CHAPTER 1

Review of Literature and Introduction

Since 1960, the world's population has doubled to 6 billion people and is projected to increase to 8-9 billion people by 2040 (Vance *et al.*, 2000). It is predicted that food production will not keep pace with the growing population demand. In nutritional terms, the greatest limiting factors to increasing world food production are nitrogen (N), phosphorus (P) and potassium (Bieleski, 1973; Vance *et al.*, 2000). Phosphorus is required for the synthesis of key molecules such as nucleic acids, phospholipids, ATP and several other biologically active compounds. It participates directly in generating the biochemical energy necessary to drive virtually every anabolic process within the cell and is a prerequisite in every phase of cellular metabolism (Goldstein, 1995). As currently practiced, it is projected that agriculture will require an additional 40 and 20 Tg (10^{12} g) of N and P fertilizer, respectively to meet the food production needs in 2040 (Bumb and Baanante, 1996; Frink *et al.*, 1999). However, agriculture is threatened by diminishing reserves of both these essential elements. Industrially synthesized nitrogen is in relatively short supply as a direct result of the energy crisis, and phosphate has become recognized as another finite, non renewable resource (Vance, 2001).

1.1: Soil Phosphorus

The concentration of total P in soils ranges from 0.02 to 0.5% (400–1200 mg/kg of soil), the variation largely being due to differences in weathering intensity and parent material composition (kucey *et al.*, 1989). The total soil P can further be categorized into organic and inorganic P. Inorganic P in acidic soils is associated with Al and Fe compounds (Gyaneshwar *et al.*, 2002) whereas calcium phosphates are the predominant form of inorganic phosphates in calcareous alkaline soils.

Organic P also makes up a large fraction, as much as 50% in soils with high organic matter content. Phytate, a hexaphosphate salt of inositol, is the major form of P in organic matter contributing between 50 and 80% of the total organic P (Molla and Chowdary, 1984). It is synthesized by microorganisms and plants and is the most stable of the organic forms of phosphorus in soil. Phytate also tends to accumulate in soils in the insoluble form as a result of forming complex molecules with Fe, Al and Ca. Other organic P compounds in soil are in the form of phosphomonoesters, phosphodiesters including phospholipids and nucleic acids, and phosphotriesters.

1.2: Plant Phosphate Availability In Soil

Despite being present in abundance in the soil, plants throughout the world experience P deficiency. This is because most of the high molecular weight inorganic P compounds must first be converted to either soluble orthophosphate anions (predominantly $H_2PO_4^-$ and HPO_4^{2-}), or low molecular weight organic phosphate, in order to be assimilated by the plants (Rodriguez and Fraga, 1999). Amount of P present in the soil solution, the form directly accessible to plants, even in fertile soils is generally not higher than 10 μM even at pH 6.5 where it is most soluble. Thus, where on an average, most mineral nutrients in soil solution are present in millimolar amounts, phosphorus is present only in micromolar or lesser quantities. These low levels of soluble P are due to high reactivity of free P with calcium (Ca), iron (Fe) or aluminum (Al) that lead to P precipitation.

The amount of P in plants ranges from 0.05% to 0.30% of total dry weight. The concentration gradient from the soil solution to the plant cell exceeds 2,000 fold, with an average free P of 1 μm in the soil solution (Bieleski, 1973; Ragothama, 1999). This concentration is well below the *K*m for plant uptake. Thus, crop yield on 40% of the world's arable land is limited by P availability. Paradoxically, few agricultural soils are actually deficient in total phosphorus present. Most of them contain sufficient reserves of phosphorus to support plant growth if such reserves were made available in forms which plants can assimilate. It is therefore that the phosphate problem is not of the *presence* but its *availability*. At any one time most of the phosphorus present consists of insoluble forms and thus it is not readily accessible to plant roots. In fact, the mineral apatite, the ultimate source of phosphorus in nature, is almost equally abundant in all varieties of igneous rocks, and phosphates are rarely deficient in soils derived from them (Vance, 2001).

To circumvent the problem of P deficiency, chemical fertilizers are added to the soils. The production of chemical phosphatic fertilizers is a highly energy intensive process requiring energy worth US$4 billion per annum in order to meet the global need (Goldstein *et al.*, 1993). The situation is further compounded by the fact that almost 75–90% of added P fertilizer is rapidly immobilized soon after application by precipitation

with Fe, Al and Ca complexes present in the soils (Vig and Dev, 1984) and becomes unavailable to plants. It has been estimated that the P accumulated in agricultural soils by this precipitation is sufficient to sustain maximum crop yields worldwide for about 100 years (Goldstein *et al.*, 1993). Thus over the years, most agricultural soils have accumulated large reserves of phosphorus as a consequence of regular applications of P fertilizers.

The phenomenon of fixation and reprecipitation of P in soil is generally highly dependent on pH and soil type. Thus, in acid soils, phosphorus 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 (Rodriguez and Fraga, 1999). 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). This also results in P loading of prime agricultural land. Runoff from a P loaded soil is a primary factor in eutrophication and hypoxia of lakes and marine estuaries in the developed world. Moreover, the precipitation ability of P has increased fertilizer use by 4-5 fold between 1960 and 2000 and is projected to increase further by 20 Tg $year^{-1}$ by 2030. An even greater reason for concern is that by some estimates inexpensive rock phosphate reserves could be depleted in as little as 60 to 80 years.

The microbial biomass in soil also contains a significant amount of P (typically 10-50 kg P/ha, but as high as 100 kg P/ha) and generally accounts for 2-5% of the total P and around 10-15% of the soil organic P. Importantly, microbial P is a dynamic component of the soil P cycle and is responsive to soil fertility, seasonal conditions and management practices (Richardson, 1994). While the P content of the microbes may vary considerably in relation to microbial C, it is evident that significant pools are maintained even in soils considered to be P deficient for plant growth (Oberson *et al.*, 2001). This indicates that microorganisms in soil are highly efficient in acquiring P to meet their own requirements. In addition, it has been shown that soil microorganisms are capable of readily assimilating P supplied from fertilizer or as plant residues. For instance, McLaughlin et al. (1988) showed that some 25% of P in labeled crop residues was incorporated into microbial biomass within 7 days. A number of studies have highlighted the potential importance of microbial P in providing available P to plants. Seasonal

dynamics indicate that significant amounts of P are released from the biomass in response to soil moisture deficiency and it is estimated that soil microbial P is completely turned over at least annually (He *et al.*, 1997). Rate of P flux through the microbial biomass has been found to be significantly high (Oberson *et al.*, 2001; Oehl *et al.*, 2001).

1.3: Need For Biofertilizers In Plant Phosphate Nutrition

According to Abelson (1999) a potential phosphate crisis looms for agriculture in the 21st century. Indiscriminate and excessive application of chemical fertilizers has led to health and environmental hazards (Khan *et al.*, 2006). Thus, there is a necessity to find alternative strategies that can ensure competitive yields while protecting the health of soils. This new approach to farming, often referred to as sustainable agriculture, requires agricultural practices that are friendlier to the environment and that maintain the long term ecological balance of the soil ecosystem. In this context, use of microbial inoculants (biofertilizers) including phosphate solubilizing microorganisms (PSM) in agriculture represents an environmentally friendly alternative to the conventional chemical fertilization. Any living microorganism which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant can be defined as biofertilizer. A continued exploration of the natural biodiversity of soil microorganisms and the optimization/manipulation of microbial interactions in the rhizosphere represents a prerequisite step to developing more efficient microbial inoculants with phosphate solubilizing ability.

Phosphorus biofertilizers in the form of microorganisms can help in increasing the availability of accumulated phosphates for plant growth by solubilization (Subba Rao, 1982; Goldstein, 1986; Tandon, 1987; Kucey *et al.*, 1989; Richardson, 1994). Under diverse soil and agro-climatic conditions, the organisms with P solubilizing abilities have proved to be an economically sound alternative to the more expensive superphosphates and possess a greater agronomic utility. In addition, the microorganisms with P solubilizing potential can enhance plant growth by increasing the efficiency of biological nitrogen fixation, enhancing the availability of other trace elements such as Fe, Zn etc. and by production of plant growth promoting substances (Kucey *et al.*, 1989).

1.4: Nature Of Phosphate Biofertilizers

Many plants have shown to have benefited from the association with microorganisms under P deficient conditions. This association could result either in better uptake of the available P, or rendering unavailable P sources accessible to the plant. The arbuscular mycorrhizae (AM) belong to the former category and the later category includes various bacteria and fungi isolated for their ability to solubilize insoluble mineral phosphate complexes.

1.4.1: Mycorrhizae

Among soil microorganisms, arbuscular mycorrhizal (AM) fungi have been found to be ubiquitous in agricultural soils and are known to be essential components of sustainable soil-plant systems (Schreiner *et al.,* 2003). AM fungi enhance P nutrition of plants by scavenging the available P mainly due to the presence of their high affinity P uptake systems and the large surface area of their hyphae (Sanders and Tinker, 1973; Hayman, 1974; 1983). Organic acid production by AM and thereby mineral phosphate solubilizing ability has also been reported (Paul and Sundara Rao, 1971; Lapeyrie, 1988). In addition to its ability to increase plant uptake of phosphate (Bolan, 1991), AM fungi are also known to provide micronutrients (Burkert and Robson, 1994), nitrogen (Barea *et al.*, 1991), facilitate soil aggregation (Tisdall, 1994), and act as antagonists against some plant pathogens (Duponnois *et al.*, 2005). Mycorrhiza develop an extensive mycelium, which aids in increasing the surface area of the root thereby increasing the phosphate absorbing sites (Bolan, 1991). Consequently, plants inoculated with AM fungi can utilize more soluble phosphate from rock phosphate as compared to non inoculated plants (Antunes and Cardoso, 1991). However AM fungi are obligate endosymbionts and hence depend completely on carbohydrates produced by the plant roots. Thus, all soil conditions that affect plant growth and physiology also influence fungal activity (Azaizeh *et al.*, 1995). It is now well established that AM fungi have also been shown to modify root functions such as root exudation, change the carbohydrate metabolism of the host plant and influence rhizosphere populations (Hobbie, 1992; Shachar-Hill *et al.*, 1995; Marshner *et al.*, 1997).

Mycorrhizal fungi are also reported to solubilize the mineral phosphates as a result of organic acid production (Lapeyrie, 1988; Lapeyrie *et al.*, 1991) and make iron

phosphates available to developing crops (Bolan *et al.*, 1987). Ectomycorrhizal fungi have been shown to possess phosphatase activity which allows them to release phosphate from soil organic matter such as inositol phosphates (Koide and Schreiner, 1992).

However, the use of AM as phosphate biofertilizer is hindered because AM infection is dependent on the plant P status and due to its inability to be cultured *in vitro* as they are obligate symbionts (Abbott *et al.*, 1984). AM fungi failed to colonize plant roots strongly under phosphorus sufficient conditions (Amijee *et al.*, 1989) and consequently the growth of certain plants has been found to be reduced by AM colonization in the presence of available phosphate (Son and Smith, 1995).

1.4.2: Mineral Phosphate Solubilizing Microorganisms

Contribution of microorganisms in mineral phosphate solubilization (mps) has been known since the beginning of the 20th century (Kucey *et al.*, 1989). PSMs are reported to be ubiquitous but their numbers vary from soil to soil. P solubilizing bacteria constitute about 1-50 % of the soil microbial and are known to out number P solubilizing fungal population (Banik and Dey, 1982; Kucey, 1983; Kucey *et al.*, 1989). Population of P solubilizing bacteria in soil and in plant rhizospheres consists of both aerobic and anaerobic strains, with a prevalence of aerobic strains in submerged soils (Sperber, 1958) The ability of different bacterial species belonging to diverse genera such as *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium, Rahnella, Enterobacter, Citrobacter, Azospirillum* and *Erwinia,* have been shown to solubilize insoluble inorganic phosphate compounds, such as tricalcium phosphate, dicalcium phosphate, hydroxyapatite, and rock phosphate (Khan *et al.,* 2006). Most of the PSMs however, show the ability to solubilize only Ca phosphates. Hence, these PSMs are effective in alkaline vertisol soils which contain Ca phosphate complexes but not in other soils such as alfisols where phosphates are present as Fe and Al ion complexes. Fluorescent Pseudomonads with high phosphate-solubilizing ability have been isolated and characterized from the alkaline and calcium-rich soils with lowP availability in the cold desert region of Lahaul and Spiti in the trans-Himalayas of India (Gulati *et al.*, 2008). There are very few reports of PSMs that can solubilize Fe–P and Al–P (Banik and Dey, 1983; Kucey *et al.,* 1989).

Phosphate solubilizing bacteria have been found to predominate in the rhizosphere as compared to that in the non rhizospheric soil. Metabolically, P solubilizing bacteria from the rhizosphere of various plants are more active than those isolated from the bulk soils (Katznelson and Bose, 1959; Baya *et al.*, 1981). In general, fungal isolates show higher P solubilizing ability than bacteria under laboratory conditions (Gaur *et al.*, 1973; Banik and Dey, 1982; Kucey, 1983). Moreover, the P solubilizing ability in bacteria was shown to be lost upon repeated subculturing while no such activity loss has been observed in the case of P solubilizing fungi (Sperber, 1958; Kucey, 1983).

The P solubilizing ability of PSMs depends on the nature of N source used in the media, with solubilization increasing in the presence of ammonium salts compared to that on nitrate as N source. The extrusion of protons during ammonium uptake, leading to a lowering of extracellular pH could be responsible for this phenomenon (Roos and Luckner, 1984). In some fungi, however, ammonium has been shown to have a negative effect on the P solubilizing ability (Reyes et al., 1999a). In addition, nutrients are also found to influence the P solubilization ability of fungal isolates (Cunningham and Kuiack, 1992). Potassium has been shown to be necessary for optimum P solubilization rates in bacteria other than *Pseudomonas* (Beever and Burns,1980; Illmer and Schinner, 1992), whereas, Mg and Na are important requirements in some fungi (Beever and Burns, 1980).

Table 1.1 summarizes the solubilization ability of different insoluble P substrates by several bacterial species. It is evident from the data that *Enterobacter*, *Rhizobium*, *Pseudomonas* and *Bacillus* species constitutes among the most powerful P solubilizers, while tricalcium phosphate and hydroxyapatite seem to be more easily solubilized as compared to rock phosphate.

1.4.3: Organic Phosphate Mineralizing Microorganisms

In addition to inorganic phosphates, soils contain a major amount of organic phosphates, which can serve as P source for plant growth. However to be able to utilize this form of P by plants as nutrition, it must be first hydrolyzed to inorganic P. Nonspecific phosphatases, which cause dephosphorylation of phosphoester or phosphoanhydride bonds in organic matter; phytases, which specifically release P from

phytic acid; phosphonatases and C–P lyases, that perform C–P cleavage in organophosphonates are some of the enzymes that carry out the mineralization of most organic phosphorous compounds in the soil.

Table 1.1: Total P accumulation in cultures of different bacterial species grown on insoluble mineral phosphate substrate (mg/l)

([1]Gyaneshwar *et al.*, 1999; Rodriguez and Fraga, 1999; [2]Hoon *et al.*, 2003; [3]Hameeda *et al.*, 2006)

Bacteria	P Substrate		
	$Ca_3(PO_4)_2$	Hydroxyapatite	Rock phosphate
[1]*Enterobacter asburiae*	nd	nd	77
[2]*Enterobacter intermedium*	nd	nd	200
Pseudomonas sp.	52	nd	nd
Pseudomonas striata	156	143	22
[3]*Pseudomonas sp.* CDB35	nd	nd	522
Burkholderia cepacia	35	nd	nd
Rhizobium sp.	nd	300	nd
Rhizobium meliloti	nd	165	nd
Rhizobium leguminosarum	nd	356	nd
Rhizobium loti	nd	27	nd
Bacillus amyloliquefaciens	395	nd	nd
Bacillus polymyxa	116	87	17
Bacillus megaterium	82	31	16
Bacillus pulvifaciens	54	65	13
Bacillus circulans	11	17	6
Citrobacter freundi	16	7	5

nd: not determined.

Among them, acid phosphatases and phytases constitutes the enzymes whose activities are known to predominate in the soil as a result of presence of their substrates in high amounts (El-Sawah *et al.*, 1993; Bishop *et al.*, 1994; Feller *et al.*, 1994). Moreover, acid phosphatases contribute significantly in the organic P mineralization, since the pH of most soils ranges in the acidic to neutral values. Microbes are considered to be an important source of phosphatase activity in soil and hence their levels show a substantial increase in the rhizosphere in contrast to the non rhizospheric soils (Tarafdar and Junk, 1987; Garcia *et al.*, 1992; Xu and Johnson, 1995). Soil bacteria expressing a significant level of acid phosphatases include strains from the genus *Rhizobium* (Abd-Alla, 1994), *Enterobacter, Serratia, Citrobacter, Proteus, Klebsiella* (Thaller *et al.*, 1995), as well as *Pseudomonas* (Gugi *et al.*, 1991) and *Bacillus* (Skrary and Cameron, 1998). Organic P mineralizing bacteria in soil have been isolated from the rhizosphere of pasture grasses (Greaves and Webley, 1965). 63% of soil bacteria could grow using phytates as carbon and P source on solid medium under laboratory conditions (Richardson and Hadobas, 1997). The numbers however, dropped to 39–44% when phytate was used as a P source in liquid medium, while a very low proportion could use it as a C source in this condition (Tarafdar and Claassen, 1988). Some soil bacteria capable of releasing P from different organic sources are shown in **Table 1.2**. Acid phosphatase and phytase genes have been cloned and characterized from fungi, plants, and bacteria and shown to improve the P utilizing ability of several plant species (Rodriguez *et al.*, 2006).

Non P solubilizing bacteria also contribute significantly as P biofertilizers because of their ability to take up the sparingly soluble P through their high affinity transporters which can than be made available to plants through mineralization as the bacteria die. P uptake and metabolism has been extensively studied in the model bacterium *Escherichia coli* (Wanner, 1994; 1996). In *E. coli,* P stress, signals a two component regulatory system PhoR and PhoB, in which PhoR is the sensor and PhoB is the cognate positive regulator, which in turn affects the expression of over 400 proteins. Under P starvation conditions, PhoR phosphorylates PhoB, that in turn binds specific DNA sequences called PHO box (Willsky and Malamy, 1976; Wanner and Chang, 1987; Wanner, 1996).

It has been reported that in *Enterobacter asburiae* PSI3, a PSM belonging to the *Enterobacteraciae* family, the enzyme glucose dehydrogenase (Gdh) implicated in P solubilizing ability was under the control of P starvation. Gdh, responsible for gluconic acid production was induced only in the presence of RP (Gyaneshwar *et al.*, 1999). In *Rhizobium*, P limitation has been shown to result in higher P transport rates and the induction of alkaline phosphatase (Al-Niemi *et al.*, 1997). *Rhziobium* has a functional homologue of PhoB (Wanner, 1996), but a PhoR counterpart has not yet been detected. As in *E. coli*, PHO box sequences are also present upstream of the genes regulated by P starvation in *Rhizobium* (Gyaneshwar *et al.*, 2002).

Table 1.2: Phosphate mineralization from organic P substrates by some soil bacteria

(Rodriguez and Fraga, 1999)

Bacterial strain	P Substrate	Enzyme type
Pseudomonas fluorescens	Non specific	Acid phosphatase
Pseudomonas sp.	Non pecific	Acid phosphatase
Burkholderia cepacia	Non specific	Acid phosphatase
Enterobacter aerogenes	Non specific	Acid phosphatase
Enterobacter cloacae	Non specific	Acid phosphatase
Citrobacter freundi	Non specific	Acid phosphatase
Proteus mirabalis	Non specific	Acid phosphatase
Serratia marcenscens	Non specific	Acid phosphatase
Bacillus subtilis	Inositol phosphate	Phytase
Pseudomonas putida	Inositol phosphate	Phytase
Pseudomonas mendocina	Inositol phosphate	Phytase
Pseudomonas fluorescens	Phosphonoacetate	Phosphonoacetate hydrolase
Bacillus licheniformis	D-α-glycerophosphate	D-α-glycerophosphatase
Klebsiella aerogenes	Phosphonates	C-P Lyase

1.5: Plant Growth Promoting Rhizobacteria (PGPR) As Phosphate Solubilizers

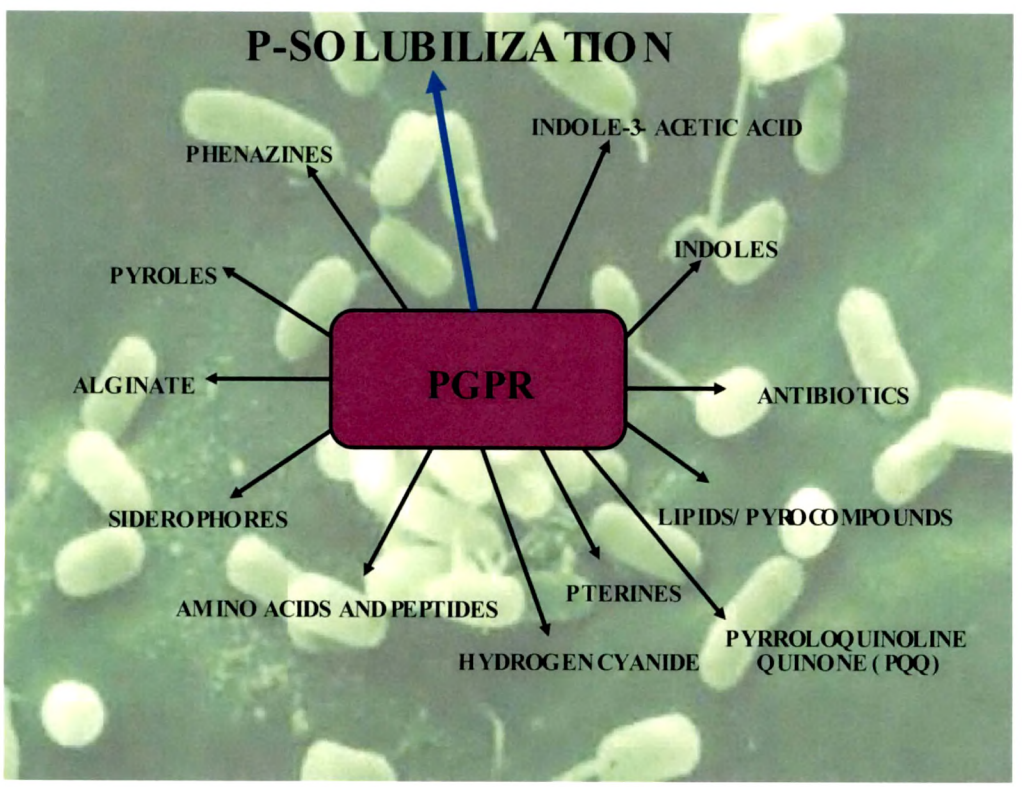
Numerous species of soil bacteria that colonize the rhizosphere of plants and stimulate plant growth by wide array of mechanisms are collectively known as PGPR. The search for newer PGPR and investigating their modes of action are increasing at a rapid pace in order to exploit them commercially as biofertilizers (Vessey, 2003). Several reports demonstrate the huge potential of PGPR as biofertilizing agents for a wide variety of crop plants in a wide range of climatic conditions (Johri *et al.*, 2003). Rhizobacterial diversity is known to significantly influence soil and plant health. The main advantage of developing PGPR as biofertilizer lies in the fact that in addition to conferring the P solubilizing ability, the bacteria can also enhance the plant survival due to additional beneficial qualities. Thus it is possible to merge more than one growth enhancing ability in a single microorganism. Among microbes, fluorescent Pseudomonads has emerged as most versatile PGPRs. Pseudomonads indirectly benefit the plant growth and development by potent biocontrol mechanisms through secretion of antibiotics, siderophores, hydrogen cyanide and other antifungal molecules (Hass and Defago, 2005). For example, *Burkholderia cepacia* simultaneously showed biocontrol activity against *Fusarium* spp. as well as stimulated growth of maize under iron poor conditions by siderophore production (Bevivino *et al.*, 1998). Similarly, *P. fluorescens* PGPR1 was shown to exhibit efficient MPS ability as well as biocontrol properties and thereby lead to the growth promotion and yield enhancement of peanut (*Arachis hypogaea* L.) (Dey *et al.*, 2004).

PGPR are known to enhance the nutrient status of the plants in a variety of ways either individually or in combination by residing in the rhizosphere of the plants. Some of the beneficial traits conferred by PGPRs include biological N fixation, increasing the availability of nutrients in the rhizosphere, inducing increase in root surface area, and enhancing other beneficial symbioses of the host. (Glick, 1995; Rodriguez and Fraga, 1999; Johri *et al.*, 2003).

Fig. 1.1 represents some of the useful growth promoting activities of an ideal rhizobacteria. Production of beneficial metabolites such as phytohormones like indole 3-acetic acid, antibiotics, alginates, siderophore production which help to facilitate the transport of ferric ion are largely responsible for the health of a plant. More recently

Pyrroloquinoline quinine (PQQ) has also been shown to serve as a plant growth promoting factor produced by *Pseudomonas fluorescens* B16 (Choi *et al.*, 2008). P solubilization is one of the important characteristics implicated for a rhizobacteria to be an efficient PGPR.

Fig. 1.1: PGPR as Phosphate solubilizer



Several studies show involvement of PGPRs in making the P available in the plant rhizosphere (Richardson, 2001). *Azotobacter chroococcum* has been shown to solubilize P in the wheat rhizosphere (Kumar and Narula, 1999), while in another report *Bacillus circulans* and *Cladosporium herbarum* showed a similar effect on wheat (Singh and Kapoor, 1999), some of the other beneficial associations include that between *Bacillus* sp. and five crop species (Pal, 1998), *Enterobacter agglomerans* and tomato (Kim *et al.*, 1998b), *Pseudomonas chlororaphis* and *P. putida* and soybean (Cattelan *et al.*, 1999), *Rhizobium* sp. and *Bradyrhizobium japonicum* and radish (Antoun *et al.*, 1998), and *Rhizobium leguminosarum* bv. *Phaseoli* and maize (Chabot *et al.*, 1998).

Although, P solubilizing bacteria are abundant in the plant rhizosphere (Nautiyal *et al.*, 2000; Vazquez *et al.*, 2000) not all show the capability to be categorized as PGPR. Cattelan *et al.* (1999) showed that only two of five rhizospheric isolates that were found to solubilize P, could positively enhance the seedling growth in soyabean. Similarly, some of the P solubilizing PGPR such as *Bacillus* sp. isolates and a *Xanthomonas maltophilia* isolated from canola (*Brassica napus* L.) rhizosphere were shown to increase plant growth performance without increasing P availability to the host plant (de Freitas *et al.*, 1997). A phosphate solubilizing *Pseudomonas putida* (B0) isolated from a sub-alpine Himalayan forest site was shown to exhibit antifungal activity against phytopathogenic fungi and produce chitinase, ß-l,3-glucanase, salicylic acid, siderophore, and hydrogen cyanide. The plant growth promotion and antifungal properties were demonstrated under greenhouse conditions to result in significant increment in plant biomass (Pandey *et al.*, 2006).

Thus, an important pre-requisite for a PGPR to be a P solubilizer is that in addition to improving P uptake by the plants by residing in the rhizosphere they should be able to enhance the plant growth and crop yield by one of the above mentioned properties. Of the 266 rhizobial strains only 54% were found to solubilize P whereas 58% produced indole acetic acid and 83% produced siderophores (Antoun *et al.*, 1998). Of the five *Pseudomonas* isolates that stimulated plant growth, three were found to be positive for ACC deaminase and phosphate solubilization activity, and two were positive for ACC deaminase activity, phosphate solubilization activity, and IAA production indicating that PGPR exhibit multiple modes of actions (Belimov *et al.*, 2001).

1.6: Mechanism Of Phosphate Solubilization

The major mechanism of mineral phosphate solubilization has been identified as the ability to produce and release organic acids by soil microorganisms **(Table 1.3 and 1.4)** (Sperber, 1958; Rodriguez and Fraga, 1999; Goldstein, 1995; Gyaneshwar *et al.*, 2002; Khan *et al.*, 2006). Organic acid secretion leads in the acidification of the surrounding vicinity resulting in the P release from mineral phosphate (Goldstein *et al.*, 1993). Gluconic acid seems to be the most frequent agent of mineral phosphate solubilization produced by bacteria such as *Enterobacter asburiae* PSI3 (Gyaneshwar *et al.*, 1999; Sharma *et al.*, 2005) *Pseudomonas* sp. (Illmer and Schinner, 1992), *Erwinia herbicola* (Liu *et al.*, 1992), *Pseudomonas cepacia* (Babu-Khan *et al.*, 1995) and

Rahnella aquatilis (Kim *et al.*, 1998a). Another potential organic acid identified in strains with P solubilizing ability is 2-ketogluconic acid, which is reported in *Enterobacter intermedium* (Hoon *et al.*, 2003), *Rhizobium leguminosarum* (Halder *et al.*, 1990), *Rhizobium meliloti* (Halder and Chakrabarty, 1993) and *Bacillus firmus* (Banik and Dey, 1982). Gram-positive bacillus such as *Bacillus liqueniformis* and *Bacillus amyloliquefaciens* have been shown to produce mixtures of lactic, isovaleric, isobutyric, and acetic acids. Some of the other organic acids identified to be involved in P solubilization include citric, glycolic, oxalic, malonic, and succinic acid (Khan *et al.*, 2006). The ability of organic acids to solubilize RP was attributed to acidification, chelation, and ligand exchange reactions (Omar, 1998).

Table 1.3: A brief summary of production of principal organic acids by phosphate solubilizing bacteria

(Khan *et al.*, 2006)

Phosphate solubilizing bacteria	Predominant acids
Enterobacter asburiae	Gluconic
Enterobacter intermedium	2-ketogluconic
Bacillus amyloliquefaciens, B. licheniformis, B. atrophaeus, Penibacillus macerans, Vibrio proteolyticus, xanthobacter agilis, Enterobacter aerogenes, E. taylorae, E. asburiae, Kluyvera cryocrescens, Pseudomonas aerogenes, Chryseomonas luteola	Lactic, itaconic, isovaleric, isobutyric, acetic gluconic
Pseudomonas cepacia	Gluconic, 2-ketgluconic
Rhizobium meliloti	2-ketogluconic
P. striata	Malic, glyoxalic, succinic, fumaric, tartaric,α-ketobutyric
Arthrobacter sp.	Oxalic, malonic
Bacillus firmus	2-ketogluconic, succinic
Micrococcus sp.	Oxalic
Bacillus subtilis, Bacillus sp.	Oxalic, succinic, citric, 2-ketgluconic

Table 1.4: A brief summary of production of principal organic acids by phosphate solubilizing fungi and actinomycetes

(Khan *et al.*, 2006)

Phosphate solubilizing fungi and actinomycetes	Predominant acids
Aspergillus flavus, A. niger, Penicillium canescens	Oxalic, citric, gluconic, succinic
A. niger	Succinic
P. rugulosum	Gluconic
P. radicum	Gluconic
P. variable	Gluconic
A. niger	Citric, oxalic, gluconic
A. awamori, A. foetidus, A. terricola, A. amstelodemi, A. tamari	Oxalic, citric
A. japonicus, A. foetidus	Oxalic, citric, gluconic, succinic, tartaric
P. bilaji	Citric, oxalic
A. niger, P. simplicissimum	Citric
A. awamori, P. digitatum	Succinic, citric, tartaric
Penicillium sp.	Oxalic, itaconic
Scwaniomyces occidentalis	Succinic, fumaric, citric, tartaric, α-ketobutyric
A. niger	Succinic
Aspergillus sp., *Penicillium* sp., *Chaetomium nigricoler*	Oxalic, succinic, citric, 2-ketogluconic
Streptomyces	Lactic, 2-ketogluconic
A. fumigatus, A. candidus	Oxalic, tartaric, citric

1.6.1: Acidification

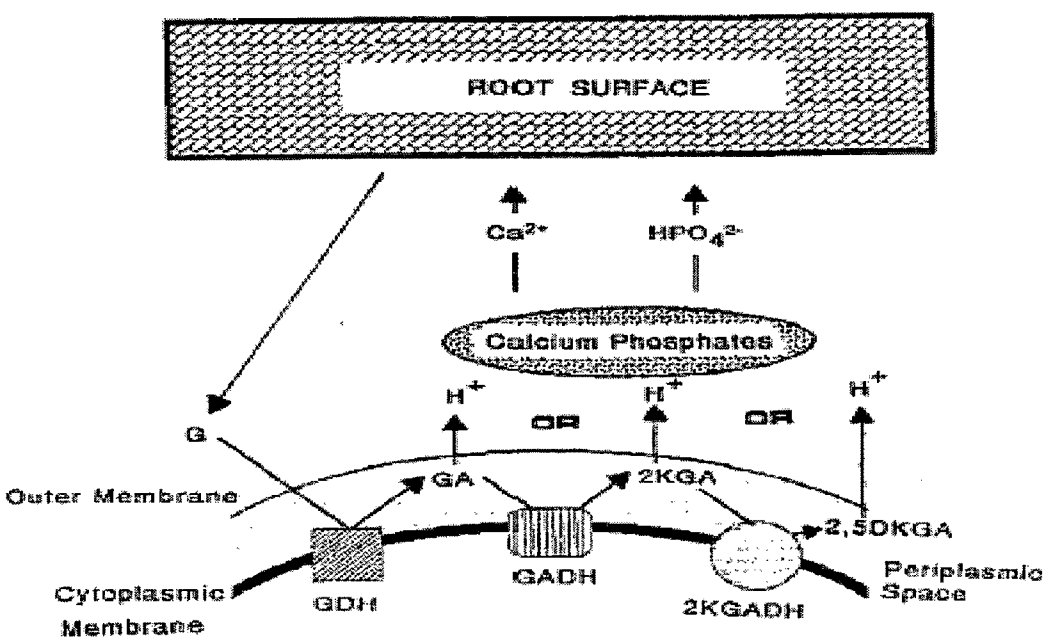
Organic acids produced by the PSMs cause reduction of the solution pH as they dissociate in a pH dependent equilibrium, into their respective anion(s) and proton(s). These H+ ions causes shift in the equilibrium of the dissolution equation thus aiding in

RP solubilization. Organic acids buffer solution pH and continue to dissociate as protons are consumed by the dissolution reaction (Welch *et al.*, 2002).

Goldstein (1995) has proposed that the direct periplasmic oxidation of glucose to gluconic acid, and often 2-ketogluconic acid, forms the metabolic basis of the mineral phosphate solubilization (mps) phenotype in some Gram negative bacteria. Mineral phosphate solubilization is the result of acidification of the periplasmic space by the direct oxidation of glucose or other aldose sugars. The enzymes of the direct oxidation pathway are located in the periplasmic space such that the organic acids may easily diffuse into the extracellular medium. Glucose is first oxidized to gluconic acid by the quinoprotein glucose dehydrogenase. Depending on the bacterial species, gluconic acid may undergo one or two additional $2e^-/2H^+$ oxidations resulting in the production of 2-ketogluconic acid/or 2, 5-ketogluconic acid **(Fig. 1.2).** 2-ketogluconic acid has often been identified as one of the potential acids responsible for bringing about efficient P solubilization among bacteria. It is one of the strongest naturally occurring organic acids with a pKa of 2.4 as compared to a pKa of 3.6 for gluconic acid.

Fig. 1.2: Direct oxidation pathway and its role in P solubilization

(Goldstein, 1995)



1.6.2: Chelation

Mineral phosphates complexed with Ca^{2+} are very easy to solubilize by lowering the pH to 5.0 (Arcand and Schneider, 2006). However, the more complex forms of mineral phosphates such as those with Fe^{2+}, Fe^{3+}, and Al^{3+} cations, which are predominant in acidic soils, are more difficult to solubilize. Simple proton extrusion cannot bring about P release from them. In addition to lowering of medium pH by acidification, organic acids also cause anion dissociation which can bring about RP solubilization through chelation reactions. The organic acid anions, in the form of hydroxyl and carboxyl groups containing oxygen, show higher affinity for the above mentioned cations and thus forms stable complexes with them resulting in the dissociation of P from the RP complex (Kucey, 1988; Jones, 1998).

Kpomblekou and Tabatabai (1994) demonstrated that the ability of organic acids to solubilize RP was affected not only by the chemical structure, but also by the type and position of functional groups in the organic acid. The number and type of functional groups on the organic acid involved as well as the specific cation determined the ability of the chelator anion to form complexes with the mineral phosphate. Organic acids with an increased number of carboxyl groups are more effective at solubilizing RP (Kpomblekou and Tabatabai, 1994; Xu *et al.*, 2004). For example, Ca^{2+} was found to form complexes more readily with tricarboxylic acids such as citric acid, over dicarboxylic acids such as malic and tartaric acids (Whitelaw, 2000). An increased number of hydroxyl (OH^-) groups has been shown to have a positive effect on the ability of an organic acid anion to dissolve RP (Kpomblekou and Tabatabai, 1994). Citric acid, a tricarboxylic acid with one α- and two β-substituted hydroxyl groups, has been shown to be superior to other acids in its RP solubilizing ability (Kpomblekou and Tabatabai, 1994; Xu *et al.*, 2004). Thus, *Penicillium bilaii*, a fungal isolate with the ability to produce significant amount of citric acid, has been reported to be an efficient RP solubilizer (Kucey, 1988; Cunningham and Kuiack, 1992). In addition, *Aspergillus niger*, involved in the industrial production of citric acid, has been recognized as one of the most effective P solubilizing organism (Sperber, 1958; Agnihotri, 1970; Omar, 1998; Abd-Alla and Omar, 2001).

However, there are some reports where oxalic and tartaric acids, which are dicarboxylic acids, have been found to release more P into solution than citric acid

indicating that tricarboxylic acids are not always superior to dicarboxylic acids in RP solubilizing ability. This is attributed to the ability of oxalic and tartaric acids to form poorly soluble precipitates with Ca^{2+}, reducing the solution saturation point (Sagoe *et al.*, 1998).

1.6.3: Ligand Exchange Reactions

Competition of organic acid anions with phosphate anions complexed with Fe^{3+} and Al^{3+} cations, can bring about P mobilization through ligand exchange reactions (Gahoonia *et al.*, 1992; Jones, 1998; Trolove *et al.*, 2003).

Mechanisms other than organic acid production have been implicated for mineral phosphate solubilization when it was observed that not all the time there was a linear correlation between pH drop and P solubilisation (Arcand and Schneider, 2006). Alternative acidification mechanisms involve H^+ release to the outer surface in exchange for cation uptake or H^+ translocation mediated by ATPase (Asea *et al.*, 1988).

1.6.4: H^+ Excretion

The type of N source present in the medium under laboratory conditions is known to influence the RP solubilizing ability of PSMs. In the presence of NH_4^+ as N source, the amount of RP solubilized was much more than when NO_3^- was used and the pH was generally lower while titratable acidity higher (Whitelaw *et al.*, 1999). Moreover, it is a well documented phenomenon in case of fungal systems that H^+ is excreted in exchange for NH_4^+ (Beever and Burns, 1980; Asea *et al.*, 1988). Microbial systems have also been shown to pump out H^+ in response to cation assimilation in a N source dependent manner (Banik and Dey, 1982).

A variety of N sources were tested for their ability to promote the maximum RP solubilization. Ammonium sulphate was found to enhance the RP solubilizing ability in some of the bacillus species such as *Bacillus circulans*, *Bacillus brevis*, and *Bacillus coagulans* (Vora and Shelat, 1998). Fungal isolates like *Penicillium bilaii* and *Penicillium fuscum* were also checked for their ability to solubilize RP in the presence of N source or without N and found that in the medium with NH_4^+, both species were able to decrease pH and solubilize RP. However, only *P. Bilaii* was able to drop the pH and solubilized RP in the absence of N which indicated that different mechanisms were

utilized by the different species of the same genus depending upon the N source (Asea *et al.*, 1988). Some microorganisms also have been shown to release H^+ ions during assimilation of NH_4^+, which serves as the sole mechanism promoting PR dissolution.

In addition to the above mentioned mechanisms, production of chelating substances by microorganisms (Sperber, 1958; Duff and Webley, 1959) as well as the production of inorganic acids, such as sulphidric (Rudolfs, 1922), nitric, and carbonic acid (Hopkins and Whiting, 1916) have also been implicated in the P solubilizing ability of PGPRs.

1.7: Mineralization Of Organic Phosphate

Mineralization of organic phosphorus is the term given to organic phosphate solubilization. Microorganisms are known to bring about organic phosphate degradation but the process is greatly affected by physicochemical and biochemical properties of these organic compounds. Nucleic acids, phospholipids, and sugar phosphates are mineralized easily whereas phytic acid, polyphosphates, and phosphonates are broken down more slowly (McGrath *et al.*, 1995). The main mechanism involves dephosphorylating reactions that hydrolyses the phosphoester or phosphoanhydride bonds with the help of different phosphatases or phosphohydrolases. These phosphohydrolases are either located in the plasma membrane as membrane bound proteins or released extracellularly. This localization facilitates easy access to the high molecular weight organic phosphate molecules such as RNA and DNA that are unable to cross the cytoplasmic membrane (McGrath *et al.*, 1998). In some cases, the DNA or RNA is first degraded to their respective low molecular weight nucleoside monophosphate via RNase and DNase, which is than further degraded by the phosphohydrolases to release P (Goldstein, 1994).

1.8: Biotechnological Approaches For Development Of Phosphate Solubilizing Bacteria

As mentionad above the mineral phosphate solubilizing ability of PSMs is chiefly due to organic acid production. Goldstein (1995) had proposed direct non phosphorylative oxidation of glucose to gluconic acid as a major mechanism for mineral phosphate solubilization in Gram-negative bacteria. The first report of cloning of a gene involved in mps comes from the Gram negative bacteria *Erwinia herbicola* (Goldstein

and Liu, 1987). The gene from *E. herbicola* was cloned into *E. coli* HB101 and it conferred the ability to solubilize hydroxyapatite by production of gluconic acid. *E. coli* on its own does not show gluconic acid production as it only synthesizes the apo-enzyme glucose dehydrogenase (GDH), but lacks the pqq synthase which acts as a cofactor for GDH. However, all *E. coli* strains do not appear to lack entire PQQ synthase gene cluster. *E. coli* K12 derivatives may contain some cryptic PQQ synthase genes.

The DNA fragment from *E. herbicola* contained a 1.8 kb locus that encoded a protein that was found to be similar to the gene III product of a pqq synthesis gene complex from *Acinetobacter calcoaceticus*, and to *pqq*E of *Klebsiella pneumoniae* indicating that the mps phenotype was a result of complementation by the single open reading frame (ORF) (Liu *et al.*, 1992). In another report, a 7.0 kb fragment from *Rahnella aquatilis* genomic DNA allowed hydroxyapatite solubilization in *E. coli* HB101 and DH5α by gluconic acid production. The mps genes expression from *R. aquatilis* into *E. coli* resulted in higher gluconic acid production and better hydroxyapatite solubilization compared to that in the donor strain. The different genetic regulation of the mps genes between the two species might be responsible for this ability. Coincidentally, the nucleotide sequence analysis of this fragment also showed two complete ORFs and a partial ORF. One of the ORFs showed similarity to *pqq*E of *E. herbicola, K. pneumoniae*, and *A. calcoaceticus* (Kim *et al.*, 1998a), while the partial ORF was similar to the *pqq*C of *K. pneumoniae*. Hence, these genes could complement the cryptic *pqq* synthase in *E. coli*, thus allowing gluconic acid production.

Another gene *gab*Y was cloned from *Pseudomonas cepacia* and it also conferred mps phenotype into the *E. coli* via gluconic acid production (Babu-Khan *et al.*, 1995). The deduced amino acid sequence showed no homology with the previously cloned gluconic acid synthesis genes, but instead showed similarity to histidine permease membrane bound components. A DNA fragment from *Serratia marcescens* has also been shown to induce gluconic acid synthesis in *E. coli*, however, no homology was found to *pqq* or *gcd* genes (Krishnaraj and Goldstein, 2001). The authors suggested that this gene acted by regulating gluconic acid production under cell signal effects. A genomic DNA fragment from *Enterobacter agglomerans* confered mps phenotype in *E. coli* JM109 in a pH independent manner (Kim *et al.*, 1997).

mps mutants of *Pseudomonas* sp. were shown to have pleiotropic effects, with apparent involvement of regulatory mps loci in some of them (Krishnaraj *et al.*, 1999). Thus, genetic transfer of any isolated gene involved in mps to induce or improve phosphate dissolving capacity in PGPR strains, would be an interesting approach. An attempt to improve mps in PGPR strains, using this approach, was carried out in our laboratory (Gyaneshwar *et al.*, 1998b). Genes responsible for conferring mps ability were cloned from *Synechocystis* PCC 6803, a unicellular cyanobacterium, which by itself does not show any mps ability. *E. coli* was transformed with the genomic DNA library of the *Synechocystis* PCC 6803 and two mps genes were selected based on the ability of the transformants to show zone of clearance on solid medium containing dicalcium phosphate (DCP) as the sole source of insoluble P. The transformants were isolated using mannitol as the C source under buffered medium conditions. The mps phenotype was found to be plasmid associated as shown by transformation of *E. coli* strain with the isolated plasmids. The transformants also showed DCP and RP solubilization with glucose and glycerol as C source under buffered conditions.

The PQQ synthase gene obtained from *E. herbicola* by Goldstein and Liu (1987), was subcloned in a broad host range vector (pKT230) and the recombinant plasmid was transferred to two PGPR strains namely *Burkholderia cepacia* IS-16 and *Pseudomonas* sp. PSS, using tri-parental conjugation. The transconjugants solubilized tricalcium phosphate which is the first report of heterologous gene expression resulting in improved mps ability to the PGPRs (Rodriguez *et al.*, 2000). Several reports of cloning and overexpression of high affinity P transporters have also been reported in plant, fungal and bacterial systems. A high affinity P transporter from mycorrhizal fungi, *Glomus vermiformis*, was cloned and its expression in resulted in improved P scavenging (Harrison and Van Buuren, 1995). **Table 1.5** summarises some of the functions of the cloned genes involved in mps.

1.9: Beneficial Composite Inoculations For Microbial Phosphate Solubilization

Microbial activities are greatly responsible for the physico-chemical properties of the soil and consist of a vast, complex and interactive community which ultimately determines the plant growth and development. Right from the germination of the seed to its development to a fully grown plant it is in close association with the soil microflora. Thus, it is of prime importance that there exists a mutual beneficial interaction between

microbe-microbe which will ultimately be helpful to the plant survival. Associations of rhizospheric microorganisms greatly influence the plant growth and their synergistic associations are of paramount importance.

Table 1.5: Cloning of genes involved in mineral phosphate solubilization (mps)

(Rodriguez *et al.*, 2006)

Microorganism	Gene or plasmid	Features
Erwinia herbicola	mps	Produces gluconic acid and solubilizes mineral P in *E. coli* HB101. Probably involved in PQQ synthesis
Pseudomonas cepacia	*gab*Y	Produces gluconic acid and solubilizes mineral P in *E. coli* JM109. No homology with PQQ genes
Enterobacter agglomerans	pKKY	Solubilizes P in *E. coli* JM109. Does not lower pH
Rahnella aquatilis	pK1M10	Produces gluconic acid and solubilizes mineral P in *E. coli* DH5α. Probably related to PQQ synthesis
Serratia marcescens	pKG3791	Produces gluconic acid and solubilizes mineral P
***Synechococcus* PCC 6803**	*ppc* gene	Synthesizes phosphoenol pyruvate carboxylase

PQQ- pyrroloquinoline quinone.

1.9.1: Efficacy Of Coinoculation Of Phosphate Solubilizing And Nitrogen Fixing Bacteria On Plant Growth

Nitrogen and phosphorus are the two major nutrients required for plant growth. Combined inoculation of N fixing bacteria and PSM may therefore be an ideal interaction for better plant development than using single organisms. N fixing bacteria and PSM when inoculated together, have been shown to colonize the rhizosphere and enhance legume growth by providing them with nitrogen and phosphate, respectively (Gull *et al.*, 2004). Similarly, when P solubilizing strains of *Pseudomonas striata* and

Bacillus polymyxa were coinoculated with a strain of *Azospirillum brasilense* which is capable of fixing N, a significant improvement of grain and dry matter yields was observed, along with a simultaneous increase in N and P uptake, in contrast to that when both the strains were inoculated individually (Alagawadi and Gaur, 1992). *Agrobacterium radiobacter* with P solubilizing ability upon coinoculation with N fixing *Azospirillum lipoferum* was shown to enhance the grain yield of barley significantly as compared to single inoculations under greenhouse conditions (Belimov *et al.*, 1995). In another report, interaction of P solubilizing *Pseudomonas striata* with N fixing *Bradyrhizobium* was shown to greatly enhance the yield of green gram (Khan *et al.*, 1997) and chickpea (Algawadi and Gaur, 1988) than that obtained by the use of *Rhizobium* alone. Furthermore, beneficial mixed culture inoculation has also been observed between *Pseudomonas striata*, *Bradyrhizobium* sp. and *Mesorhizobium ciceri* (Sarojini *et al.*, 1989). Similar interaction was observed between *Laccaria laccata*, *Agrobacterium radiobacter* and beech roots which influenced the P, K, Mg and Fe mobilization from soil and improved plant growth (Leyval and Berthelin, 1989). Similar result was obtained when phosphate solubilizing strain of *Agrobacterium* was cocultured with N fixing *Rhizobium* in the case of French bean (*phaseolus vrisulga*). Thus it could be suggested that mixed culture inoculants or coinoculation are more preferred over single inoculation treatments in providing balanced nutrition for the plant growth. This evidence points to the advantage of the mixed inoculations of PGPR strains comprising P solubilizing bacteria.

Synergistic interactions between N fixing microorganisms and PSMs not only improves N and P status of the soil but is also highly beneficial to the plants. There are several reports of coinoculations of *Rhizobium* and P solubilizing fungi. *Aspergillus awamori* was shown to enhance chickpea crop yield under field conditions (Dudeja *et al.*, 1981). Synergistic interactions between three P solubilizing fungi: namely, *Aspergillus niger*, *A. fumigatus* and *Penicillium pinophilum* and N fixing *Rhizobium leguminosarum* biovar viciae showed significant increase on the growth and yield of *Vicia faba* along with improved uptake of nitrogen and phosphorus (Mehana and Wahid, 2002). However, in contrast to several beneficial interactions between P solubilizing rhizobacteria and N fixing bacteria that contributed significantly to the plant growth and yield, there are a few reports of negative effects of this cointeractions. When phosphate solubilizing *Penicillum baliji* was cocultured with *R. phaseoli* in autoclaved soil, they

showed no significant increase in growth and crop yields of beans or total uptake of phosphate (Kucey, 1987). Moreover, a reduction in total N fixation was seen in peas due to dual inoculation of *P. bilaji* and *Rhizobium leguminosarum* (Downey and Van Kessel, 1990). This negative effect of P solubilizing *P. bilaji* on N fixation can be attributed to the strong organic acid producing ability of the fungus as rhizobia are known to require neutral or alkaline pH during nodulation (Venkateswarlu *et al.*,1984).

1.9.2: Efficacy Of Coinoculation Of Phosphate Solubilizing Microorganisms And Arbuscular Mycorrhizal Fungi On Plant Growth

AM fungi are ubiquitous in the soil and survive in symbiosis with the plant where the plant provides nutrients to the AM fungi via root exudates which in turn increase the P availability to the plants (Jeffries, 1987). AM fungi increase their mycelial growth towards the presence of P source, far away from the root thus allowing P quenching from areas beyond the reach of the plant root rather than solubilizing P from the RP ores present in the nearer vicinity by secreting organic acids (Remy *et al.*, 1994). Thus if a P solubilizing microorganism can interact with AM fungi, the synergistic relationship can be even more beneficial to the plant. PSMs can release some P from otherwise sparingly soluble P sources, which can be taken up by the high affinity P transporters of AM fungi and translocated to the plant.

It has been shown that in the soils with RP, the survival of PSM increases in the presence of AM fungi, in contrast to the absence of mycorrhizal roots and stimulates plant growth and P uptake. It has also been shown that the rhizospheric microbial population is affected in response to the development of AM fungus on the plant roots due to altered root exudation in phosphorus deficient soils (Ames *et al.*, 1984).

RP application or inoculation with P solubilizing rhizobacteria was shown to improve root infection of AM fungi. When *Glomus fasciculatum* was coinoculated with P solubilizing and N fixing *Azotobacter chroococcum,* it was shown to stimulate root infection of AM fungi, enhance plant growth, and increase nitrogen, phosphorus uptake in maize and wheat (Elgala *et al.*, 1995). Similarly, enhancement in the yield of wheat plants was observed as a result of dual inoculation of RP solubilizing fungi such as *Aspergillus niger* and *Penicillium citrinum* and *Glomus constrictum* (Omar, 1998).

1.9.3: Efficacy Of Coinoculation Of Phosphate Solubilizing Microorganisms, Nitrogen Fixing Bacteria And Arbuscular Mycorrhizal Fungi On Plant Growth

An ideal consortium for promoting plant growth would consist of a N fixing bacteria, a P solubilizing PGPR and AM fungus. Coinoculation of plants with the above mentioned combination could improve N and P status of the soil thereby enhancing plant growth and crop yields. On the other hand, AM fungus could enhance the accessibility of P to the plants. Association of PSMs and AM fungus together improves P solubilization and uptake resulting in higher P concentration in the plant which in turn benefits N fixing bacteria thus leading to an increase in their N fixing ability which in turn enhances mycorrhizal and root development. Coinoculation of *Bradyrhizobium japonicum*, AM fungus and P solubilizing microbes was shown to drastically increase root colonization and nodulation in soybean (Singh and Singh, 1993) whereas no significant response of soybean to dual inoculation was observed (Kloepper *et al.*, 1980). Inoculation of *Rhizobium*, *Bacillus polymyxa* and *Glomus faciculatum* resulted in significant increase in the crop production and P uptake (Poi *et al.*, 1989).

Addition of RP along with triple inoculation of *Glomus mosseae*, *Bacillus* sp. and *Rhizobium* sp was shown to dramatically influence *Pisum sativum* cultivation. Similarly, combined inoculation of *Rhizobium* and *Glomus etunicatum* along with RP application in presence of PSM was shown to significantly increase the yield of clovers (Leopold and Hofner, 1991), whereas inoculation of PSM and AM fungus along with *Rhizobium* resulted in increased nodulation in mungbean (Zaidi *et al.*, 2004) and cowpea (Thiagarajan *et al.*, 1992). Thus, a complex positive interaction was found to exist between the three microorganisms, which resulted in increased available soil phosphate, grain yield, nodulation efficacy, nitrogen uptake, root colonization, phosphorus uptake and growth promotion (Zaidi *et al.*, 2003; Zaidi and Khan, 2005).

1.10: Factors Affecting The Survival Of Phosphate Solubilizing Microorganisms In Field Conditions

The survival of a P solubilizing microorganism in natural conditions is known to be influenced by a variety of biotic and abiotic factors (Bashan *et al.*, 1995). Though a lot is known about the performance of a PSM in the natural field conditions, it is difficult to predict the behavior and efficacy of the inoculated PSM in a particular climatic and environmental location. It has been observed that the population or density of artificially

inoculated PSM rapidly decreases in the soil (Ho and Ko, 1985). The variations in the effectiveness of the inoculated PSMs on P availability to the plants can be attributed to several factors such as:

1) Survival and colonization of inoculated PSM in the rhizosphere
2) Competition with the resident native rhizobacteria
3) Nature and properties of soils and plant varieties
4) Insufficient nutrients in the rhizosphere to produce enough organic acids to solubililize phosphorus
5) Physiological status, temperature, pH, moisture content (Van Elsas *et al.*, 1991)
6) Presence of natural plasmids (Van Veen *et al.*, 1997)

Thus, despite the fact that PSMs are present in abundance in the rhizosphere as well as the bulk soils, phosphorus is still one of the major factors limiting the plant growth and crop yields. Several doubts arise regarding the efficacy of PSM to solubilize P under natural environmental conditions. An important question to be asked is, Why is phosphorus limited for plants when PSM are abundant in soils? Understanding of this part of the use of PSM is the most limiting factor in its efficacy as a P solubilizer.

It was found that PSMs which showed high P solubilizing ability under laboratory conditions failed to release P in alkaline vertisol soils. This failure to solubilize P could not be overcome even when the soil was supplemented externally with carbon and nitrogen sources. The inability of the PSM to solubilize mineral phosphorus was attributed to the high buffering capacity of the alkaline vertisol soils (Gyaneshwar *et al.*, 1998a). Another major reason is the inability of the PSMs to produce high concentrations of organic acids in the field conditions. The nature and type of organic acid secretion plays an important role in determining the efficacy of a PSM to solubilize P. The nature and type of organic acid synthesized by the PSM is influenced by the type of carbon source utilized by the PSM and the nature and type of carbon source utilized by the PSMs is in turn influenced by the nature and quantity of the root exudates since it is a fact that the rhizobacteria utilize the root exudates as the source of carbon and energy. However, in the natural conditions there exists very little scope to manipulate the type of carbon source availability for the PSMs. Thus, use of proper screening strategies such as incorporating buffering ability into the screening medium which mimicks the

alkaline vertisol buffering conditions would be highly beneficial in selecting for the PSMs with high concentration of organic acid producing ability.

Along with sugars, several weak organic acids of the tricarboxylic acid cycle form an integral part of plant root exudates. These organic acids are preferentially utilized over sugars as C sources by some bacterial genera. Thus, the type of bacterial strain used can also have a drastic effect on the P solubilizing ability of any PSM. As a result the organic acid producing ability of the PSMs in the soils could be repressed. This is due to the fact that bacteria differ in their metabolic activities. The phenomenon of carbon catabolite repression which allows preferential utilization of carbon sources when a mixture of them are present could also be one of the major causes of variable response of bacterial inoculations on plant growth.

Thus for widespread application of PSMs for sustainable agriculture, there is a need to develop strategies which can impart or enhance the efficiency of P solubilization to the existing rhizobacteria under field conditions.

1.11: Strategies For Developing Efficient Phosphate Solubilizing Rhizobacteria

Majority of the PSMs are isolated using glucose as the carbon source and hence this results invariabily in isolation of gluconic acid producing PSMs. In soils, however, glucose is not the major source of carbon and energy. Thus, one of the approaches could be to screen for bacteria with an ability to utilize alternative carbon sources such as sucrose for organic acid secretion and P solubilization since sucrose is by far the most commonly found disaccharide in nature with its distribution being universal among photosynthetic plants.

Genetic engineering can help overcome the various factors that affect the field performance and enhance the efficacy of the PSM under soil conditions. Genetically engineered *E. coli* with P solubilizing ability along with some rhizobacterial strains have been reported **(Table 1.5).** Transferring the mps ability to various bacteria that are competent of colonizing a particular rhizosphere by genetic engineering would be an important approach for developing efficient PSMs under natural conditions. Thus, an alternative approach would be to screen for the mps genes randomly by using a cDNA library which allows for heterologous overexpression of genes, followed by the selection of transformants with mineral phosphate solubilizing ability under buffered conditions.

1.12: The Objectives Of The Present Study Involves

(i) Biochemical characterization of variation in the nature of organic acid secretion and rock phosphate solubilization by rhizobacteria, *Citrobacter* sp. DHRSS on various carbon sources

(ii) Repression of mineral phosphate solubilising (mps) phenotype in the presence of weak organic acids in plant growth promoting fluorescent Pseudomonads

(iii) Screening of *Arabidopsis thaliana* cDNA library for mineral phosphate solubilizing (mps) ability

Fig. 1.3: **Global distribution of phosphorus in soil**

(Fairhurst *et al.*, 1999)