CHAPTER 1

Introduction and Review of literature

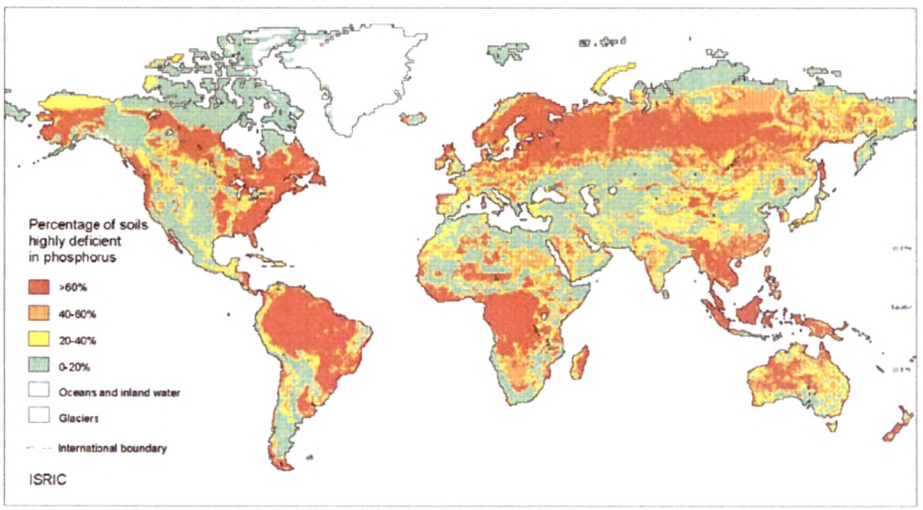
1. INTRODUCTION

Demand which is placed on agriculture for future supply of food will be one of the greatest challenges of 21th century with the increase of human population. The effort to focus on the soil biological system and agro-eco system as a whole has become a necessity in order to understand complex interactions governing the stability of agricultural land so as to meet the problem of this food insecurity. Green revolution especially in developing countries like India had led extensive use of chemical fertilizers and other chemicals which have resulted in reduction in soil fertility and to environmental degradation (Gyaneshwar et al., 2002). The chemical fertilizers usage has gone to theoretical maximum beyond which there will be no further increase in yields (Ahmed, 1995). According to some reports 8-9 billion people will be added by 2040 necessitating the need for second green revolution since food production will not keep pace with the growing population demand (Leisinger, 1999; Vance et al., 2000). After nitrogen, phosphorous is the chief macronutrient required for plant growth and crop yield (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).

1.1 P STATUS IN SOILS – INORGANIC AND ORGANIC P

The concentration of Phosphorus in many rocks is very less. Total phosphorous concentration in soil ranges from 0.02 to 0.5% (400–1200 mg kg^{-1} of soil) depending upon the weathering of rocks and other environmental factors (Kucey et al., 1989). P in soil exits in inorganic and organic forms. Although most of the 40% arable soils are not limited by P abundance (**Fig. 1.1**), they do not support optimal plant growth due to P limitation. Inorganic P in soil is present as salts of calcium in case of calcareous alkaline soils while Al and Fe form predominant in acidic soils (Gyaneshwar et al., 2002). Most inorganic soil P is made up of poorly soluble mineral phosphate precipitates. Soluble P also gets adsorbed to the soil particles making them in accessible to plants. Although most soils contain large amounts of total phosphorus, they are deficient in phosphate available to plants. As indicated in the study of Fairhurst et al., (1999) more than 60% of the soils are highly deficient in phosphorus.

Fig. 1.1: Global distribution of phosphorus in soil (Fairhurst et al., 1999)



1.1.1 Organic P

Organic phosphate constitutes about 20-50% of the total phosphate available in the soil and is an important potential source of P for plants in most agricultural soils (George et al., 2005). The three principal groups of organic P compounds are the inositol phosphates (e.g. phytate), phospholipids (e.g. lecithin) and nucleic acids (e.g. RNA) and their degradation products or derivatives (Stevenson and Cole, 1999). Insoitol phosphates (IPx where x = 1-6) are found widely in the natural environment and major form is phytic acid (myoinositol hexakisphosphate). They are synthesized in terrestrial ecosystems by plant soils, where they accumulate to form the dominant class of organic phosphorus (Turner et al., 2002). Soil phytate is mainly contributed by decaying seeds, pollen and other vegetative tissues, and undigested phytic acid from plants in the manure of non-ruminant animals. The availability to plants of various forms of organic P in soil depends upon their liability to phosphatase enzymes released by plant roots or microorganisms in soil. Availability of these forms to plants decrease with lecithin > RNA > phytate (Stevenson and Cole, 1999).

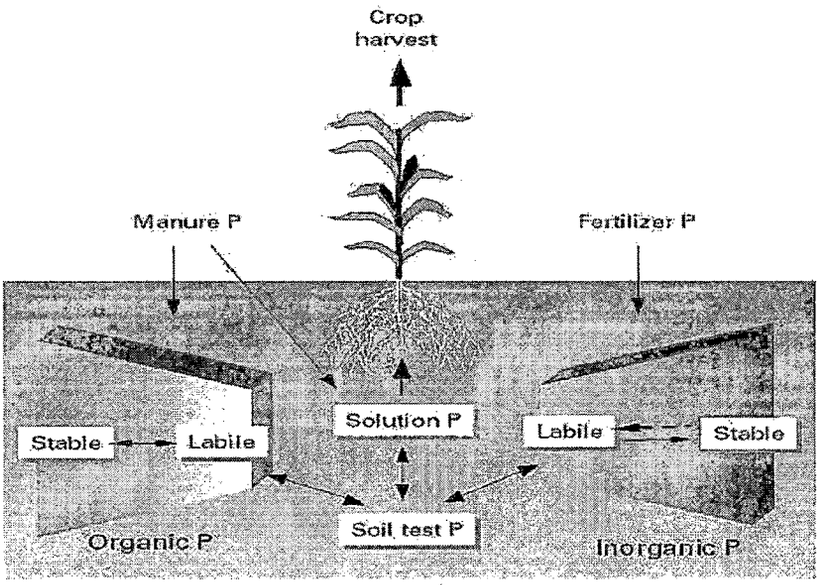
1.2 PHOSPHOROUS AVAILABILITY TO PLANTS

Phosphorous being the 10^{th} most abundant element on earth and the agricultural soils are postulated to contain enough amount to sustain the agriculture for 100years

(Goldstein, 1993). In majority of soils, including the soils categorized as highly fertile, P limits plant growth. Soluble P even in fertile soil is in micro molar ranges as compared to other elements which are present in millimolar concentration (Khan et al., 2006). Soluble P in the form of $H_2PO_4^-$ and HPO_4^{2-} is only taken up by the plants. Hence, inorganic and organic P complexes need to be converted to either soluble orthophosphate anions or low molecular-weight organic phosphate (Rodriguez and Fraga, 1999).

Only 10% of the applied chemical fertilizer is available to the plants rest gets fixed due to interaction with metals such as Al, Ca and Fe (Vig and Dev, 1984; Stevenson, 1986). 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, where the concentration of P in plants cells is around 10mM. Phytate has dense negative charges, hence they are precipitated as insoluble salts with positive charged metal ions (e.g. Fe, Al and Ca phytate) or adsorbed into soil colloids, thus rendering them highly invulnerable to biodegradation (Celi et al., 1999; Tang et al., 2006). In fact, only a limited number of soil bacteria can utilize soil phytate as a sole source of C and P (Richardson and Hadobas, 1997). Conversion of unavailable to available forms of soil P usually occurs too slowly to meet crop P requirements (**dashed line on Fig. 1.2**).

Fig. 1.2: The P cycle in soil



1.3 NEED FOR BIOFERTILIZERS IN PLANT PHOSPHATE NUTRITION

Excessive addition of phosphate to sustain the plant growth and crop yield has resulted in degradation in health of the soil and other environmental problems such as eutrophication, and hypoxia of lakes and marine estuaries (Bennett et al., 2001). Along with environmental hazards and soil health, economical burden has also led the agronomists to find an alternate for chemical fertilizers which are environmental friendly, economical and can sustain agriculture for long time (Khan et al., 2006). Therefore the use of microbial inoculants (biofertilizers) which included phosphate solubilizing microorganisms (PSMs) in agriculture represents an environmentally friendly alternative to the conventional chemical fertilization.

Phosphorus biofertilizers in the form of microorganisms can help in increasing the availability of accumulated phosphates for plant growth by solubilization (Subba Rao, 1982; Tandon, 1987; Kucey et al., 1989; Goldstein, 1993; Richardson, 1994). 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; Vessey, 2003).

1.4 NATURE OF PHOSPHATE BIOFERTILIZERS

1.4.1 PSMs

PSMs are ubiquitous, and their numbers varies depending upon the soil (**Fig. 1.2**). In soil, P-solubilizing bacteria (PS bacteria) out numbers P solubilising fungi (PS fungi) with a factor of 2-150 fold (Banik and Dey, 1982; Kucey, 1983, 1989). P solubilising bacteria constitute 1–50% and fungi 0.5%–0.1% of the total respective population. However, these PSMs may be effective even in these soils when supplemented with rock phosphate (RP) (Kucey, 1983). Most P-solubilizing bacteria were isolated from the rhizosphere of various plants and are known to be metabolically more active than those isolated from bulk soil (Katznelson and Bose, 1959; Baya et al., 1981). Among the bacterial genera with P solubilising capacity are *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium,*

Microccocus, Aereobacter, Flavobacterium, Rahnella, Enterobacter, Azospirillum and *Erwinia.* In general, fungal isolates exhibit greater P-solubilizing ability than bacteria in both liquid and solid media (Kucey, 1983; Gyaneshwar et al., 2002; Khan et al., 2006). Among the fungal genera with this capacity are *Aspergillus, Penicillium,* and *Rhizopus.*

The P-solubilizing ability of PSMs also depends on the nature of N source used in the media, with greater solubilization in the presence of ammonium salts than when nitrate is used as N source. This has been attributed to the extrusion of protons to compensate for ammonium uptake, leading to a lowering of extracellular pH (Roos and Luckner, 1984). In some cases, however, ammonium can lead to decrease in P solubilization (Reyes et al., 1999). In addition, other media components were also found to affect the P solubilization ability (Cunningham and Kuiack, 1992).

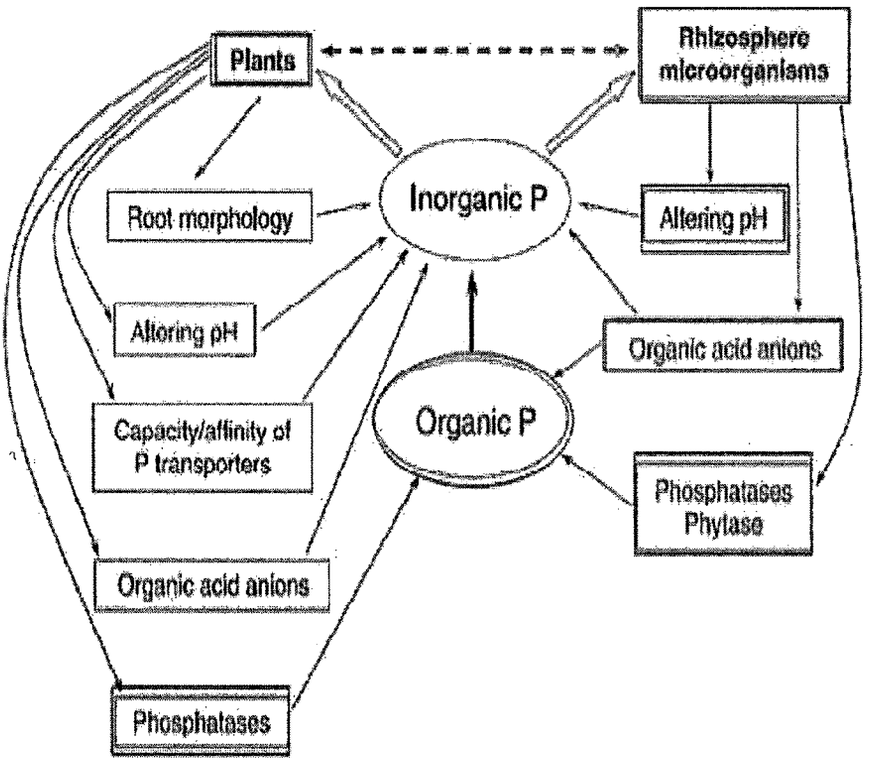
1.4.1.1 Mechanism of P Solubilisation

1.4.1.1.1 Acidification

PSM ability to solubilize insoluble phosphates has been attributed to reduce the pH of their surroundings either by the release of organic acids or protons (Gyaneshwar et al., 1999; Whitelaw, 2000; Patel et al., 2008) (**Fig. 1.3**). The organic acids secreted can either directly dissolve the mineral phosphate as a result of anion exchange of PO_4^{2-} by acid anion i.e. acid hydrolysis, chelation of both Fe and Al ions associated with phosphate or by competing with phosphate for adsorption sites in soil (Gyaneshwar et al., 2002; Khan et al., 2006). PSMs secrete variety of organic acids when grown on simple carbohydrates such as gluconic acid (GA), oxalic, citric, malic, etc. (**Table 1.1 A and B**). In certain cases phosphate solubilization is induced by phosphate starvation (Gyaneshwar et al., 1999). Among nodule bacteria (e.g., *Rhizobium/ Bradyrhizobium*), the phosphate-solubilizing activity of *Rhizobium* was associated with the production of 2-ketogluconic acid which was abolished by the addition of NaOH, indicating that the phosphate-solubilizing activity of this organism was entirely due to its ability to reduce the pH of the medium (Halder and Chakrabarty, 1993). Inorganic acids such as hydrochloric acid can also solubilize

phosphate but they are less effective compared with organic acids at the same pH (Kim et al., 1997).

Fig 1.3: Plant- microbe mechanism to increase Phosphorus availability in rhizosphere (adapted from Richardson, 1994).



1.4.1.1.2 Chelation

Acidification, however, does not seem to be the only mechanism of solubilisation. The chelating ability of organic acids is also important, as it has been shown that the addition of 0.05M EDTA to the medium has the same solubilizing effect as inoculation with *Penicillium bilaii* (Kucey, 1988). 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 (Kpomblekou and Tabatabai 1994; Xu et al. 2004). 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). *P. bilaii*, a citric acid producer and *Aspergillus niger*, an industrial citric acid producing strain, have been effective P solubilizers (Sperber 1958; Agnihotri, 1970; Kucey, 1988; Cunningham and Kuiack, 1992; Omar, 1998; Abd-Alla and Omar, 2001). Apart from tricarboxylic acids, dicarboxylic acids are also show potent RP solubilizing ability. Oxalic and tartaric acids release more P into solution than citric acid which was attributed to the fact that oxalic and tartaric acids form poorly soluble precipitates with Ca^{2+}, effectively lowering the solution saturation point (Sagoe et al., 1998). Oxalic acid is also better in releasing P from alfisols than citric acid (Srivastava et al., 2006).

1.4.1.1.3 H^+ excretion

Microbial excretion of H^+ occurs in response to the assimilation of cations, has also been attributed to solubilisation of insoluble P. This phenomenon is well documented in fungi which excrete H^+ in exchange for NH_4^+ (Beever and Burns 1980; Banik and Dey, 1982; Asea et al., 1988). This is also been shown under laboratory conditions, where it has been observed that more RP is solubilized using NH_4^+ rather than NO_3^- as a source of N and therefore pH was generally lower but higher titratable acidity, on NH_4^+ (Whitelaw et al., 1999). Similarly, ammonium sulphate was found to promote RP solubilization for bacterial species *Bacillus circulans*, *Bacillus brevis*, and *Bacillus coagulans* on a variety of N sources (Vora and Shelat, 1998). For some microorganisms, the release of H^+ ions due to the assimilation of NH_4^+ seems to be the sole mechanism promoting RP dissolution. *Penicillium bilaii* and *Penicillium fuscum* could decrease pH and solubilize RP when media contained NH_4^+ compared to *P. billai* which could maintain the ability to lower the pH and solubilize RP when there was no N in the media (Asea et al., 1988). This result suggested that different mechanisms were utilized by the different species; one mechanism required the presence of NH_4^+ in the medium and the other did not.

Table 1.1: Nature of organic acids secreted by PSMs

(Khan et al., 2006)

A: Nature of organic acids secreted by PS fungi and actinomycetes

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

B: Nature of organic acids secreted by PS bacteria

([1]Patel et al., 2008)

Organism	Predominant acids	Organism	Predominant acids
Phosphate-solubilizing bacteria			
Citrobacter sp. DHRSS[1]	Acetic, gluconic	*Pseudomonas cepacia*	Gluconic, 2-ketgluconic
Enterobacter intermedium	2-ketogluconic	*Rhizobium meliloti*	2-ketogluconic acid
Arthrobacter sp.	Oxalic, malonic	*P. striata*	Malic, glyoxalic, succinic, fumaric, tartaric, α-ketobutyric
Bacillus firmus	2-ketogluconic, succinic	*Bacillus subtilis*, *Bacillus* spp.	Oxalic, succinic, citric, 2-ketgluconic
Micrococcus spp.	Oxalic	*E. asburiae*	Gluconic
Bacillus amyloliquefaciens, *B. licheniformis*, *B. atrophaeus*, *Penibacillus macerans*, *Vibrio proteolyticus*, *xanthobacter agilis*, *Enterobacter aerogenes*, *E. taylorae*, *Kluyvera cryocrescens*, *Pseudomonas aerogenes*, *Chryseomonas luteola*	Lactic, itaconic, isovaleric, isobutyric, acetic, gluconic		

1.4.1.1.4 Other P solubilisation mechanisms

Other P solubilisation mechanisms have been summarized by Yadav and Dadarwal, (1997). Some metabolic end products like CO_2 and H_2S produced by

organotrophs also play a role to a limited extent in solubilisation of inorganic phosphates purely by chemical reactions. The CO_2 produced in the rhizosphere by the microorganisms has been reported to be involved in increase in P availability to the plants. Mechanism involved could be the formation of carbonic acid which reacts with Ca phosphate forming $CaHPO_4$ or Ca $(H_2PO_4)_2$ with formation of $CaCO_3$. Hydrogen sulfide (H_2S) which is produced by fermentative microorganisms from sulphur containing amino acids as well as by sulphur respiring bacteria like *Desulfovibrio* and *Desulfotomaculum*, reduces ferric phosphate to ferrous sulphide leading to release of H_2PO_4 ions, which has been reported in water logged soils amended with sulphur. *Nitrosomonas* and *Nitrobacter* producing HNO_3 from ammonium salts and the iron bacteria *Ferrobacillus ferrooxidans* and *Thiobacillus ferrooxidans* which oxidise iron and forms H_2SO_4 or convert Fe $(OH)_2.H_2PO_4$ to $Fe(OH)_3$ releasing $H_2PO_4^-$ ions in solution has also been reported.

1.4.1.2 Relation between nature of organic acids and solubilization of mineral phosphates.

P-solubilisation by PSMs depend upon the secretion of organic acid, but amount of P release need not correlate with the amount of organic but also on the nature of organic acid (Thomas, 1985; Asea et al., 1988). Therefore nature of organic acid includes the chelation ability and strength of the acid to be effective in P solubilisation ability. Oxalic acid is more effective in acidic soils supplemented with RP than citric acid but *vice versa* is true in alkaline vertisols. Acetic acid even in very high concentration was not able to release P in both alkaline vertisols as well as alfisols (Gyaneshwar et al., 1998; Srivastava et al., 2006).

1.4.1.3 Molecular mechanism of GA secretion in PSMs

Mineral phosphate solubilizing capacity has been shown to be related to the production of organic acids. Direct oxidation of glucose to GA has been a major mechanism for mineral phosphate solubilization (MPS) in Gram-negative bacteria (Goldstein, 1995) (**Fig. 1.4**) Gene responsible for MPS phenotype has been cloned from *Erwinia herbicola* in *E. coli* HB101 by its ability to solubilize hydroxyapatite (Goldstein and Liu, 1987) (**Table 1.2**). This gene was similar to gene III of a PQQ synthesis gene complex from *Acinetobacter calcoaceticus*, and to *pqq*E of *Klebsiella*

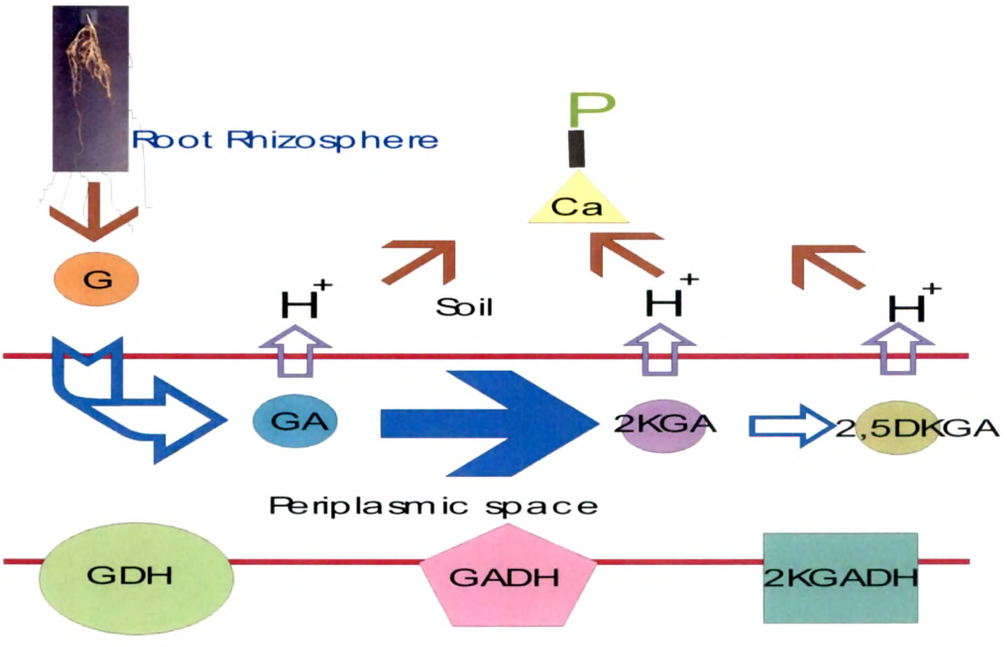
pneumoniae (Liu et al., 1992). Since *E. coli* does not have genes for PQQ synthesis and therefore cannot produce GA, it was suggested that the *E. herbicola* DNA fragment functions as a PQQ synthase gene, and that probably, some *E. coli* strains contain some cryptic PQQ synthase genes that could be complemented by this single open reading frame (ORF) isolated by them. Similarly, nucleotide sequence analysis of a 7.0 kb fragment from *Rahnella aquatilis* genomic DNA that induced hydroxyapatite solubilization in *E. coli*, showed two complete ORFs and a partial ORF showing similarity to *pqqE* of *E. herbicola, K. pneumoniae, A. calcoaceticus* and to *pqqC* of *K. pneumoniae* respectively (Kim et al., 1998b). These genes were proposed to complement cryptic PQQ in *E. coli* genes, thus allowing GA production. Similarly expression of 396bp ORF (*gab*Y) could induce MPS phenotype and production of GA in *E. coli* JM109. The deduced amino acid sequence showed no homology with previously cloned direct oxidation pathway (GA synthesis) genes, but was similar to histidine permease membrane bound components. GA production was seen when the gene expression was done in presence of external PQQ (Babu-Khan et al., 1995). Mutant of *gdh* gene resulted in MPS^- phenotype. DNA fragment from *Serratia marcenses* induces GA synthesis in *E. coli*, but showed no homology to *pqq* or *gdh* genes because the *E. coli* did not produce GA when the *S. marcenses* was replaced by another PQQ producing strain (Krishnaraj and Goldstein, 2001). The gene product could act as an inducer since *gdh* mutant did not show the phenotype. Other isolated genes involved in the MPS phenotype seem not to be related with *pqq* or *gdh* biosynthetic genes. A genomic DNA fragment from *Enterobacter agglomerans* showed MPS activity in *E. coli* JM109 without altering the pH of the medium (Kim et al., 1997). Expression in *E. coli* of the mps genes from *R. aquatilis* supported a much higher GA production and hydroxyapatite dissolution in comparison with the donor strain (Kim et al., 1998b). These results suggested that different genetic regulation of the mps genes might occur in both species. MPS negative or delayed phenotype mutants of *Pseudomonas* spp. showed pleiotropic effects, indicating the involvement of regulatory mps loci or structural gene which is coordinately regulated (Krishnaraj et al., 1999). All these results indicate the complex regulation of various metabolic traits which could be influenced by genetic background of the recipient and metabolic interactions in the recipients.

Table 1.2: Cloning of genes involved in MPS phenotype

Microorganism	Gene / plasmid	Features	Reference
E. herbicola	*mps*	Produces GA and solubilizes mineral P in *E. coli* HB101 Probably involved in PQQ[a] synthesis	Goldstein and Liu, 1987
P. cepacia	*gabY*	Produces GA and solubilizes mineral P in *E. coli* JM109 No homology with PQQ genes	Babu-Khan et al., 1995
E. agglomerans	*pKKY*	Solubilizes P in *E. coli* JM109 Does not lower pH	Kim et al., 1997
R. aquatilis	*pK1M10*	Solubilizes P and produces GA in *E. coli* DH5α Probably related to PQQ synthesis	Kim et al., 1998b
S. marcescens	*pKG3791*	Produces GA and solubilizes mineral P	Krishnaraj and Goldstein, 2001

a: PQQ: pyrroloquinoline quinone.

Fig. 1.4: Molecular mechanism of P solubilisation by direct oxidative pathway



1.4.2. Mycorrhizae

Ubiquitous nature of mycorrhizae in agriculture soils with symbiosis association with terrestrial plants are believed to enhance P nutrition of plants by scavenging the available P due to the large surface area of their hyphae, and by their high affinity P uptake mechanisms (Sanders and Tinker, 1973; Hayman, 1974, 1983; Jeffries, 1987). In association with nitrogen fixers, arbuscular mycorrhizal fungi (AMF) increase nitrogen and phosphatic nutrients of plants, especially in phosphorus-deficient soil (Cruz et al., 1988). In addition, PSM interact well with AMF in phosphorus deficient soils or soils with RP (Poi et al., 1989). P released from sparingly soluble or insoluble phosphorous by PSMs are tapped and translocated by the AMF hyphae to the plant (Azcon-Aguilar et al., 1986; Barea et al., 1991; Toro et al., 1997). PSMs survive longer around mycorrhizal roots compared with non-mycorrhizal roots and act synergistically with the AMF, leading to increased plant growth, especially where RP is applied to soil (Singh, 1990; Bolan, 1991). AMF have been reported to function as intermediaries between plants and soil microbes by moving C and other phytoexudates from the plant to the rhizosphere and transferring microbe mobilised nutrients to the plant (Garbaye, 1994; Toro et al. 1997; Copley, 2000; Duponnois, 2006; Frey-Klett et al., 2007). Therefore dual inoculation of AMF with PSM has been shown to stimulate plant growth more than inoculation of either microorganism alone in certain situations when the soil is phosphorus-deficient (Piccini and Azcon, 1987).

Apart from the P uptake, mycorrhiza improves the plant growth by improved uptake of micronutrients (Burkert and Robson, 1994) and nitrogen (Barea et al., 1991). AMF is also known to improve soil quality by forming soil aggregates (Tisdall, 1994), alleviate metal toxicity (Hildebrandt et al., 2007; Pozo and Azcón-Aguilar, 2007) and show antagonist effect against plant pathogen (Duponnois et al., 2005). AMF also secretes organic acids that could solubilize the insoluble mineral phosphates due to the exploration by the external hyphae of the soil beyond the root-hair zone where phosphorus is depleted (Paul and Sundara Rao, 1971; Lapeyrie, 1988; Cui and Caldwell, 1996). AMF also helps in development of a superior root system, increased photosynthetic efficiency, increased water conducting capacity, alleviate environmental stresses (Kapoor et al., 2008). AMF modify root functions (i.e. root exudation) (Marshner et al., 1997), change the carbohydrate metabolism of the host plant (Schachar et al., 1995) and influence rhizosphere populations (Hobbie, 1992).

AMF also produce plant hormones and increase the activity of nitrogen-fixing organisms in the root zone (Bagyaraj, 1984).

Infection of AMF depends upon the P status of the plant (Abbott et al., 1984; MacFall et al., 1991; Mortimer et al., 2008) and therefore in general do not colonize plant roots strongly under P sufficient conditions leading to reduced growth of certain plants in the presence of available phosphate (Amijee et al., 1989; Son and Smith, 1995; Vivas et al., 2003). However, a more thorough understanding of interactions between soil microorganisms is needed for an optimal utilization of these interactions with respect to the growth and development of plants.

Centrosema macrocarpum plants were inoculated with *Rhizobium* strains and the AMF *Glomus manihotis* or *Acaulospora longula*, a significantly greater dry matter production, mineral absorption, nodulation and infection by AMF was recorded (Satizabal and Saif, 1987). However, a small amount of nitrogen fertilizer was suggested for application at the time of sowing. The dual inoculation of *Azotobacter chroococcum* and *Glomus fasciculatum* enhanced root infection of AMF, stimulated plant growth, and increased nitrogen, phosphorus and zinc contents in maize and wheat (Elgala et al., 1995).

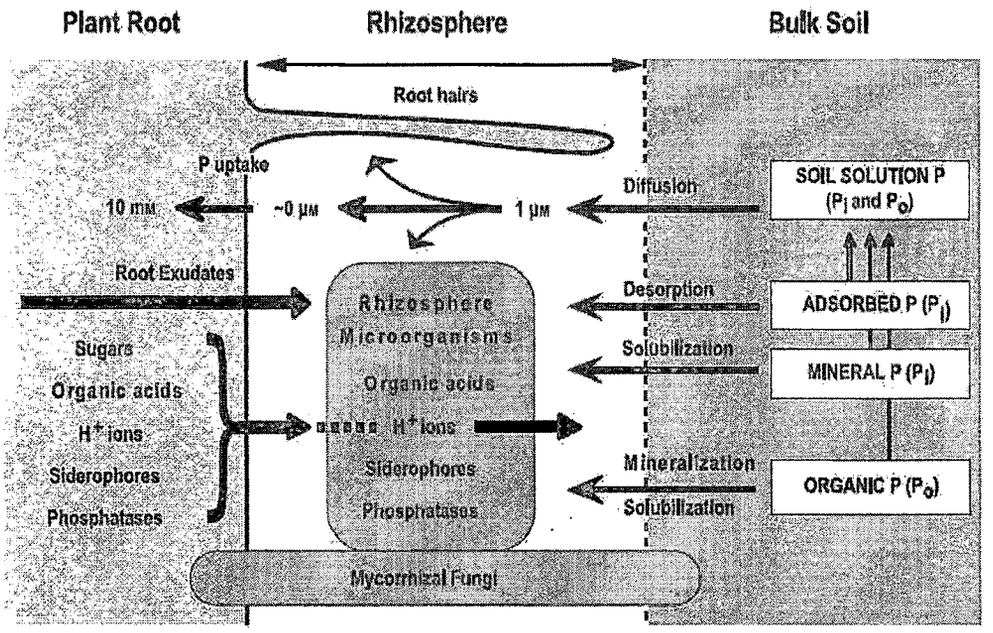
1.4.3 Utilisation of Organic P

Release of root exudates, that either directly influence P availability or support the growth of microbial populations within the rhizosphere, and the production of phosphatase enzymes are important, as are mycorrhizal associations (Richardson, 1994) (**Fig.1.5**).

Organic P which constitutes about 30-80% of total soil P is one of the major P source. Utilisation of organic P by the microorganisms through enzymes are known to bring about organic phosphate degradation and this process is greatly affected by physicochemical and biochemical properties of these organic compounds. These enzymes can be categorized into three groups: (1) Nonspecific phosphatases, catalysing dephosphorylation of monophosphoester or phosphoanhydride bonds in organic matter, (2) Phytases, which specifically cause P release from phytic acid, and

(3) Phosphonatases and C–P Lyases, catalysing C–P cleavage in organophosphonates (Rodriguez and Fraga, 1999).

Fig. 1.5: Schematic representation of the physiological and chemical processes that influence the availability of phosphorus in the rhizosphere (adapted from Richardson, 1994).



1.4.3.1 Non specific phosphatases

Non specific phosphatases and phytases are predominant because of the presence of large amount of their substrates in soil (El-Sawah et al., 1993; Bishop et al., 1994; Feller et al., 1994). The major source of phosphatase activity in soil is considered to be of microbial origin especially in the rhizosphere (Gracia et al., 1992; Xu and Johnson, 1995; Richardson, 2001). Acid phosphatases which play the major role in organic P utilization have been found in various soil bacteria, such as *Rhizobium* (Abd-Alla, 1994), *Enterobacter, Serratia, Citrobacter, Proteus, Klebsiella* (Thaller et al., 1995), *Pseudomonas* (Gugi et al.,1991) and *Bacillus* (Skrary and Cameron, 1998). The *acpA* gene isolated from *Francisella tularensis* expresses an acid phosphatase with optimum pH 6.0 and with a wide range of substrate specificity (Reilly et al., 1996). Similarly, genes encoding nonspecific acid phosphatases class A (PhoC) and class B (NapA) isolated from *Morganella morganii* show P-irrepressibility and

showing broad substrate action and high activity around pH 6.0 (Thaller et al., 1994, 1995b; Rodriguez and Fraga, 1999). Outer membrane protein gene from *Burkholderia cepacia* a rhizobacterial strain, facilitating phosphatase activity has been isolated, which enhances synthesis of protein in the absence of soluble phosphates in the medium (Rodriguez et al., 2000a). Two nonspecific periplasmic acid phosphatase genes (*napD* and *napE*) from *Rhizobium* (*Sinorhizobium*) *meliloti* have been cloned (Deng et al., 1998, 2001). Some examples of soil bacteria capable of P release from different organic sources are shown in **Table 1.3**.

Table 1.3: List of soil bacteria capable of P release from lower forms of inositol phosphates (Rodríguez 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
P. fluorescens	Phosphonoacetate	Phosphonoacetate hydrolase
Bacillus licheniformis	D-α-glycerophosphate	D-α-glycerophosphatase
Klebsiella aerogenes	Phosphonates	C-P Lyase

1.4.3.2 Phytase

General non specific phosphatases, secreted by soil microorganisms as well as by the roots of many plants, are unable to initiate attack on the phosphomonoester bonds of phytate and hence most plants are unable to efficiently use phytates as P-source (Turner et al., 2002; Mudge et al., 2003). Phytase have been well characterized from microbial phytases, especially from filamentous fungi such as *Aspergillus ficuum*, *A. fumigatus*, *Mucor piriformis*, *Rhizopus oligosporus*, and *Cladosporium* species. Phytate degrading enzymes from yeasts such as *Schwanniomyces occidentalis*, *Pichia anomala*, *Arxula adeninivorans*, gram-negative bacteria such as *Escherichia coli*, *Pseudomonas* species, *Klebsiella* species, and gram-positive bacteria such as various *Bacillus* species were also identified and characterized (Rodríguez and Fraga, 1999; Haefner et al., 2005). The enhanced utilization of inositol phosphate by plants by the presence of soil microorganisms has also been reported (Richardson et al., 2001b; Idriss et al., 2002; Unno et al., 2005). Therefore, developing agricultural inoculants with high phytase production would be of great interest for improving plant nutrition and reducing P pollution in soil.

Phytase genes have been cloned from fungi, plants, and bacteria (Lei and Stahl, 2001). The bi-functionality of these enzymes makes them attractive for solubilization of organic P in soil. Neutral phytase genes have been cloned from *B. subtilis* and *B. licheniformis* (Tye et al., 2002). *phyA* gene coding for extracellular phytase has been cloned from the FZB45 strain of *B. amyloliquefaciens* which stimulates the growth of maize seedlings under limited phosphate and in the presence of phytate (Idriss et al., 2002). This was further supported by mutant studies where culture filtrates obtained from a phytase-negative mutant strain, did not stimulate plant growth. The occurrence and the biochemical properties of phytase are reviewed in detail by Oh et al. (2004) and Konietzny and Greiner (2002). Some examples of soil bacteria capable of P release from phytate are shown in **Table 1.4.**

Table 1.4: List of soil bacteria capable of P release from phytase

(Rodríguez and Fraga, 1999; [1]Unno et al., 2005)

Bacterial strain	P Substrate	Enzyme type
Bacillus subtilis	Inositol phosphate	Phytase
Pseudomonas putida	Inositol phosphate	Phytase
Pseudomonas mendocina	Inositol phosphate	Phytase
Bacillus amyloliquefaciens	Inositol phosphate	Phytase
Pseudomonas spp.	Inositol phosphate	Phytase
Burkholderia spp.[1]	Inositol phosphate	Phytase

1.4.4 P solubilisers as PGPR

The solubilization of P in the rhizosphere is one of the PGPR activities that increase nutrient availability to host plants (Richardson, 2001). PGPRs having P solubilisation ability have been studied with respect to plants which include *Azotobacter chroococcum* with wheat (Kumar and Narula, 1999), *Bacillus circulans* with *Cladosporium herbarum* and wheat (Singh and Kapoor, 1999), *Bacillus* sp. with five crop species (Pal, 1998), *Enterobacter agglomerans* with tomato (Kim et al., 1998b), *Pseudomonas chlororaphis* and *P. putida* with soybean (Cattelan et al., 1999), *Rhizobium* sp. and *Bradyrhizobium japonicum* with radish (Antoun et al., 1998), and *Rhizobium leguminosarum* bv. *Phaseoli* with maize (Chabot et al., 1998). However, the ability to solubilize P by no means indicates that a rhizospheric bacterium will constitute a PGPR and similarly vice-versa. Cattelan et al., (1999)

found only two of five rhizospheric isolates positive for P solubilization actually had a positive effect on soybean seedling growth. Similarly de Freitas et al., (1997) found a number of P-solubilizing *Bacillus* sp. isolates and a *Xanthomonas maltophilia* isolate from canola (*Brassica napus* L.) rhizosphere which had positive effects on plant growth, but no effects on P content of the host plants.

1.5 EFFICACY OF PSMS – FIELD STUDIES

1.5.1 Single inoculation studies

Microorganisms have been extensively investigated for plant growth in field condition (Subba Rao, 1982; Goldstein, 1986; Tandon, 1987; Kucey et al., 1989). Phosphobacterin increased 10-15% crop yield in 30% of the experiments (Yadav and Dadarwal, 1997). *Penicillium bilaii* and *Bacillus megatarium* are considered the most effective PSMs according to field experiments (Asea et al., 1988; Kucey, 1988). *Bacillus megatherium* has been shown to release P from organic phosphates, but does not solubilize mineral phosphates. In 35 papers dealing with potential PSMs, 60 different microbial sp. have been reported to solubilise P. Of this 13 reports described laboratory experiments, 8 studies tested organisms on plants in sterile soil or sand, 8 with non sterile soil and only 6 described field trials. The PSM-plant inoculations resulted in 10–15% increases in crop yields in 10 out of 37 experiments (Tandon, 1987; Zoysa et al., 1998). These studies also demonstrated an increase in P uptake by plants. The results of PSM-plant inoculations, however, may not conclusively show a direct role for the PSMs in supplementing soil phosphates for plants because: (i) No increase was found in about 70% of the experiments; (ii) the increase in crop yields were not compared with crop yields with addition of superphosphates; (iii) the plant growth promoting activity of PSMs, other than P solubilization, has not been determined; and (iv) enhancement in P uptake mediated by AMF as a consequence of PSM inoculation was not considered in interpreting the results. Single successful bacterial inoculants (PSMs) have been summerised in **Table 1.5** (Kucey et al., 1989; Yadav and Dadarwal, 1997).

Table 1.5: Single bacterial inoculation field studies on various crops

(Kucey et al., 1989; Yadav and Dadarwal, 1997).

Crop/ conditions	Inoculant used	Effect observed
Red pine	*Pseudomonas* spp.	Increased plant weights and P uptake in soils with added Ca phosphate
Tomato	*Bacillus circulans*	No effect
Soybean	Unidentitified	Increase VAM colonization
Millet	*B. circulans*	Increased plant weight and P uptake, increase in P available in soil
Rice	*Bacillus* spp.	Increase available P in soil increase plant P uptake
Peas	*B. megatarium* var. phosphaticum	Increase plant weight and P uptake
Fava bean	*B. megatarium* var. phosphaticum	Increased plant weight and P uptake and concentration from superphosphate fertilized soil
Lavender	*Pseudomonas* spp.	Increased plant weight and P
	Agrobacterium spp.	Uptake and concentration from soil amended with RP
Soybean	*B. megatarium*	No effect
Rice	*B. firmus*	Increased plant weights from soil with added RP
Oat	Soil containing PSM	Increase in yield and P uptake
Wheat	Phosphobacterin	Increase in yield, P uptake, Protein content and gluten content. Better germination and plant growth
	Pseudomonas striata	Increase in yield and P uptake
Barley	Phosphobacterin	Increase in grain yield, P uptake, Protein P and Lipid P; Better germination and increase in yield
Maize	Phosphobacterin	Increase in yield and P uptake
Peas	Phosphobacterin	Increase in yield
Paddy	Phosphobacterin	Increase in grain yield

Single inoculation bacterial PSM *Bacillus subtilis* inoculant increased biomass, grain yield and P and N uptake in mung bean (Gaind and Gaur, 1991). Similar results with *B. thuringiensis* increased number and weight of the pods and seed yield in canola (de Freitas et al., 1997). P solubilising *Rhizobium* increased P uptake in lettuce and maize (Chabot et al., 1996). Most of the organisms chosen for inoculant studies have been bacteria, although fungi are most often more effective P solubilisers (Nahas, 1996; Khan et al., 2006). Compared to bacteria fungi are active at a wide pH range and can utilize wide variety of sugars from hexoses, pentoses as well as disaccharides. *Penicillium billai* was the most successful fungal PSM known so far secreting ~10mM citric and ~10mM of oxalic acid. *P. billai* was effective with various crops and was successful in around 55 sites in Canada (**Table 1.6**; Zoysa et al., 1998).

Table 1.6: ***P. billai* inoculation field studies on various crops**

(Zoysa et al., 1998)

Crop/ conditions	Effect observed
Wheat/ greenhouse	Enhanced P uptake and increased dry matter at early stages of growth
Wheat/ greenhouse	Increased dry matter and P uptake
Wheat/ greenhouse	Increased plant weights and P, Cu Zn uptake, increased P availability in soil added with RP
Wheat/ Field	Increased grain yield supplemented with RP
Wheat/ Field	Increased grain yield
Bean/ Greenhouse	Increased dry matter and P uptake
Canola/greenhouse	Increased P uptake but not dry matter
Canola/ field	Increased grain yield with rock P in field
Pea/Greenhouse	Increased dry matter, nitrogen uptake and P uptake
Pea and Lentil/field	Increase in growth, P uptake, N uptake and grain yield
Alfalfa/Field	Increased dry matter and P uptake at three trifoliate leaf stage
Alfalfa/Field	Increased forage yields

1.5.2 Microbial consortia

Varying and inconsistence results of single PSM inoculation studies led to multiple or mixed culture approach (MCA), often called co-inoculation, where PSM are used along with other beneficial rhizospheric microorganisms proved more beneficial.

1.5.2.1 PS bacterium and PS fungus

Co-inoculation studies on various crops with bacterial PSMs along with PS fungus or multiple bacterial inoculants have resulted in increase plant growth, grain yield and increased total P uptake than single bacterial inoculation (**Table 1.7**).

Table 1.7: Co-inoculation studies of bacterial PSMs with fungal PSMs or multiple bacterial inoculants. (Yadav and Dadarwal, 1997)

Crop/Conditions	Inoculant used	Effect observed
Rice/ Greenhouse	*P. striata, A. awamorii*	Increased plant weight and P uptake and concentration
Wheat/ Field	*P. striata, A. awamorii*	Increased plant weights in soils with added RP and N
Rice/ Greenhouse	*Bacillus* spp. *Penicillium* spp. *Aspergillus* spp.	Increased plant weight and P uptake and concentration increased P available in soil
Wheat	*B. megatarium, P. Striata, Penicillium* spp	Increase in grain yield
	Pseudomonas sp., *A. awamorii*	Increase in yield and P uptake
Barley	*P. striata, A. awamorii*	Increase in yield
	P. striata, B. megatarium, A. awamorii	Increase in yield and P uptake
Potatoes	*P. striata, B. polymyxa*	Increase in yield
Cow pea	*P. striata, B. circulans*	Increase in uptake
	P. striata, B. polymyxa, A. awamorii	Increase in dry matter production and nitrogen fixation

Increase in yields of legumes after inoculation with rock phosphate and the PS bacteria, *B. megaterium* and *P. striata*, and the PS fungus *Aspergillus awamori* in alkaline and neutral soils (Dubey and Billore, 1992; Dubey, 1996). Improved nodulation, available P_2O_5 content of soil, root and shoot biomass, straw and grain yield and nitrogen and phosphorus uptake by moongbean (*Vigna raiata* (L.) wilczek) plants upon inoculation with thermo-tolerant species of phosphate-solubilizing *Bacillus subtilus*, *B. circulans* and *Aspergillus niger* (Gaind and Gaur, 1991).

1.5.2.2 PSM with N fixers

Combined inoculation of nitrogen fixers and PSM may benefit the plants better than either group of organisms alone. Interaction studies have been done both in vitro and in vivo (Sarojini et al., 1989). Phosphate solubilization was observed by mixed cultures (e.g., *P. striata*, *Bradyrhizobium* sp. and *M. ciceri*), suggesting that they could be used as mixed microbial inoculum and antagonistic behavior of one organism towards another was not observed. Nitrogen fixers and PSM when inoculated together colonized the rhizosphere and enhanced the growth of legumes by providing them with nitrogen and phosphate, respectively (Gull et al., 2004). PS bacteria *P. striata* and *Rhizobium* gave significantly higher yield in greengram (Khan et al., 1997) and chickpea (Algawadi and Gaur, 1988). *Rhizobium* and PS fungi *A. awamorii*, when used as seed inoculant, increased the grain yield of chickpea under field conditions (Dudeja et al., 1981). Similarly, the effect of interactions between three PS fungi, *A. niger*, *A. fumigatus* and *Penicillium pinophilum* and nitrogen- fixing *Rhizobium leguminosarum* biovar viciae showed significantly greater positive effects on growth, N and P and consequently, the yield of *Vicia faba* under field conditions (Mehana and Wahid, 2002). Long term trials using phosphate-solubilizing bacteria and nitrogen-fixing organisms enhanced the seed production in soybean crops and were found to be more effective compared with superphosphate alone (Dubey, 2001). In a similar study, single or combined inoculation with PSM and nitrogen fixers had a positive effect on the yield and nutrient uptake of cereals and legume crops (Sarojini and Mathur, 1990; Whitelaw, 2000; Kumar et al., 2001). A beneficial effect of phosphate solubilizer in combination with nitrogen fixer on cotton (Kundu and Gaur, 1980) and wheat (Zaidi and Khan, 2005) has also been reported. Similar results were obtained for *phaseolus vrisulga* when inoculated with *Agrobacterium*, a phosphate

solubilizer. In contrast, adverse effect of *P. bilaii* on nitrogen fixation has been observed. Beans grown in autoclaved soil inoculated with phosphate-solubilizing *P. bilaii*, and *R. phaseoli* showed no significant increase in dry matter or total uptake of phosphate (Kucey, 1987). Decrease in total nitrogen fixation in peas due to dual inoculation of *P. bilaji* and *R. leguminosarum* has also been reported (Downey and Van Kussel, 1990). Organic acids secretion by PS fungi could also be attributed to incompatibility with neutral or alkaline conditions required for nodulation (Venkateswarlu et al., 1984). Therefore, the compatibility between the two associates must be checked *in vitro* before going for field studies. Combined inoculation of *Rhizobium* and phosphate-solubilizing *P. striata* or *B. polymyxa* with or without added fertilizers on chickpea yielded increased nodulation, increased availability of phosphate of the soil, increased dry matter of the plants, grain yield and phosphorus and nitrogen uptake by the plants. The inoculation effects, however, were more pronounced in the presence of added fertilizers (Algawadi and Gaur, 1988). In a pot experiment, lentil seeds were inoculated with *R. leguminosarum* along with increasing doses of rock phosphate with or without a 1:1 mixture of elemental sulfur presence PS bacteria resulted in increased plant dry weight, increased N, P, iron, zinc, manganese, copper and sulfur uptake. Dry matter yield and nutrient uptake was slightly higher with sulfur application (Saber and Kabesh, 1990). A combination of *Azotobacter chroococcum* GA-1 and GA-3 with *Penicillium* HF-4 and HF-5 and *Aspergillus* GF-1 and GF-2 increased radicle and plumule length, but the remaining culture combinations decreased radicle/ plumule length. A significant increase in mungbean yield and groundnut yield was observed with the inoculation of *Rhizobium* spp. and phosphate-solubilizing bacteria along with phosphatic fertilizers (Khan et al., 1997, 1998). Increase in yield of various legumes has been observed following seed or soil inoculation with nitrogen-fixing organisms and PSM (Zaidi, 1999; Perveen et al., 2002) or PSM and arbuscular mycorrhizal fungus (Mukherjee and Rai, 2000). About 50% of phosphatic fertilizer requirement could be saved by the combined inoculation of *Rhizobium* strain Tt 9 with *B. megaterium* var. phosphaticum in groundnut. *Rhizobium* strain Tt 9 along with phosphobacteria recorded higher nodule number, root length and shoot length and increased pod yield (Natarajan and Subramanian, 1995). No significant increase in phosphate contents in pigeonpea plants inoculated with *Rhizobium* (CCI) with *B. megaterium* var. phosphaticum was observed (Gunasekaran and Pandiyarajan, 1995). About 37% increase in the grain yield of

blackgram was reported following the co-inoculation of *Rhizobium* and *B. megaterium* (Prabakaran et al., 1996). Co-inoculation of P-solubilizing *Pseudomonas* and *Bacillus* spp with *Mesorhizobium* on chickpea significantly increased legume grain yield, concentration and uptake of N and P (Wani et al., 2007). Inoculation with *M. ciceri, A. chroococuum* and P solubilizing *Bacillus* tripled the seed yield and resulted in highest grain protein, ~2 fold increase in P concentration.

1.5.2.3 PSM with Mycorrhizae

Dry matter yield of wheat plants increased significantly following dual inoculation of RP-solubilizing fungi (*Aspergillus niger* and *Penicillium citrinum*) and *Glomus constrictum* (Omar, 1998). Combined inoculation of *Glomus intraradices* and PS fungi *A. niger* increased significantly the urease, protease and phosphatase activities of the rhizosphere soil of the lettuce plants along with foliar P and K contents (Kohler et al., 2007). Addition of superphosphate further increased plant growth due to the enhanced P-uptake efficiency when applied along with AMF and/or P-solubilizer rhizobial strains. Establishment of AMF on the root system can alter the rhizospheric microbial populations, which in turn affects the competitive interaction between introduced and native rhizobia for nodulation sites (Ames et al., 1984). On the other hand, it has been postulated that some P solubilizing bacteria behave as mycorrhizal helper bacteria (Garbaye, 1994; Frey-Klett et al., 2007).

1.5.2.4 PSM, Nitrogen fixing bacteria with Mycorrhizae

During the inter-generic interaction, nitrogen-fixing microorganisms provide nitrogen to the plants and consequently improve the nitrogen status of the soil, while PSM enhance plant growth by providing it with phosphates. Where nitrogen and phosphorus are limiting, AMF may improve phosphate uptake for plants; the higher phosphorus concentration in the plant benefits the nitrogen fixers and the functioning of its nitrogenase, leading to increased nitrogen fixation, which in turn promotes root and mycorrhizal development. Associative effect of *Bradyrhizobium japonicum*, arbuscular mycorrhizal fungus and phosphate-solubilizing microbes has been established on soybean in a mollisol (Singh and Singh, 1993).

Application of RP with triple inoculation significantly increased grain yield, nodulation, nitrogen uptake and available soil phosphate. Impact of *Glomus mosseae*, *Bacillus* sp. and *Rhizobium* sp. resulted in drastic increase in plant growth and soil aggregation under *Pisum sativum* cultivation (Bethlenfalvay, 1994). It is evident from the earlier studies that a positive interaction exists between root colonization, phosphorus uptake and growth promotion, as observed by a few researchers (Zaidi et al., 2003; Zaidi and Khan, 2005). Inoculation of *Rhizobium*, *Bacillus polymyxa* and *Glomus faciculatum* resulted in significantly greater dry matter production and phosphate uptake as compared with single or double inoculation (Poi et al., 1989).

Combined inoculation of *Rhizobium* and *Glomus etunicatum* and application of RP or PSM gave the greatest yield and had variable effects on nodulation in clovers (Leopold and Hofner, 1991), mungbean (Zaidi et al., 2004), cowpea (Thiagarajan et al., 1992) and chickpea (Poi et al., 1989). Co-inoculation study of P solubilising strain of *Mesorhizobium ciceri* with *Glomus mosseae and Glomus intraradices* increased significantly dry matter of lentil shoots and seeds, its P and N contents. Rhizobial strain with P solubilising ability had beneficial effect on plant growth and nutrient uptake (Mehdi et al., 2006).

1.6 FACTORS INFLUENCING MPS PHENOTYPE IN FIELD CONDITIONS

Many successful field studies with consortium of microorganisms have resulted in enhancement of plant growth directly due to phosphate availability or directly due to enhancement in other properties such as nitrogen fixation, increase in nutrient uptake, alleviation of metal toxicity and other plant growth promotion activities such as phytohormones, biocontrol molecules etc. The competitiveness of a P solubilizing microorganism in natural environments will depends upon its ability to survive and multiply in soil (Van Veen et al., 1997). The effective level of the density and activity of introduced microorganisms depends on the ecological conditions required by the application. When the introduced microorganisms are to be involved in a process which will give them a selective (nutritional or spatially protective) advantage in the soil system, only a minimal number of active cells is initially necessary for the application to be effective. Only 300 rhizobial cells per seed have been reported to be required for optimal nodulation (Giddens et al., 1982). Rhizobia can also be

ecologically favored by amending soil with specific substrate. Ground soybean added to soil enhanced the viable numbers of a specific *B. japonicum* serogroup 1,000-fold, but the nodulation behavior of this subgroup in competition with other groups was hardly affected (Viteri and Schmidt, 1996). However, no significant studies are reported to understand the factors influencing the survival and efficacy of PSMs in field conditions.

In general, the population sizes of the introduced microbe decline rapidly upon the introduction in soils (Ho and Ko, 1985). The survival of the inoculant strain depends upon various abiotic as well as biotic factors. Parameters such as such as soil composition (Heijnen et al., 1993, 1995; Bashan et al., 1995), temperature and the presence of the recombinant plasmids (Van Veen et al., 1997) also affect the efficacy of PSMs in the soil. The biotic factors play a very important role in the survival of the inoculated strains as the decline observed in non-sterile soils can often be abolished in sterile soils (Heijnen et al., 1988; Heijnen and Van Veen, 1991). Additionally, an increase in the population of the introduced microbe can also be observed (Postma et al., 1988).

1.6.1 Effect of soil type on the fate of bacterial inoculants

Soils of different texture differ in particle size composition and, thus, in pore size distribution. Pore size distribution strongly determines the fate of introduced microorganisms, and differences in the behavior of bacteria released into different textured soils may be related to differences in the protective pore spaces present in these soils (van Veen et al., 1997). *P. fluorescens* inoculant strain when introduced into loamy sand and silt loam soils and monitored for 3 years showed better survival in finer-textured soil i.e. silt loam soil than in the sandy soil (van Elsas et al., 1986). Phosphate solubilisation varies with respect to the composition of the medium and to the nature of the mineral phosphate provided. This phenomenon is attributed to the buffering effect of these components (Cunningham and Kuiack, 1992). Efficacy of PSMs is drastically reduced in soil due to the high buffering capacity of soils such as alkaline vertisol (Gyaneshwar et al., 1998).

1.6.2 Substrate Availability in Soil

Availability of the utilizable substrates often limits the success of the microbial inoculants. In most of the natural environment, nutrients are present in small amounts (Paul and Clark, 1988; Gottschal et al., 1992; Koch et al., 2001). The effectiveness of the introduced microbes also depends upon their physiological status. In a recent study, the survival of inoculant strains of various bacteria were shown to be not dependent on the availability of C or on selective predation but only on the initial inoculum density (Jjemba and Alexander, 1999). Scarcity of the carbon in the bulk soil compared to the rhizosphere has been shown by using reporter gene technology where carbon limitation dependent gene expression induced by σ^{s} sigma factor promoter of *E. coli* was fused with *lacZ* was introduced in *Pseudomonas fluorescens* in soil (Koch et al., 2001).

In the rhizosphere, organic carbon is considered as the driving force for microbial density and activity (Bowen and Rovira, 1999; Lugtenberg and Dekkers, 1999). During plant growth, roots actively or passively release a range of organic compounds in micromolar amounts. Among them exudates, mainly carbohydrates, carboxylic acids and amino acids, are passively released along concentration gradients (Lynch and Brown, 2001; Walker et al., 2003; Bais et al., 2006). Specific exudate releasing sites on plant roots have been identified at sub-apical zone, root-hair zone and emerging sites of secondary ramifications (Curl and Truelove, 1986; Bais et al., 2006). These exudates are of prime importance for microorganisms since they are readily assimilable without synthesis of exoenzymes (Bremer and van Kessel, 1990; Bremer and Kuikman, 1994). Thus, these exudates represent a convenient source of carbon (and possibly nitrogen) and energy and are likely to favor fast growing microbes in the rhizosphere, provided they have the corresponding metabolic abilities.

In most cases, the quantity of carbon used in the laboratory studies (several mg C per gram soil) is unrealistic when compared to carbon concentrations usually calculated for rhizosphere soil (Kozdroj and van Elsas, 2001; Schutter and Dick, 2001; Griffiths et al., 2004). Daily carbon input to the rhizosphere can be estimated at around 50-100 mg C g^{-1}soil (Trofymow et al., 1987; Iijima et al., 2000). Diversity of carbon sources in rhizosphere ranges from high concentration of organic acids such as

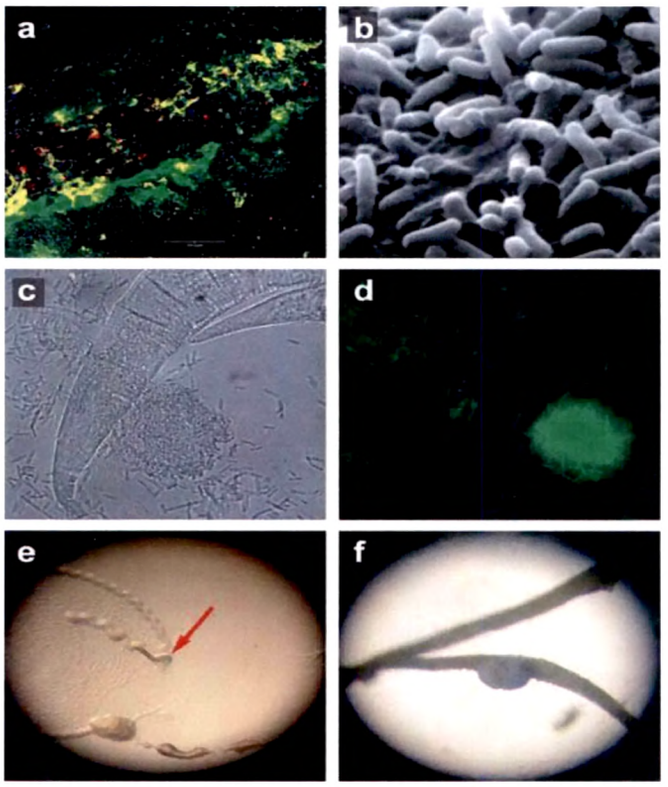
malate, citrate, acetate, formate oxalate etc (Jones, 1998), sugars such as glucose, fructose, maltose, ribose, sucrose, arabinose, mannose, galactose, and glucuronic acid (Jaeger III et al., 1999; Lugtenberg et al., 1999, 2001; Kupier et al., 2002 ; Bacilio-Jimenez et al., 2003 ; Bais et al., 2006; Kamilova et al., 2006) amino acids such as histidine, proline, valine, alanine, and glycine (Bacilio-Jimenez et al., 2003; Phillips et al., 2004). Therefore, the growth and survival of PSMs in rhizosphere depends upon the versatility of utilization of carbon sources.

1.6.3 Root colonization ability

Root colonization ability is one of the major factor which accounts for the efficacy of the PSMs in the soil condition. Bennett and Lynch (1981) showed that three bacterial species, independent of their inoculum size, colonized sterilized barley roots up to similar final population sizes. Similar studies with biocontrol *B. cepacia* cell populations colonizing the roots of peas to grow to characteristic densities (King and Parke, 1996). The final population sizes probably represented the carrying capacity (defined as the ecological "space" available for maintenance and persistence) of the rhizosphere, in terms of nutrient supply and physical colonizable space, for this organism. This can be substantiated by the fact that one of the factors governing the biocontrol activity is by blocking the sites on the root for the attacking fungi. Colonization of the root by *B. subtilis* biofilms limits the root space available for *P. syringae* to infection (**Fig. 1.6**; Bais et al., 2006). The capacity to colonize the rhizosphere of a host plant could be favored and even increased by several components of the root exudates, which could, in turn, induce some temporary modifications in the structure of bacterial lipopolysaccharides (Dekkers et al., 1998; Begonia and Kremer, 1999; Song and Lin, 1999). The exact mechanisms that allow to colonize as well as to identify the specific host organisms have not been clearly identified. Chemotactic response towards amino acids, sugars, or organic acids is fundamental for bacterial behavior both *in vitro* and *in situ* (Barak et al., 1983; Bashan and Holguin, 1994) and represents, very probably, the first step in root colonization (Zheng and Sinclair, 1996). Once bacteria are in the vicinity of the root, attachment to target cells on the plant surface can be mediated by a network of fibrillar material (Bashan et al., 1991; Vande Broek and Vanderleyden, 1995). Colonization of the roots is a complex phenomenon in which one of the first steps is the migration of

microorganisms towards the roots. Other characteristics that participate are movement along the root (Schippers et al., 1987); agglutinability by root exudates (Chao et al., 1988; Glandorf et al., 1994) and adherence (De Weger et al., 1987; Vesper, 1987).

Fig.1.6 Colonization of the root of *Arabidopsis thaliana* by *B. subtilis* biofilms limiting the outer space for *Pseudomonas syringae* to infection (Bais et al., 2006).



1.6.4 Competition with native micro organisms

Competition with other microorganisms in the soil is also one of the major factors governing the efficacy of the PSMs. Introduced organism should be either is able to resist the factors or molecules released by other native bacteria or should be able to produce them so that the population is sustained for a longer period (Van Veen et al., 1997; Zoysa et al., 1998). Organic acid utilization by rhizobacteria is crucial for competitive tomato root tip colonization, a process which is often essential for biocontrol (Woeng et al. 2000; Kamilova et al. 2005). The population of certain kind of the bacteria increases with the amendments along with inoculations such as ground

soybean added to soil enhanced the viable numbers of a specific *Bradyrhizobium japonicum* serogroup 1,000-fold, (Viteri and Schmidt, 1996).

1.6.4.1 Metabolic load

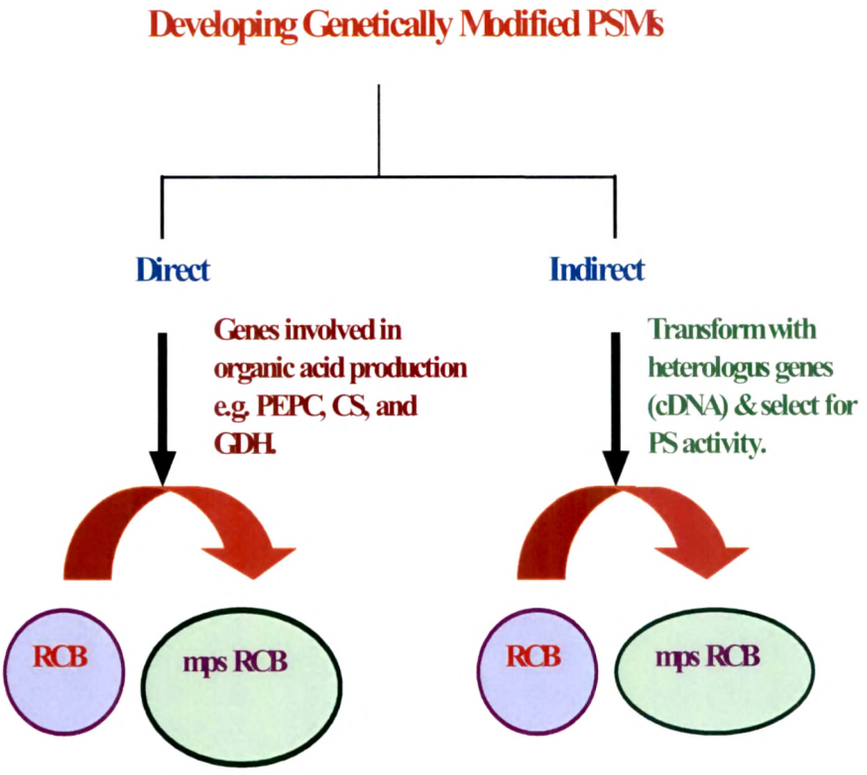
Attempts have been made to either enchance the P solubilisation property of the microorganisms (Gyaneshwar et al 1999a; Rodriguez and Fraga, 1999) or to incorporate P solubilisation ability in PGPR strains so that multiple traits can be incorporated in single kind of bacteria to increase its PGPR efficacy (Rodriguez and Fraga, 1999; Rodriguez et al., 2006). Attempts were also made to increase plant growth promoting activity in microorganisms such as degradation of ethylene (Ma et al., 2004; Bloemberg and Lugtenberg, 2001; Rodriguez et al., 2006) by genetic modifications. These genetic modifications though help in increasing the efficacy of the strains, could compromise the fitness of the microorganism in the soil (DeLeij et al., 1998; van Veen et al., 1997; Holguin and Glick, 2001; Eisenlohr and Baron, 2003)

1.7 APPROACHES/STRATEGIES FOR THE DEVELOPMENT OF PHOSPHATE BIOFERTILIZERS

1.7.1 Metabolic engineering for enhanced organic acid secretion

Molecular mechanism of P solubilisation phenotype has been so far attributed to GA production by direct oxidation of glucose (Goldstein, 1995). Since, apart from quantity of organic acid secretion qualitative nature of organic acid plays an important role in phosphate solubilisation phenotype (Asea et al., 1988). Till now fungi are known to be the best P solubiliser compared to other microorganisms, has been attributed to citric and oxalic acid secretion by them. This has been substantiated *in vitro* with the amount of P release from alkaline vertisol and alfisol (Gyneshwar et al., 1999; Srivastava et al., 2006) by adding various organic acids in different concentrations. The results indicated that low amount of citric and oxalic acids are required to release same or more amount of P compared to gluconic, malic or succinic acid. Many attempts were made to genetically modify microorganisms and plants to either citric acid or to convert non PSMs to PSMs **(Fig. 1.7).**

Fig. 1.7: Molecular Strategies for the development of phosphate biofertilizers (Gyaneshwar et al., 2002)



1.7.1.1 Gluconic acid secretion

pqq synthase gene from *E. herbicola* when expressed in *Burkholderia cepacia* and *Pseudomonas* sp. resulted in increase in MPS phenotype, became the first report to convert non PSM into PSM (Rodríguez et al., 2001). Similarly, *pqq* synthase gene from *Deinococcus radiodurans* when expressed in *E. coli* not only showed MPS phenotype but also may protect the cell membrane components and DNA from oxidative damage by acting as a mixed ROS scavenger either directly as an antioxidant or indirectly by inducing antioxidant enzymes (Khairnar et al., 2003).

1.7.1.2 Other organic acids

Genomic DNA library of a non-P solubilizing *Synechocystis* PCC 6803 in *E. coli* was screened for P solubilisation ability on dicalcium phosphate (DCP) as well as on RP with mannitol, glycerol and glucose as sole carbon source (Gyaneshwar et al.,

1998). Transformants with P solubilisation phenotype were isolated on glycerol and mannitol, which are non GDH substrates. These transformants could be secreting organic acids which were not produced by GDH enzyme. The mps phenotype was found to be plasmid borne. Direct attempts were also made in citrate secretion in plants by over expression of citrate synthase gene but the effectiveness of the strategy could be limited (Koyama et al. 2000; Delhaize et al., 2001; Anoop et al., 2003).

1.7.2 Improved phytate mineralization

1.7.2.1 Phytase

Organic P compounds are hydrolyzed by phosphatases or phytases which may be of plant or microbial origin. Phytases (EC 3.1.3.8 and EC 3.1.3.26) hydrolyse phosphate ions from phytic acid (myo-inositol 1,2,3,4,5,6 hexakisphosphate, IP6) sequentially to inositol phosphate IP3. Extensive studies were carried on microbial phytases originating from filamentous fungi such as *Aspergillus ficuum*, *Aspergillus fumigatus* or *Rhizopus oligosporus*, and *Cladosporium* species (Haefner et al., 2005). In addition, phytate-degrading enzymes were characterized from yeasts such as *Schwanniomyces castellii*, *Pichia anomala, Arxula adeninivorans*, gram-negative bacteria such as *Escherichia coli*, *Pseudomonas*, *Klebsiella*, and gram-positive bacteria such as various *Bacillus* species.

Phytase from many organisms have been cloned and checked for their expression on Po release from phytate, but there are only one or two reports which deal with the phytase overexpression and utilization of Po by the plants. Phytase gene from *Bacillus amyloliquefaciens* FZB45 was cloned and expressed in *B. subtilis* which secreted the phytase extracellularly and culture filtrates of this strain stimulated growth of maize seedlings under phosphate limitation in the presence of phytate (Idriss et al., 2002). Phytases from microbial origin are also expressed in various plants such as *Arabidopsis*, soybean, tobacco, potato etc and have shown to utilize Po for their growth (Lung et al., 2005; George et al., 2005; Bilyeu et al., 2008; Hong et al., 2008).

Therefore, considering all the parameters which affect the PSMs the objectives of the present study involves the following:

1.8 OBJECTIVES

1. Substrate specificity of Glucose dehydrogenase (GDH) of *Enterobacter asburiae* PSI3 and RP solubilisation with GDH substrates as C sources
2. Effect of the presence of plasmids on the P-solubilization by *E. asburiae* PSI3
3. Overexpression of *Escherchia coli appA* gene in different rhizobacteria - Effect of acidification on phytate utilization and growth promotion of maize
4. Effect of heterologous overexpression of *cs* and *ppc* genes in *Enterobacter asburiae* PSI3