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 µg 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 µg 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-solubilzation 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 Kuiack 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 moicroorganisms, 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 Txzon-e database revealed that most closely related type strain for PSB-3 is *Pantoea cypripedii* 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. cypripedii* 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. cypripedii* 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 Simine 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 Kuiack 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 *Schwanntomyces 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 $(NH_4)_2SO_4$ 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 Freites 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. cypripedii* 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. cypripedii* 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 μg ml^{-1}) and *Trichoderma harzianum* (68 μg 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 μg 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 hydoxamates, catecholate, 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). Microrganisms 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. cypripedii* 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 multilocational 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), intercroping, 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. correegata* (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. cypripedii*, *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. cypripedii, *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 agrocilmatic 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. cypripedii*, *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. cypripedii*, *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. cypripedii*, *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. cypripedii*, *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 ºC 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 inoulum 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 ºC compared to 37 ºC 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 ºC and 37 ºC. 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 (Rodrı´guez 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. cypripedii*, *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-inoulaion along with RRP fertilization in organic farming and at multilocational 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 cypripedii* (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 increased 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. cypripedii* 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. cypripedii*, *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. cypripedii, 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. cypripedii, 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. cypripedii*, *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. cypripedii*, *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 ºC and 37 ºC) 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.