and Reddy (2012) showed a significant relationship between quantities of phosphate solubilized and drop in pH of culture filtrate. The P-solubilizing activity was also determined by the biochemical ability of bacteria to produce and release organic acids. Both isolates used in this study produced significant amount of organic acids in RP-amended medium.

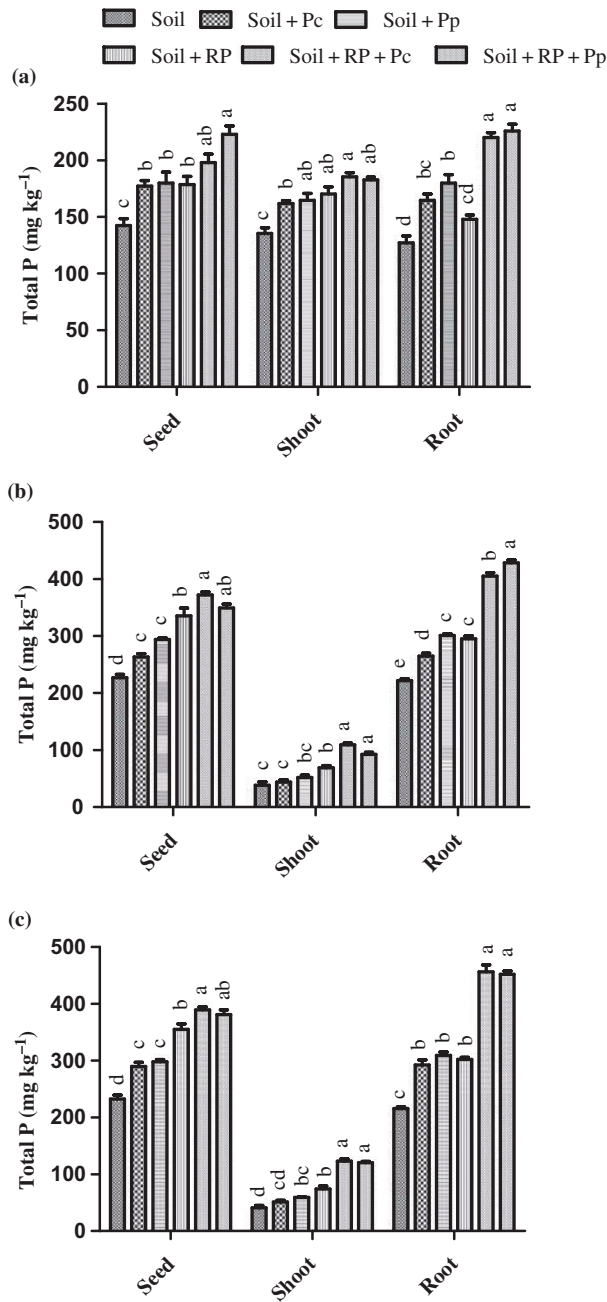


Figure 3. Effect of *Pantoea cypripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) on P uptake of (a) maize and (b) wheat crop grown in the first year, and (c) wheat crop grown in the second year supplemented with and without RP in organic field. Bars sharing a common letter within the treatments are not significant at $P < 0.05$.

Table 2. Effect of *Pantoea cypripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) on rhizosphere soil characteristics of maize plants grown during the first year of field trial in organic field.

Treatments	pH	Organic carbon (%)	Available P (mg kg ⁻¹)	Total P (mg kg ⁻¹)	Total nitrogen (%)	Acid phosphatase activity (μmol PNP g ⁻¹ hr ⁻¹)	Alkaline phosphatase activity (μmol PNP g ⁻¹ hr ⁻¹)	Phytase activity (μmol phosphate g ⁻¹ hr ⁻¹)	Dehydrogenase activity (mg formazan kg ⁻¹)	No. of PSB (cfu g ⁻¹ soil)
Soil	8.33 ± 0.03a	0.38 ± 0.05b	4.0 ± 0.2c	237 ± 8.3b	0.035 ± 0.00a	370 ± 1.2f	530 ± 1.8b	6237 ± 500b	8.60 ± 0.90b	1.8 × 10 ⁶ ± 6c
Soil + Pc	7.84 ± 0.02c	0.52 ± 0.02a	5.6 ± 0.2b	241 ± 4.2b	0.039 ± 0.00a	471 ± 2.8d	712 ± 13a	11904 ± 1703a	10.4 ± 0.10ab	9.4 × 10 ⁷ ± 7b
Soil + Pp	7.85 ± 0.01c	0.55 ± 0.05a	5.6 ± 0.1b	256 ± 2.4b	0.040 ± 0.01a	479 ± 3.6c	699 ± 62a	11665 ± 1663a	9.80 ± 0.10ab	1.0 × 10 ⁸ ± 8b
Soil + RP	7.94 ± 0.02b	0.46 ± 0.01a	5.3 ± 0.1b	465 ± 8.7a	0.042 ± 0.01a	425 ± 2.7e	703 ± 2.9a	8735 ± 126b	9.90 ± 2.80ab	3.5 × 10 ⁶ ± 9c
Soil + RP + Pc	7.78 ± 0.02d	0.54 ± 0.01a	8.1 ± 0.2a	480 ± 35a	0.039 ± 0.01a	490 ± 3.8b	746 ± 4.0a	12446 ± 183a	12.1 ± 0.09a	2.3 × 10 ⁸ ± 13a
Soil + RP + Pp	7.76 ± 0.01d	0.55 ± 0.02a	8.5 ± 0.3a	504 ± 17a	0.048 ± 0.01a	523 ± 1.5a	762 ± 1.3a	12960 ± 287a	12.1 ± 0.07a	2.4 × 10 ⁸ ± 7a
LSD	0.03	0.06	0.34	29	0.02	4.96	46	1787	2.16	1.5
(<i>P</i> < 0.05)										

Notes: Means sharing a common letter within the column are not significant at *P* < 0.05. Values are mean ± SD (*n* = 10).

Table 3. Effect of *Pantoea cyripredii* (Pc) and *Pseudomonas plecoglossicida* (Pp) on rhizosphere soil characteristics of wheat plants grown during the first year of field trial in organic field.

Treatments	pH	Organic carbon (%)	Available P (mg kg ⁻¹)	Total P (mg kg ⁻¹)	Total nitrogen (%)	Acid phosphatase activity (μmol PNP g ⁻¹ hr ⁻¹)	Alkaline phosphatase activity (μmol PNP g ⁻¹ hr ⁻¹)	Phytase activity (μmol phosphate g ⁻¹ hr ⁻¹)	Dehydrogenase activity (mg formazan kg ⁻¹)	Number of PSB (cfu g ⁻¹ soil)
Soil	8.30 ± 0.02a	0.46 ± 0.04a	4.9 ± 0.18d	204 ± 7.2b	0.044 ± 0.005a	439 ± 1.4d	329 ± 0.9e	6484 ± 84c	12.3 ± 0.1d	2.0 × 10 ⁶ ± 5c
Soil + Pc	7.67 ± 0.02c	0.55 ± 0.02ab	6.7 ± 0.18b	234 ± 25b	0.050 ± 0.008a	540 ± 1.6b	467 ± 0.4c	8055 ± 97b	14.0 ± 0.1bc	2.1 × 10 ⁸ ± 16b
Soil + Pp	7.61 ± 0.01d	0.57 ± 0.05ab	6.9 ± 0.18b	245 ± 8.3b	0.055 ± 0.011a	541 ± 1.0b	466 ± 1.1c	8212 ± 99b	14.2 ± 0.1b	2.2 × 10 ⁸ ± 9b
Soil + RP	8.13 ± 0.02b	0.47 ± 0.08ab	5.9 ± 0.18c	455 ± 6.4a	0.045 ± 0.010a	465 ± 1.2c	402 ± 0.4d	8092 ± 89b	13.9 ± 0.1c	4.1 × 10 ⁶ ± 6c
Soil + RP + Pc	7.55 ± 0.03e	0.57 ± 0.02ab	9.1 ± 0.11a	445 ± 8.3a	0.061 ± 0.010a	575 ± 1.4a	491 ± 1.1b	9837 ± 55a	14.1 ± 0.1bc	3.6 × 10 ⁸ ± 25a
Soil + RP + Pp	7.55 ± 0.01e	0.59 ± 0.06a	8.8 ± 0.11a	434 ± 28a	0.060 ± 0.016a	574 ± 1.0a	495 ± 1.3a	9938 ± 136a	15.6 ± 0.1a	3.8 × 10 ⁸ ± 18a
LSD	0.03	0.09	0.28	29	0.16	2.18	1.73	172	0.17	2.6
(<i>P</i> < 0.05)										

Notes: Means sharing a common letter within the column are not significant at *P* < 0.05. Values are mean ± SD (*n* = 10).

Table 4. Effect of *Pantoea cypripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) on rhizosphere soil characteristics of wheat plants grown during the second year of field trial in organic field.

Treatments	pH	Organic carbon (%)	Available P (mg kg ⁻¹)	Total P (mg kg ⁻¹)	Total nitrogen (%)	Acid phosphatase activity (μmol PNP g ⁻¹ hr ⁻¹)	Alkaline phosphatase activity (μmol PNP g ⁻¹ hr ⁻¹)	Phytase activity (μmol phosphate g ⁻¹ hr ⁻¹)	Dehydrogenase activity (mg formazan kg ⁻¹ soil)	Number of PSB (cfu g ⁻¹ soil)
Soil	8.32 ± 0.03a	0.46 ± 0.04b	4.73 ± 0.11e	211 ± 5b	0.04 ± 0.01a	441 ± 1.7f	328 ± 1.3d	6494 ± 69c	12.5 ± 0.06d	2.4 × 10 ⁶ ± 4.5e
Soil + Pc	7.63 ± 0.02c	0.63 ± 0.02a	7.89 ± 0.16c	237 ± 21b	0.05 ± 0.01a	571 ± 1.2d	471 ± 1.3b	8193 ± 42b	16.0 ± 0.12b	3.6 × 10 ⁸ ± 9.1c
Soil + Pp	7.59 ± 0.01c	0.61 ± 0.04a	8.39 ± 0.09b	241 ± 15b	0.06 ± 0.01a	581 ± 1.0c	473 ± 1.5b	8258 ± 177b	15.9 ± 0.09b	3.7 × 10 ⁸ ± 6.7c
Soil + RP	7.93 ± 0.08b	0.51 ± 0.05b	6.40 ± 0.07d	458 ± 4a	0.05 ± 0.02a	473 ± 1.2e	430 ± 16c	8157 ± 83b	14.4 ± 0.17c	4.4 × 10 ⁶ ± 6.2d
Soil + RP + Pc	7.42 ± 0.02d	0.66 ± 0.04a	11.5 ± 0.09a	454 ± 8a	0.06 ± 0.01a	606 ± 0.7b	503 ± 2.0a	9957 ± 135a	17.2 ± 0.20a	4.9 × 10 ⁸ ± 5.9b
Soil + RP + Pp	7.44 ± 0.02d	0.68 ± 0.01a	11.7 ± 0.26a	440 ± 8a	0.06 ± 0.02a	620 ± 7.0a	508 ± 1.6a	9975 ± 120a	17.3 ± 0.04a	5.2 × 10 ⁸ ± 9a
LSD (<i>P</i> < 0.05)	0.06	0.06	0.29	27	0.02	5.69	12	202	0.22	1.3

Notes: Means sharing a common letter within the column are not significant at *P* < 0.05. Values are mean ± SD (*n* = 10).

Gluconic acid was found to be predominant in both of the isolates. Gluconic acid seems to be the most frequent agent of mineral phosphate-solubilization (Illmer & Schinner 1992). The major microbiological mean by which the insoluble P compounds are mobilized is the production of organic acids accompanied by acidification of the medium. Both isolates produced acid phosphatase enzymes in the RP-amended medium. Positive correlation between phosphate-solubilization capacity and phosphatase activity was observed. Relwani et al. (2008) suggested that acid phosphatase plays a major role in P-solubilization, apart from other phosphate-solubilization mechanisms. It is desirable that P-solubilizers have additional plant growth-promoting properties like IAA and siderophore production ability. The present study clearly revealed that both isolates used in this study have the ability to produce IAA and siderophores. Several plant growth-promoting bacteria are known to secrete siderophores in culture medium (Park et al. 2009). Production of IAA, even without addition of precursor indicated that bacteria were actively involved in the synthesis of IAA in pure culture. Production of IAA by *Pseudomonas fluorescens* RAF15 was observed only in presence of L-tryptophan (Park et al. 2009). Contrary to this we observed that both of isolates were able to produce IAA without tryptophan also.

In the present study, a significant increase was found in the biometric parameters (shoot height, shoot and root dry biomass) of maize and wheat plants after treatment with PSBs. The effect was more pronounced when PSBs inoculation was done along with RP fertilization. PSBs inoculation along with RP fertilization showed a stimulatory effect on P uptake of plants. This might be due to better utilization of P from the pool of soil nutrients by the action of P-solubilizing bacteria (Mamta et al. 2010). The treatments with PSBs showed a significant increase in total P content of plant tissues. The total P level was more pronounced in phosphate-solubilizing bacterial seed inoculations along with RP fertilization. The highest increase in total P content in plant shoot, root, and grains was observed in the treatments showing highest available P in soil. These results suggested that a subsequent crop will reap the benefits imparted by PSBs to the soil in terms of available P content, physical and biological characteristics of the soil (Mittal et al. 2008). Results showed that RP amendment along with seed inoculations had promising positive effects on the yield of maize and wheat crops as compared with the control treatments.

Rhizosphere colonization by microbial inoculants has been described as a crucial factor for plant growth promotion (Lugtenberg 2001). Results of the present study are also in agreement with this statement. Stimulatory effect of PSBs on growth, yield, and nutrient uptake in maize and wheat crops can be correlated with increased population of PSBs in rhizospheric soil by PSBs seed inoculation along with RP fertilization. This may be subjected to the increase in population of PSBs and possibly also the indirect increment in total native microbial populations resulting from altered root exudation. Addition of RP to the seed inoculated treatment soil raised the total soil population of PSBs, suggesting that RP acts as a substrate for the tested PSBs, and is obviously beneficial for proliferation and survival of these isolates. Similar results were also reported by Yu et al. (2011) with TCP amendments.

It was apparent from the results that various treatments had an inherent potential for the improvement of soil properties especially related to organic carbon and available P that directly affect the plant growth, yield, and nutrient uptake. Maximum amount of soluble P was observed in the soil with PSBs inoculation along with RP fertilization treatment. The organic carbon level was significantly increased in all the treatments in comparison with the initial values. Our results are in agreement with Himani and Reddy (2011), who reported an increase in soil organic carbon level in bio-inoculated and RP-

fertilized soil as compared with control soil. Increase in organic carbon in seed inoculated and RP-amended treatments may be due to the enhanced growth and increase in bacterial population in the inoculated treatment than control. The changes in soil organic carbon contents directly associated with changes in microbial biomass carbon and biological activity in soil (Nakhro & Dkhar 2010). Little decrease in soil pH was observed in all the treatments compared with control soil. Inorganic P is solubilized by the action of organic and inorganic acids secreted by P-solubilizing bacteria in which hydroxyl and carboxyl groups of acids chelate cations and decrease the pH in basic soils and increase the concentration of available phosphorous in soil (Stevenson 2005).

Soil enzymes have been suggested as potential indicators of soil quality because of their relationship to soil biology, ease of measurement, and rapid response to changes in soil management (Dick et al. 1996). Inorganic P is released from organic matter by hydrolysis of C–O–P ester bonds by phosphatases, which are, therefore, important in the P nutrition of plants. Dehydrogenases represent a class of enzymes that give us information about the influence of natural environmental conditions on microbial activities of the soil (Schaffer 1993). Activities of enzymes such as dehydrogenase, acid phosphatase, alkaline phosphatase, and phytase in all the treatments were higher than the control soil. In all the treatments, the phytase activity was observed to be more than phosphatase activity. This may be due to the higher extracellular phytase enzyme activity of selected bacterial isolates as compared with extracellular phosphatase enzyme activity similar to which was reported by Aseri et al. (2009) that microbes execute extracellular phytase activity many times more than extracellular phosphatase activity. The potential role of soil microorganisms for increasing the amount of available P from phytase activity has been reported (Richardson 2001). The alkaline phosphatase and phytase activities were slightly decreased after wheat harvest as compared with the initial values. This may be due to the slight reduction in soil pH, a fact which has been also observed by Richardson et al. (2005). Higher enzyme activities in soils indicated the potential of soil to affect the biochemical transformations necessary for the maintenance of soil fertility (Rao et al. 1990).

Conclusion

The results of the present study revealed that the use of PSBs along with RP fertilization is an efficient approach for the improvement of growth, yield, and nutrient uptake in maize and wheat crops and also for the improvement in physiochemical properties of soil especially for the maintenance of phosphorous level in an organic farming. The inoculation of P-solubilizing bacteria along with RP fertilization significantly increased the growth and yield of maize and wheat crops over 2 years in an organic field. Uniqueness of this study lies in the fact that this is an early report on the RP-solubilization by *P. cyripedii* and *P. plecoglossicida* isolated from an organic field and on their significant effect as bio-inoculant along with RP fertilization on improvement of crop yields and soil properties. These treatments could ensure the long-term fertility of the soil in relation to P and would provide a cost effective, sustainable, and environmentally friendly production system for crops in organic farming.

Acknowledgments


The authors are thankful to University Grants Commission (UGC), Government of India for financial assistance and TIFAC-CORE for providing facilities.

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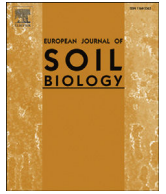
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Highlights

- A field study was conducted at multilocational sites to test the efficacy of P solubilizing bacteria.
- *Pantoea cyripedii* and *Pseudomonas plecoglossicida* significantly increased the yield of wheat and maize in all sites.
- Supplementation of rock phosphate along with bacteria improved the soil fertility at different sites.

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Original article

Influence of P-solubilizing bacteria on crop yield and soil fertility at multilocational sites

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ARTICLE INFO

Article history:

Received 23 October 2013

Received in revised form

13 December 2013

Accepted 28 December 2013

Available online xxx

Handling editor: Kristina Lindström

Keywords:

Bio-inoculants

Maize

Pantoea cyripedii

Phosphorous

Pseudomonas plecoglossicida

Wheat

ABSTRACT

Phosphate-solubilizing bacteria (PSBs) *Pantoea cyripedii* (PSB-3) and *Pseudomonas plecoglossicida* (PSB-5) isolated from organic field were studied for improvement of maize and wheat crop at multilocational sites with and without rock phosphate (RP) fertilization during two year of field study under different agroclimatic regions. Application of both the bacterial strains as bio-inoculants showed significant effect on maize and wheat grain yield and on soil fertility at all different agroclimatic regions. Grain yield, phosphorous (P) uptake, soil organic carbon, available P, enzyme activities and P-solubilizing bacterial population in all the experimental crop fields at different sites significantly increased due to inoculation. Improvement in crop yield and soil fertility at three different sites was more pronounced and significant when RP was supplemented along with seed inoculation compared to uninoculated control treatment. Present study results suggest that *P. cyripedii* (PSB-3) and *P. plecoglossicida* (PSB-5) along with RP fertilization play an important role in plant growth promotion and improvement of soil fertility in different agroclimatic regions.

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1. Introduction

Phosphorus is an essential macronutrient required by the plants for their growth and development. Added P fertilizers undergo fixation due to the complex exchanges within the soil [1]. The nutrient reservoirs in the soil shrink when crops are removed from the field at harvest. This nutrient export creates a P deficit, necessitating regular P addition to replace the harvested P. This leads to the need of frequent application of phosphate fertilizers, but its use on a regular basis has become a costly affair and also environmentally undesirable [2]. Chemical fertilizers are also adversely affecting the soil microbial population [3]. Natural phosphate rocks have been recognized as a valuable alternative for P fertilizers. In India, it is estimated that there are almost 260 million tons of phosphate rock deposits and this material should provide a cheap source of phosphate fertilizer for crop production [4]. Unfortunately, rock phosphate (RP) is not readily available to the plants in soils with a pH >5.5–6.0. Because of this, extension services are reluctant to be recommended and farmers are hesitant to utilize RP directly. Several P-solubilizing microorganisms have the ability to convert insoluble low grade rock phosphates into soluble forms

available for plant growth [5]. PSMs solubilize insoluble form of phosphates by acidification, chelation and exchange reactions and also by production of organic acids [6]. This process not only compensates for higher cost of manufacturing fertilizers in industry; it also mobilizes the fertilizers applied to the soil. Biological systems are therefore preferred over chemical fertilizers, as they are not only ecofriendly and economical in approach, but also involved in improving the soil quality and maintenance of natural flora [7]. An increase in P availability to plants through the inoculation of PSMs has been reported in pot experiments and under field conditions [8,9]. The use of PSBs with the aim of improving P nutrient availability for plants is an important practice and necessary for agriculture. However the potential benefits of these PSBs are not fully realized because of the limitations like inconsistent performance at different sites. There is no doubt that bacterial inocula can increase the yield of various crops significantly, but the performance has generally been inconsistent. A key factor involved in the lack of success has been the rapid decline of the size of populations of active cells, to levels ineffective to achieve the objective, following introduction into soil. Potential of bacterial inoculums may be determined in a single experiment, but the consistent of performance can only be determined in multiple trails [10]. The aim of this study was to evaluate the efficiency of PSBs strains (isolated from rhizosphere of *Stevia* plants grown in organic farm) single and along with RP fertilization on crop yield and soil

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fertility of maize and wheat crop at three different sites comes under different agroclimatic regions.

2. Material and methods

2.1. Bacterial strains

Bacterial strains *Pantoea cyripedii* (PSB-3) and *Pseudomonas plecoglossicida* (PSB-5) isolated from rhizospheric soil samples of *Stevia rebaudiana* growing in organic field at Pojewal, Punjab, India were used in this study. These isolates were selected based on their plant growth promotion activities and their efficiency to improve the crop yield and soil fertility as shown in our previous study [11].

2.2. Experimental field preparation and cultivation practices

Field trials were conducted at three different sites (Table 1). As these sites come under different agroclimatic region that have different climatic conditions and soil fertility level (Table 1) and were selected for field study to check the effect of selected PSB isolates on improvement of crop productivity and soil fertility in these different regions. Their survival, effect on crop productivity and soil fertility in these different agroclimatic regions will provide us the consistent performance of these isolates in different agroclimatic regions. Organic field used in this study was a field where from last ten years no chemical fertilizer was used. Mainly animal manure, vermi-compost and green manure were used to maintain the soil fertility. During first year of field study, maize variety DKC-9106 (20 kg/ha) was cultivated in the rainy season. Field trials were conducted in a completely randomized block design; each plot size was 4 m × 4 m (16 m²) and included six treatments, soil; soil + *P. cyripedii*; soil + *P. plecoglossicida*; soil + RP; soil + RP + *P. cyripedii*; soil + RP + *P. plecoglossicida*, each with three replicates. Rock phosphate was amended in respective plots at the rate of 59 kg P₂O₅/ha before seeding only once during maize cropping. Bacterial strains were inoculated as seed treatment. At the time of seeding, size of inoculum per maize seed was 2.5–3.0 × 10⁵ cfu per seed. All the plots were irrigated once before the sowing to ensure proper germination of seeds and then regularly during crop growth as per agronomic practices. Nitrogen fertilizer of 272 kg/ha was used in the form of urea as per agronomic practices. No other chemical fertilizer was applied to the crop. The crop was harvested in Oct, 2011. The experimental field was left empty for one year.

Wheat variety PBW-621 (99 kg/ha) was sown in the same field in winter season (Nov 2012) to check the effect of inoculum on wheat crop in second year without addition of any phosphate fertilizer to check the effects of previously added (in maize field) rock phosphate on wheat crop growth in second year. The field was tilled and irrigated without disturbing the experimental design. At the time of sowing size of the inoculum per wheat seed was 1.7–

Table 1
Some climatic and soil physiochemical properties of experimental sites.

Sites	Agroclimatic region	Soil type	Location	Climate	pH	Organic carbon (%)	Available P (mg kg ⁻¹)	Total P (mg kg ⁻¹)	Organic P (mg kg ⁻¹)	Total nitrogen (%)
Patiala	Central plain region	Sandy loam (Typic Ustifluvents)	30.30°N 76.38°E	Tropical hot and dry	8.27 ± 0.05	0.33 ± 0.05	3.9 ± 0.17	251 ± 14	138 ± 42	0.020 ± 0.013
Balachaur	Sub mountain undulating central region	Sandy loam (Typic Ustifluvents)	31.07°N 76.32°E	Dry sub humid	8.18 ± 0.02	0.31 ± 0.09	3.6 ± 0.16	237 ± 15	124 ± 24	0.019 ± 0.007
Pojewal	Sub mountain undulating region	Loamy sand (Typic Ustorthents)	31.65°N 76.26°E	Dry sub humid	8.37 ± 0.02	0.42 ± 0.08	4.3 ± 0.09	245 ± 8.33	207 ± 24	0.035 ± 0.008

Values are mean ± SD (n = 3).

Soil + Pc Soil + Pp Soil + RP
Soil + RP + Pc Soil + RP + Pp

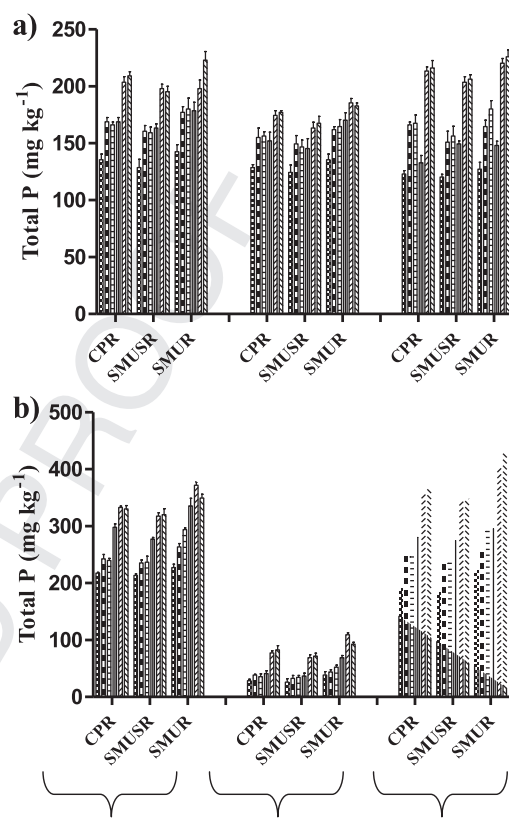


Fig. 1. Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) single or along with rock phosphate (RP) fertilization on total P uptake in grain, shoot and root of a) maize and b) wheat crop grown at Central plain region (CPR), Sub mountain undulating central region (SMUCR) and Sub mountain undulating region (SMUR).

2.0 × 10⁵ cfu. Urea was added at the rate of 272 kg/ha as per agronomic practices. Wheat was harvested in April 2013.

2.3. Plant and soil analysis after harvesting

During each crop harvest, entire observation plot (4 m × 4 m (16 m²)) of each treatment was harvested to record the grain yield. After harvesting root, shoot and seed samples of ten randomly selected plants of each plot were oven dried at 65 °C for 72 h and were ground to pass through a 0.5 mm sieve and analyzed for total P content. Total phosphorus content in plant roots, shoot and seed were determined by vanado-molybdophosphoric yellow color

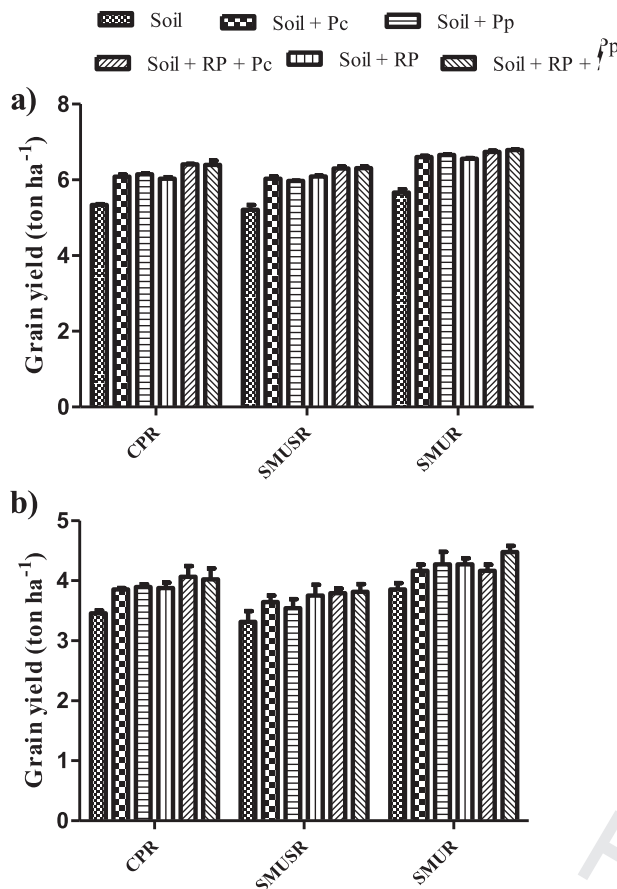


Fig. 2. Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) single or along with rock phosphate (RP) fertilization on grain yield of a) maize and b) wheat crop grown at Central plain region (CPR), Sub mountain undulating central region (SMUCR) and Sub mountain undulating region (SMUR).

method described by Kitson and Mellon [12]. After harvesting soil samples to the depth of 5–10 cm were drawn carefully from rhizosphere of 10 randomly selected plants from each plot and homogeneous composite sample was prepared for each plot, stored at 4 °C and analyzed within week for soil enzyme activities and for PSBs population. For physiochemical analysis soil samples were air dried under shade and then passed through 2.0 mm sieve and stored in labeled polythene bag before analysis. Soil samples of each plot were analyzed for its pH, organic carbon [13], available P [14], total phosphorus [12], Organic P by ignition method [15], acid and alkaline phosphatase activity [16], phytase activity [17], dehydrogenase enzyme activity [18], and PSBs population by serial dilution method on Pikovskayas agar plates.

2.4. Statistical analysis

The data were analyzed by analysis of variance (ANOVA) and the means were compared with Tukey's test at $P < 0.05$. All the analyses were performed by using Graph Pad Prism 5.1 software.

3. Results

3.1. Effect of PSBs and RP fertilization on maize and wheat crop

A significant increase in grain yield and total P uptake in seed, shoot and root of maize and wheat crop was observed by introduction of *P. cyripedii* and *P. plecoglossicida* during two year of field

study compared to control (Figs. 1 and 2). Stimulatory effects of PSBs on yield and P uptake were found to be more pronounced when inoculated along with RP fertilization. Enhancement in yield and P uptake in maize and wheat was more pronounced and significantly higher at sub mountain undulating region compared to central plain region and sub mountain undulating central region.

3.2. Effect of PSBs and RP fertilization on soil fertility at different sites

Physiochemical properties of maize and wheat rhizospheric soils were significantly improved in all the treatments but the effects were more pronounced when seed inoculation was done along with RP fertilization (Tables 2 and 3). Bacterial inoculation slightly decreased the soil pH in all the treatments compared to control in all field trials. During first year in maize crop, inoculation along with RP fertilization increased the soil organic carbon content 41%, 40% and 40% at central plain region, sub mountain undulating central region and at sub mountain undulating region respectively compared to control treatments. Available P level was significantly increased at central plain region (38%), sub mountain undulating central region (38%) and at sub mountain undulating region (40%) due to inoculation but the effect was more pronounced (112%, 113% and 113%) when RP was supplemented along with seed inoculation compared to control. During second year in wheat crop, inoculation of bacteria along with RP fertilization increased the soil organic carbon content 27%, 27% and 30% and available P level 84%, 84% and 80% at central plain region, sub mountain undulating central region and at sub mountain undulating region respectively. Acid phosphatase, alkaline phosphatase, phytase and dehydrogenase enzyme activities were significantly increased in all the treatments compared to control treatment in maize and wheat crop fields at different sites (Fig. 3). Results showed that negative correlation were observed between total organic phosphorous and soil enzyme activities (Phytase, Acid phosphatase and alkaline phosphatase). For phytase, acid phosphatase and alkaline phosphatase enzymes at central plain region in maize correlation was 0.292, -0.937 and -0.869, in wheat correlation was -0.897, -0.753 and -0.929, in sub mountain undulating central region for maize it was -0.912, -0.881 and -0.890 and in wheat correlation was -0.836, -0.892 and -0.802 and in sub mountain undulating region in maize correlation was -0.777, -0.749 and -0.929 and in wheat it was -0.884, -0.985 and -0.950. Maximum population density of PSBs was observed in inoculation treatments along with RP fertilization compared to control in all field trials of maize and wheat crops (Tables 2 and 3). Results of two way ANOVA showed that significant interactions existed between experimental sites and treatments in case of available P, soil enzyme activities and P-solubilizing bacterial population in both maize and wheat crop field trials. Organic carbon, available P, soil enzyme activities and PSBs population was significantly higher at sub mountain undulating region followed by central plain region and sub mountain undulating central region. Multiple regression analysis showed a significant correlation between maize grain yield and organic carbon (0.871, 0.932 and 0.868) and enzyme activities (acid phosphatase: 0.838, 0.807 and 0.824; alkaline phosphatase, 0.923, 0.850 and 0.976; phytase, 0.711, 0.5000 and 0.455) in all three sites, with organic P (0.805) in sub mountainous undulating region and with available P (0.762 and 0.739) in central plain region and sub mountain undulating central region. Multiple regression analysis also showed a significant correlation between wheat grain yield and organic carbon (0.848 and 0.724), available P (0.836 and 0.741), alkaline phosphatase (0.917 and 0.788) and phytase enzyme (0.813 and 0.693) activities in central plain region and sub mountain undulating central region.

Table 2
Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) single or along with rock phosphate (RP) fertilization on rhizosphere soil characteristics of maize plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	pH	Organic carbon (%)	Available P (mg kg ⁻¹)	Total P (mg kg ⁻¹)	Organic P (mg kg ⁻¹)	Dehydrogenase activity (ppm)	No. of PSB (CFU g ⁻¹ soil)
Central plain region							
Soil	8.24 ± 0.03b	0.36 ± 0.07cd	3.4 ± 0.2i	251 ± 15c	151 ± 24ab	8.3 ± 0.14de	0.3 × 10 ⁵ ± 1i
Soil + Pc	8.11 ± 0.02d	0.50 ± 0.05abc	4.7 ± 0.1efg	256 ± 6c	124 ± 24ab	9.3 ± 0.05cde	5.3 × 10 ⁷ ± 6efg
Soil + Pp	8.03 ± 0.02e	0.46 ± 0.08abcd	4.6 ± 0.1fg	260 ± 9c	138 ± 42ab	9.5 ± 0.04bcde	6.5 × 10 ⁷ ± 9def
Soil + RP	7.8 ± 0.01hij	0.44 ± 0.01abcd	5.1 ± 0.1def	483 ± 8a	151 ± 48ab	9.5 ± 0.14bcde	1.7 × 10 ⁵ ± 2i
Soil + RP + Pc	7.73 ± 0.02lm	0.49 ± 0.05abcd	7.09 ± 0.3bc	494 ± 17a	110 ± 24ab	11.5 ± 0.09ab	8.9 × 10 ⁷ ± 6bc
Soil + RP + Pp	7.74 ± 0.01klm	0.51 ± 0.03ab	7.2 ± 0.1b	493 ± 10a	96 ± 42ab	11.6 ± 0.05ab	9.3 × 10 ⁷ ± 5bc
Sub mountain undulating central region							
Soil	8.19 ± 0.02c	0.35 ± 0.08d	3.2 ± 0.2i	227 ± 15c	138 ± 42ab	7.9 ± 0.10e	N.D × 10 ⁵ i
Soil + Pc	7.87 ± 0.02g	0.45 ± 0.05abcd	4.3 ± 0.1gh	240 ± 10c	124 ± 24ab	8.5 ± 0.01de	3.6 × 10 ⁷ ± 4gh
Soil + Pp	7.85 ± 0.01gh	0.46 ± 0.03abcd	4.4 ± 0.2gh	244 ± 6c	124 ± 24ab	8.9 ± 0.04cde	4.7 × 10 ⁷ ± 5fg
Soil + RP	7.82 ± 0.02hi	0.44 ± 0.06abcd	4.9 ± 0.1efg	447 ± 21b	138 ± 42ab	9.0 ± 0.04cde	0.7 × 10 ⁵ ± 1i
Soil + RP + Pc	7.72 ± 0.0lm	0.47 ± 0.04abcd	6.6 ± 0.2c	436 ± 15ab	82 ± 24b	11.0 ± 0.1abc	6.8 × 10 ⁷ ± 6de
Soil + RP + Pp	7.7 ± 0.01m	0.49 ± 0.04abcd	6.8 ± 0.2bc	447 ± 15ab	96 ± 72ab	10.9 ± 0.23abc	7.5 × 10 ⁷ ± 6cd
Sub mountain undulating region							
Soil	8.33 ± 0.03a	0.39 ± 0.05bcd	4.0 ± 0.2h	237 ± 8.3c	221 ± 42a	8.60 ± 0.90de	1.8 × 10 ⁶ ± 6hi
Soil + Pc	7.84 ± 0.0gh	0.52 ± 0.02ab	5.6 ± 0.2d	241 ± 4.2c	179 ± 42ab	10.4 ± 0.10abcd	9.4 × 10 ⁷ ± 7bc
Soil + Pp	7.85 ± 0.01gh	0.55 ± 0.05a	5.6 ± 0.1d	256 ± 2.4c	193 ± 24ab	9.80 ± 0.10bcde	1 × 10 ⁸ ± 8b
Soil + RP	7.94 ± 0.02f	0.46 ± 0.01abcd	5.3 ± 0.1de	465 ± 8.7ab	179 ± 41ab	9.90 ± 2.80bcde	3.5 × 10 ⁶ ± 9gh
Soil + RP + Pc	7.78 ± 0.02ijk	0.54 ± 0.01a	8.1 ± 0.2a	480 ± 35a	165 ± 87ab	12.1 ± 0.09a	2.3 × 10 ⁸ ± 13a
Soil + RP + Pp	7.76 ± 0.01jkl	0.55 ± 0.02a	8.5 ± 0.3a	504 ± 17a	179 ± 72ab	12.1 ± 0.07a	2.4 × 10 ⁸ ± 7
Two way ANOVA analysis							
Treatments (T)	***	***	***	***	ns	***	***
Sites (S)	**	**	***	***	***	***	***
Interaction (T*S)	***	ns	***	*	ns	ns	***

N.D. Not detected.

Values are mean ± SD (n = 10). Means sharing a common letter within the column are not significantly different at P < 0.05. ns: not significant at P < 0.05; *, **, *** significant at P < 0.05, 0.01 and 0.001, respectively.

Table 3
Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) single or along with rock phosphate (RP) fertilization on rhizosphere soil characteristics of wheat plants grown in Central plain region (Thapar university, Patiala, Punjab), Sub mountain undulating central region (Balachaur, Punjab) and Sub mountain undulating region (Pojewal, Punjab).

Treatments	pH	Organic carbon (%)	Available P (mg kg ⁻¹)	Total P (mg kg ⁻¹)	Organic P (mg kg ⁻¹)	Dehydrogenase activity (ppm)	No. of PSB (CFU g ⁻¹ soil)
Central plain region							
Soil	8.23 ± 0.02b	0.39 ± 0.11bc	3.9 ± 0.09f	225 ± 13b	151 ± 24ab	8.9 ± 0.21gh	2.0 × 10 ⁵ ± 0.6i
Soil + Pc	7.62 ± 0.03ghi	0.47 ± 0.04abc	5.5 ± 0.21cd	238 ± 17b	138 ± 72ab	10.0 ± 0.05ef	8.4 × 10 ⁷ ± 4def
Soil + Pp	7.66 ± 0.01fgh	0.48 ± 0.05abc	5.4 ± 0.31cde	233 ± 8b	124 ± 64ab	10.0 ± 0.13ef	7.7 × 10 ⁷ ± 2def
Soil + RP	8.14 ± 0.05c	0.44 ± 0.05abc	5.05 ± 0.34de	468 ± 15a	124 ± 48ab	9.12 ± 0.05g	4.0 × 10 ⁵ ± 1i
Soil + RP + Pc	7.60 ± 0.02hij	0.48 ± 0.04abc	7.10 ± 0.07b	465 ± 16a	96 ± 42ab	11.3 ± 0.09d	9.8 × 10 ⁷ ± 4d
Soil + RP + Pp	7.59 ± 0.04ij	0.49 ± 0.08abc	7.16 ± 0.07b	466 ± 15a	96 ± 64ab	11.2 ± 0.17d	1.3 × 10 ⁸ ± 12c
Sub mountain undulating central region							
Soil	8.19 ± 0.02bc	0.37 ± 0.07c	3.7 ± 0.11f	213 ± 6b	138 ± 42ab	8.3 ± 0.18h	0.7 × 10 ⁵ ± 0.6i
Soil + Pc	7.73 ± 0.02ef	0.44 ± 0.03abc	5.1 ± 0.26de	211 ± 19b	96 ± 42ab	9.4 ± 0.13fg	6.7 × 10 ⁷ ± 2fg
Soil + Pp	7.74 ± 0.03e	0.45 ± 0.04abc	4.9 ± 0.07de	206 ± 17b	110 ± 64ab	9.3 ± 0.11g	6.9 × 10 ⁷ ± 2efg
Soil + RP	8.0 ± 0.02d	0.43 ± 0.06abc	4.9 ± 0.24e	434 ± 15a	138 ± 72ab	9.10 ± 0.20g	2.7 × 10 ⁵ ± 1i
Soil + RP + Pc	7.70 ± 0.01ef	0.47 ± 0.02abc	6.82 ± 0.13b	429 ± 8a	68 ± 24b	10.5 ± 0.17e	8.4 × 10 ⁷ ± 6def
Soil + RP + Pp	7.67 ± 0.02fg	0.46 ± 0.02abc	6.81 ± 0.18b	426 ± 6a	82 ± 24ab	10.49 ± 0.11e	9.6 × 10 ⁷ ± 7de
Sub mountain undulating region							
Soil	8.30 ± 0.02a	0.46 ± 0.04abc	4.9 ± 0.18de	204 ± 7.2b	207 ± 24a	12.3 ± 0.1c	2.0 × 10 ⁶ ± 5hi
Soil + Pc	7.67 ± 0.02fg	0.55 ± 0.02ab	6.7 ± 0.18b	234 ± 25b	151 ± 48ab	14.0 ± 0.1b	2.1 × 10 ⁸ ± 16b
Soil + Pp	7.61 ± 0.01ghij	0.57 ± 0.05a	6.9 ± 0.18b	245 ± 8.3b	138 ± 42ab	14.2 ± 0.1b	2.2 × 10 ⁸ ± 9b
Soil + RP	8.13 ± 0.02c	0.47 ± 0.08abc	5.9 ± 0.18c	455 ± 6.4a	193 ± 24ab	13.9 ± 0.1b	4.1 × 10 ⁶ ± 6gh
Soil + RP + Pc	7.55 ± 0.03ij	0.57 ± 0.02a	9.1 ± 0.11a	445 ± 8.3a	124 ± 24ab	14.1 ± 0.1b	3.6 × 10 ⁸ ± 25a
Soil + RP + Pp	7.55 ± 0.01j	0.60 ± 0.06a	8.8 ± 0.11a	434 ± 28a	110 ± 24ab	15.6 ± 0.1a	3.8 × 10 ⁸ ± 18a
Two way ANOVA analysis							
Treatments (T)	***	***	***	***	**	***	***
Sites (S)	***	***	***	***	**	***	***
Interaction (T*S)	ns	ns	***	ns	ns	***	***

Values are mean ± SD (n = 10). Means sharing a common letter within the column are not significantly different at P < 0.05. ns: not significant at P < 0.05; *, **, *** significant at P < 0.05, 0.01 and 0.001, respectively.

4. Discussion

The potential of PSBs isolated from rhizospheric soil of organic farming in contrast to conventional farming, in promoting the crop production and soil fertility at different sites is a promising area of research.

Pantoea cyripedii and *Pseudomonas plecoglossicida* were first time reported in our present study for their significant effect on improvement of crop productivity and soil fertility of maize and wheat crop fields at multilocal sites of different agroclimatic regions. The grain yield was improved significantly in both maize and wheat crops due to inoculation treatments. Many reports are available

indicating yield improvement due to inoculation of phosphate-solubilizing microorganisms [19,20]. The effect of PSBs on improvement of yield and P uptake in maize and wheat crop was more pronounced when RP was supplemented along with seed inoculation. This might be due to better utilization of P from the pool of soil nutrients by the action of P-solubilizing bacteria [21]. Khalimi et al. [22] showed that *Pantoea agglomerans* effectively promoted the rice growth and increased the yield.

Rhizosphere colonization by microbial inoculants has been described as a crucial factor for plant growth promotion [23]. Results of present study are also in agreement with this statement. A significant increase in population density of PSBs was observed in rhizospheric soil of maize and wheat plants at different sites in all the treatments compared to control but the RP amended soil showed more pronounced effects compared to control. Addition of RP to the seed inoculation treatment soil raised the total soil population of PSBs, suggested that RP acts as a substrate for the tested PSBs, and is obviously beneficial for proliferation and survival of these isolates. Similar results were also reported by Yu et al. [24] with TCP amendments.

It was apparent from the results that various treatments had inherent potential for the improvement of soil properties especially related to organic carbon and available P that directly affect the crop yield and nutrient uptake. Maximum available P was observed in soil with PSBs inoculation along with RP fertilization treatments. The organic carbon level was significantly increased in all the treatments in comparison with the initial values at all three different regions. Our results are in agreement with Himani and Reddy [9], who reported an increased in soil organic carbon level in bio-inoculated and RP fertilized soil compared control. Increased in organic carbon in seed inoculated and RP amended treatments may be due to the enhanced growth and increase in bacterial population in inoculated treatments than control. The changes in soil organic carbon contents directly associated with changes in microbial biomass and biological activity in soil [25]. All the treatments also showed a drop in pH values of the soil compared to control soil at all the three sites. Inorganic P is solubilized by the action of organic acids secreted by PSBs in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe and Ca) and decreases the pH in basic soils and increases the concentration of phosphorous in soil [26].

Acid phosphatase, alkaline phosphatase, phytase and dehydrogenase enzyme activities were significantly increased in maize and wheat crop fields in inoculated treatments along with RP fertilization compared to control. In the soil, 20–80% of phosphate is in organic form and plants may poorly/not possess an innate ability to acquire phosphorous directly from the phytate [27]. The soil samples of maize and wheat crop field trial were tested for phytase enzyme activities. In all the treatments, phytase activity was more than phosphatase activity that might be due to the higher extracellular phytase activity of bacterial isolates as compared to extracellular phosphatase enzyme activity as reported by Aseri et al. [28]. Simultaneous exudation of phosphatases could increase P solubility, by releasing bound organic phosphates and its mineralization by increasing the rate of hydrolytic cleavage [29]. The Organic P level was decreased in all the treatments as the level of enzyme activities was increased in all the treatments. Results also showed a negative correlation between total organic phosphorous and soil enzyme activities. This showed that phytase, acid and alkaline phosphatase enzymes are significantly transforming the organic form of P into plants available inorganic forms. A strong relationship between phytase activity and depletion of soil organic P has been shown by Yadav and Tarafdar [30]. Tarafdar and Jungk [31] report that the concentration of soil organic phosphorous was depleted in soil within 1 mm of clover and wheat roots with high activity levels of phosphatase and microbial populations.

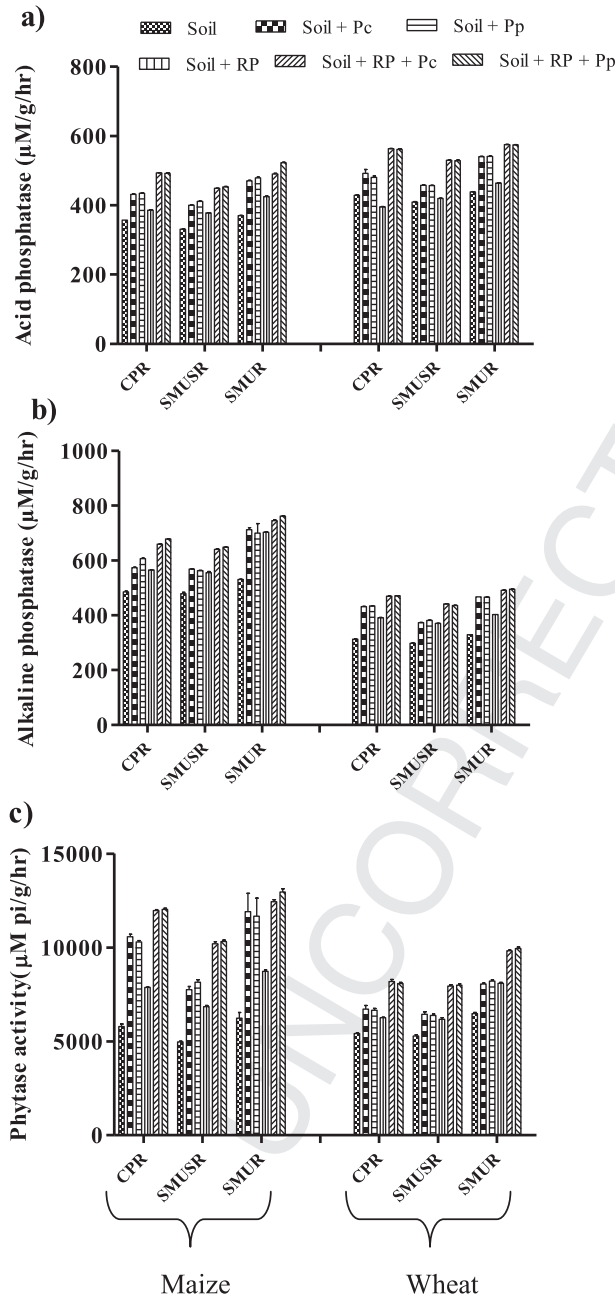


Fig. 3. Effect of *Pantoea cyripedii* (Pc) and *Pseudomonas plecoglossicida* (Pp) single or along with rock phosphate (RP) fertilization on a) acid phosphatase b) alkaline phosphatase and c) phytase enzyme activity of rhizospheric soil of maize and wheat crop grown at Central plain region (CPR), Sub mountain undulating central region (SMUSR) and Sub mountain undulating region (SMUR).

Dehydrogenase activity of soil in all the treatments in maize and wheat crop field at different sites was significantly higher compared to uninoculated control soils. The dehydrogenase activity measured in soil actually represents instant metabolic activities of soil microorganisms [7]. Two year field study of maize and wheat crop showed that considerable crop yield and soil fertility was improved when *P. cyripedii* and *P. plecoglossicida* were applied as bio-inoculants along with RP fertilization at different sites under different agroclimatic regions. This indicated that both the test strains were equally effective in all different sites that are different in their agroclimatic conditions. A very few reports are available on the effect of PSBs as a plant growth promoter and for improvement of soil fertility at different agroclimatic regions. Cakmakci et al. [32] investigated the effectiveness of PGPR in sugar beet at two soils organic matter contents in field and showed that *Bacillus megaterium* and *Pseudomonas putida* has positive effects on growth and sugar yield. The plant growth promotion ability of PGPR inoculations varied with soil organic matter content. Free living bacteria depends on organic carbon content as a food source, addition of organic matter to the soil will enhance the organic carbon content in soil may be increased the plant growth promoting activity of PGPR [32]. Chabot et al. [33] reported that two strains of *Rhizobium leguminosarum* bv. *Phaseoli* solubilizing soil P stimulates the growth of lettuce and maize at three sites having different high to low levels of available P. However no reports are available on the effect of phosphate-solubilizing bacteria along with RP fertilization that showed their effect as a plant growth promoter at different sites comes under different agroclimatic regions with different soil physiochemical properties. Results of present study revealed that the *P. cyripedii* and *P. plecoglossicida* used as bio-inoculants during two years of field study have the ability to adapt to different agroclimatic conditions and equally improve yield and soil fertility in different sites in different agroclimatic regions.

In conclusion inoculation of *P. cyripedii* and *P. plecoglossicida* significantly increased the yield and total P uptake in maize and wheat crop and improved the soil fertility during two year of field study but the effects were more pronounced and significant when RP was supplemented along with seed inoculation. This is an early report on *P. cyripedii* and *P. plecoglossicida* (isolated from an organic field) for their significant effect as a bio-inoculant along with RP fertilization on improvement of yield and soil fertility at different sites in different agroclimatic regions with different physiochemical properties of soil. In long term, this approach would ensure cost effective, sustainable and environmental friendly production system for maize and wheat crop at several sites comes under different agroclimatic regions.

Acknowledgments

The authors are thankful to University Grant Commission (UGC) for financial assistance and TIFAC-CORE for facilities.

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