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 $H_2PO_4^{-1}$ 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.
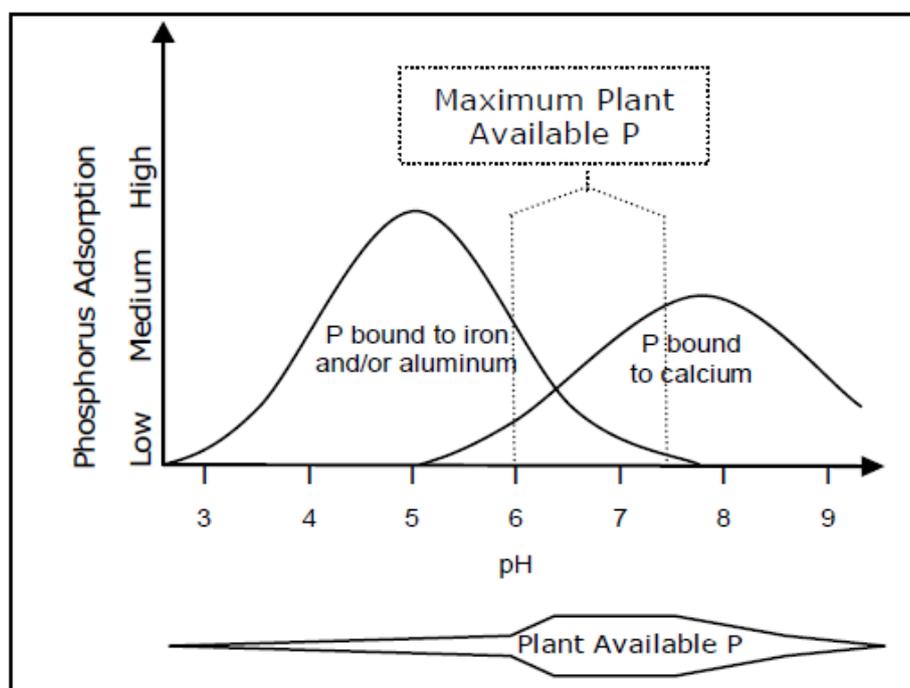

Fig. 2.1 Soil pH impacts P availability (Sauchelli 1951).

2.4 Phosphorus problem in soil

The soils being poor in organic matter (1.5- 4.2 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; $H_2PO_4^{-1}$, HPO_4^{-2} and PO_4^{-3} and generally the availability of these ions to the plants is in the order of $H_2PO_4^{-1} > HPO_4^{-2} > PO_4^{-3}$. 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 $H_2PO_4^{-1}$ 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.
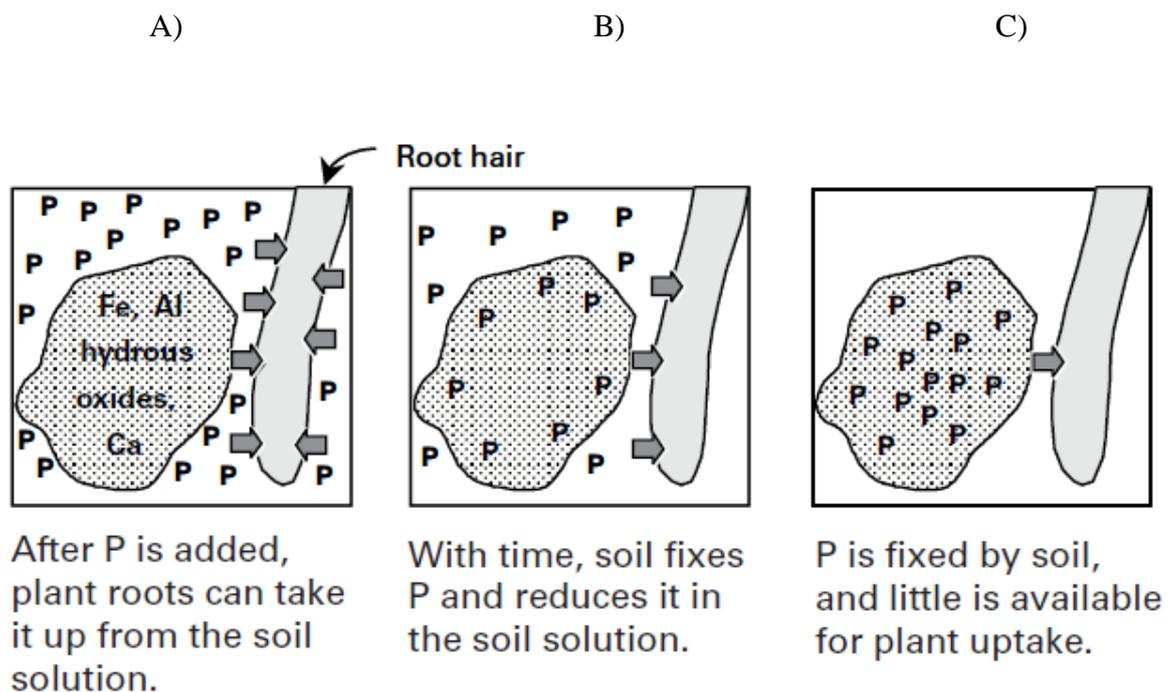

Fig. 2.2 Picture shows how phosphorus (phosphates) is tied up by soil minerals. A) A large percentage of the P is available for root uptake immediately after fertilization application. B) P in solution binds rapidly to the surface of soil minerals. Roots may still use this P. C) eventually; most of the 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 Equation1.

$$Ca_{10}F_2(PO_4)_6 + 12H^+ \rightarrow 10Ca^{2+} + 6H_2PO_4^- + 2F^- \qquad (1)$$
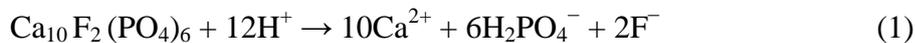
Engelstad et al (1974) found that lower the soil pH, more available the P from RP. The addition of RP will increase total soil P with the potential to replenish labile P and plant-available P. By utilizing microbial mechanisms that can effectively extract P from RP and release it into the soil solution 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 (Gaind 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	
Azospirillium	Seshadri et al. 2000.

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

Penicillium spp.	Whitelaw et al. 1999; Reyes et al. 2002; Pradhan and Sukla 2006; Babana and Antoun 2006. Himani and Reddy 2012.
Trichoderma spp.	Altomare et al. 1999; Zayed and Motaal 2005.
Eupenicillium parvum	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 NH_4^+ 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.
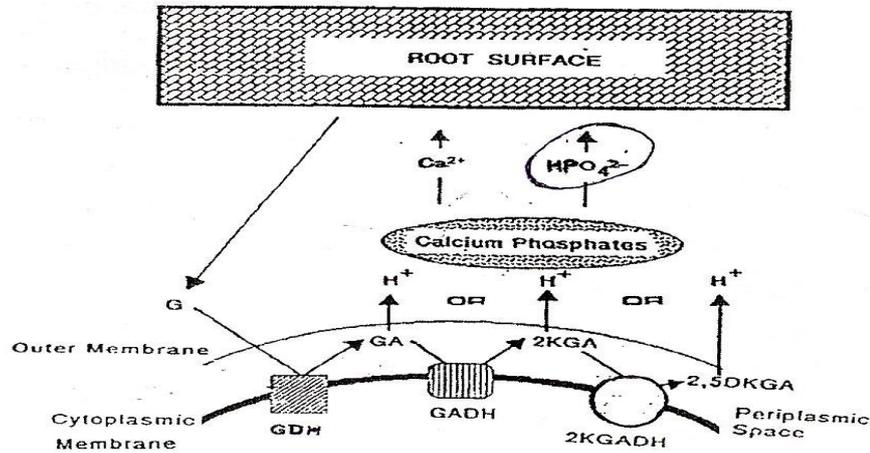

Fig. 2.3 Role of oxidative pathway in release of Pi from tri-calcium phosphate at or near rhizosphere (Goldstein, 1995). G = glucose; GA = gluconic acid; 2KGA = 2-ketogluconic acid; 2,5 DKGA = 2,5 di Ketogluconic acid; GDH = glucose dehydrogenase; GADH = glukonate dehydrogenase; 2KGADH = 2-ketogluconate dehydrogenase.
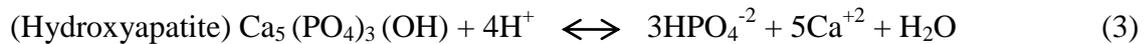
These acids are produced by the pathway depending on the physiological conditions in the periplasmic space and extra cellular environment. Solubilization of phosphate is result of acidification of the periplasmic space which in form, results in acidification of the region adjacent to the cell or colony 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
Aspergillus fumigatus, Bacillus firmus, Micrococcus spp.	Oxalic acid, Tartaric acid, Citric acid	Banik and Dey 1982
Aspergillus sp*., Penicillium* sp*., Bacillus spp.*	Oxalic acid, Succinic acid, Citric acid, 2-keto gluconic acid	Banik and Dey 1983
Aspergillus niger	Succinic acid	Venkateshwarlu et al. 1984
Aspergillus awamori, Penicillium digitatum	Succinic acid, Citric acid, Tartaric acid	Gaur 1990
Aspergillus niger, Penicillium simplicissimum.	Citric acid	Burgstaller et al. 1992
Penicillium bilaji	Citric acid, Oxalic acid	Cunningham and Kuiack 1992.
Aspergillus niger	Citric acid, Oxalic acid, Gluconic acid	Illmer et al. 1995
Pseudomonas cepacia	Gluconic acid, 2-keto-gluconic acid	Bar-Yosef et al. 1999
Aspergillus sp*., Bacillus spp., Arthrobacter* sp*., Enterobacter spp., Pseudomonas spp.*	Succinic acid	Vazguez et al. 2000
Enterobacter intermedium	2-ketogluconic acid	Hwangbo et al. 2003
Burkholderia cepacia	Gluconic acid	Lin et al. 2006.
Aspergillus niger	Citric acid, Oxalic acid, Gluconic acid	Illmer et al. 1995

Pseudomonas cepacia	Gluconic acid, 2-keto-gluconic acid	Bar-Yosef et al. 1999
Aspergillus sp., *Bacillus spp.,* *Arthrobacter* sp., *Enterobacter* *spp., Pseudomonas spp.*	Succinic acid	Vazguez et al. 2000
Enterobacter intermedium	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.

(Di-calcium phosphate) $CaHPO_4 + H^+ \longleftrightarrow H_2PO_4^{-1} + Ca^{+2}$ (2)

(Hydroxyapatite) $Ca_5 (PO_4)_3 (OH) + 4H^+ \longleftrightarrow 3HPO_4^{-2} + 5Ca^{+2} + H_2O$ (3)

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 Fe^{3+} and Al^{3+} 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).

(Strengite) $FePO_4.2H_2O \longleftrightarrow HPO_4^{-2} + chelate\text{-}\{Fe^{3+}\} + OH^- + H_2O$ (4)

(Variscite) $AlPO_4.2H_2O \longleftrightarrow HPO_4^{-2} + chelate\text{-}\{Al^{3+}\} + OH^- + H_2O$ (5)

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 Kpomblekou-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, Kpomblekou-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 (Kpomblekou-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, $CaCO_3$ 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) phosphonatases 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 sulpher 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 on 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 similultaneous solubilization of $411\mu g\ ml^{-1}$ 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: $KNO_3 > (NH_4)_2SO_4 > NH_4NO_3 >$ aspargine > 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 (*Bacillius 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 (*Sacchrum 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
Glomus sp., *Bacillus circulans*, *Cladosporium herbarum*	*Triticum aestivum*	Improved growth and yield	Singh and Kapoor 1998
Rhizobacteria	Wheat and Rice	Increased yield, nutrient uptake and IAA production	Khalid et al. 2004
Bacillus subtilis, Aspergillus awamori, Aspergillus niger, Pseudomonas fluorescens	*Solanum lycopersicum*	Increased nutrient uptake and P bioavailability	Khan et al. 2002
Bacillus cereus MJ-1	Red Pepper	Increased P uptake, and plant biomass	Joo et al. 2005
Pseudomonas sp., *Bacillus* sp., *Aspergillus* sp.	*Gossypium* sp.	Increased P uptake	Narula et al. 2005.

Enterobacterium	*Pisum sativum* and *Cicer arietinum*	Increased P uptake and biomass	Hynes et al. 2008.
Thiobacillus	*Brassica napus*	Increased yield	Salimpour et al. 2010
Aspergillus niger, Aspergilius tubingensis	Wheat, Maize	Increased yield and total P uptake	Richa et al. 2007; Himani and Reddy 2011, 2012.
Pantoea agglomerans	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 P_2O_5 as SSP can safely be replaced with 30 kg P_2O_5 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 P_2O_5

as RP in combination with PSB (*Bacillus magaterium*) was superior to 40 kg P_2O_5 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 pumullus* and *Glomus deserticola* have higher efficiency than chemical phosphorus fertilizer in production of dry weight. Phosphorus is one of the necessary elements (Trolove 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.