CHAPTER - 1

ISOLATION AND CHARACTERIZATION OF PHOSPHATE SOLUBILIZING MICROORGANISMS SUITABLE AS BIOFERTILIZERS IN ALKALINE SOILS

1.0 INTRODUCTION

The world today is able to feed itself despite an increase in population by 50% in the last 3 decades. In India there has been 80% increase in agriculture yields from 1965 to 1990 resulting in the transformation from food deficit to food surplus country. In addition to high yielding varieties of plant and better management practices, chemical fertilizers have played a major role in the self sufficiency in food. The nutrient inputs are considered as important as better plant varieties and much more important than improved agronomic practices and water management (El- Swaify *et al.*, 1985). The total world consumption of chemical fertilizers is estimated to be 41.25 billion US $ while that of Asia is 16.92 billion US$. In India there has been 1000% increase in fertilizer consumption during 1965 to 1990 (Ahmed, 1995). 13 million tones of plant nutrients in the form of chemical fertilizers were used in India in 1994-95. Of the total, nitrogen fertilizers contributed 70% while the phosphorus and potassium fertilizers were 23% and 7% respectively (Tandon, 1995).

Nitrogen (N) and Phosphorus (P) are the major nutrients whose availability determines the plant growth and crop yields. For legumes, which forms symbiotic relationship with nitrogen fixing bacteria, the growth is mainly limited by P availability in the soil. An increase in tissue nitrogen as well as overall plant growth has been recorded in response to improved P status (Andrew & Robbins, 1969;

Israel, 1987; Singleton *et al.*, 1985). Improved P status of plant in soil was associated with an enhancement in nitrogenase activity of nodules (Bethlenfalvay & Yoda, 1981; Jakobsen, 1985; Israel, 1987, Bardin *et al.*, 1996). The continuous P deficient treatment of soybean resulted in decrease of whole plant dry mass, P and N by 62, 90 & 78% respectively (Sa & Isreal, 1991). A P transporter mutant of *Rhizobium meliloti* was found to be defective in nodule development and nitrogen fixation (Bardin *et al.*, 1996).

Although P is abundant in soils in both inorganic and organic forms, many soils throughout the world are P deficient because free phosphorus concentration, even in fertile soils is generally not higher than 10 μM (Arnon, 1953; Fried & Shapiro, 1961). On an average, most mineral nutrients in soil solution are present in millimolar amounts, however, phosphorus is present only in micromolar or lesser quantities (Ozanne, 1980). In the soils of U.S.A. the inorganic P concentration was never higher than 8 μM with an average of 1.5 μM (Bieleski, 1973). In India, the extent of P deficiency has been assessed in extensive surveys, both as the available P status of the soils and of the responses of crops to added P. Of the 372 districts surveyed, 45% were found to be deficient and 50% were moderate in available P, respectively (Tandon, 1987).

1.1 WHY IS P DEFICIENCY SO WIDESPREAD ?

P deficiency arises not because of it's low abundance but because of low availability. As a result of it's high reactivity P gets precipitated with Ca, Fe or Al depending on the soil composition and thus becomes unavailable for plants. P in soils is present in both inorganic and organic form. Inorganic P in acidic soils is associated with Al and Fe compounds (Sharpley *et al.*, 1984) whereas calcium phosphates are the predominant form of inorganic phosphate in calcareous soils and in Vertisols and

Inceptisols of India along with Al and Fe phosphates. Generally, soils in India with pH higher than 7.0 have a high proportion of their inorganic P as Ca-P (Vig & Dev, 1984). The organic P, may also make up a large fraction of soluble P, as much as 50% in soils with high organic matter (Barber, 1984). Phytate, a hexaphosphate salt of inositol, is the major form of organic matter contributing between 50-80% (Alexander, 1977). Although microorganisms are known to produce phytases, which can hydrolyze phytate, phytate tend to accumulate in virgin soils because it is insolubilized as a result of complexation with Fe, Al and Ca (Alexander, 1977). Phospholipids and nucleic acids form the labile pool of P in soil which is easily available to most of the organisms present in the soil (Molla & Chowdary, 1984).

1.2 HOW DO PLANTS RESPOND TO P DEFICIENCY?

In plants, adaptation to P deprivation can occur at morphological, physiological and metabolic levels. P deficiency could lead to substantial decrease in the level of cytoplasmic Pi. It can be as low as < 0.01 mM - 0.23 mM as compared to 5-8 mM for Pi sufficient leaves (Lauer *et al.*, 1989). At the morphological level some plants form special root structures (Lamont, 1972; Gardner *et al.*, 1981). At the metabolic level, plants show an alternative glycolytic pathway and changes in mitochondrial respiration. This pathway allow the plant to bypass the adenylate and Pi dependent reactions of respiration (Theodorou & Plaxton, 1993).

P deficiency also leads to increased cellular levels of phosphatase which release free P by hydrolyzing phosphate esters. Acid phosphatase activity of both cell wall and extracellular solution increased upon P starvation in subterranean clover (Dracup, 1984). In *Brassica napus*, acid phosphatase increased five fold in the rhizospheric soils as a result of P deficiency (Hadely, 1982). P deficient treatment led to an increase in the levels of excreted acid phosphatases of both the suspended cultured

cells and the plant of *Lycopersicon esculentum* (Goldstein *et al.*, 1988 a & b). The role of acid phosphatases in P nutrition of plants has been reviewed extensively (Stephen *et al.*, 1994). Two cDNAs coding for high affinity P transporters have been cloned from P starved *Arabidopsis thaliana*. These cDNAs AtpT1 and AtpT2 are root specific and their expression increases upon P starvation (Muchhal *et al.*, 1996). The genes encoding these transporters have also been cloned and shown to be expressed under P deficient conditions (Smith *et al.*, 1997).

Some plant species are known to be especially effective in obtaining P from P deficient soils *e.g. Lupinus albus* (Gardener *et al.*, 1981), *Fagophysarum esculatum* (McLachlan, 1976), *Brasicca napus* (Grinsted *et al.*, 1982), *Cicer arietinum* (chickpea) and *Cajanas cajan* (pigeon pea) (Ae *et al.*, 1990,1991). Each of these species acidifies it's rhizosphere by simple proton extrusion and/or organic acid secretion. *B. napus cv. Emerald* could lower the pH of the soil near the root surface from 6.5 to 4.1 when nitrate was provided as sole nitrogen source (Grinsted *et al.*, 1982). The pH change was induced by phosphate deficiency and was attributed to excretion of H^+ to compensate for cation absorption unbalanced by anion uptake (Hadely *et al.*, 1982). This could have increased P availability to the plant. The roots of *Cicer arietinum* also changed the pH of the soil from 6.0 to 4.5 with either nitrate or ammonium as nitrogen source whereas *Zea mays* could reduce the pH only with ammonium as nitrogen source (Marschner, 1983 cf. Clarkson, 1985).

Lupinus albus is known to secrete high amount of citric acid from proteiod roots under P deficiency (Marchner *et al.*, 1986). It has been proposed that citric acid forms high molecular weight complexes with Fe-P, causing P to be released on reduction of ferric to ferrous (Gardener *et al.*, 1982). The citrate secreted can be as high as 20% of the total dry weight of the plant. The proteiod roots secreting citric

acid show altered metabolism with increase in the activities of phosphoenolpyruvate carboxylase, citrate synthase and malate dehydrogenase. A portion of the carbon for citrate synthesis is derived from nonautotrophic CO_2 fixation by proteiod roots (Johnson *et al.*, 1994).

On the other hand, the P efficient crops growing in the Alfisols, where there is no acid soluble P are known to secrete specific chelators of iron. Pigeon pea secretes Piscidic acid which chelates ferric ion thereby liberating P (Ae *et al.*, 1991). Chickpea, another P efficient crop in both alkaline and acid soils, is known to secrete citric acid at high concentration. Citrate is well known for its capacity to resorb phosphate from sesquioxides surfaces by anion exchange (Prafitt, 1979). A mechanism involving both desorption and chelation in mobilization of insoluble P by citrate has been proposed (Marchner *et al.*, 1986).

1.3 HOW IS P SUPPLEMENTED IN SOIL?

To circumvent the problem of P deficiency, chemical fertilizers are added to the soils. In most of the chemical fertilizers, P is present as water soluble forms of calcium and ammonium phosphates. Super phosphate contain 16% water soluble P whereas nitrophosphates have 30-40% P in water soluble forms (Tandon, 1987). Even this low amount of soluble phosphate is not truly available for plants as almost 75-90% of it again gets precipitated due to complexation with Fe, Al and Ca complexes present in the soils (Vig & Dev, 1984; Stevenson, 1986). The supplementation of P fertilizers usually exceed crop requirements substantially which has resulted in the increase in total P concentration in many soils markedly over time (Barber, 1984).

Approximately 3 million tones of super phosphate was used in India in 1990 and it is estimated to reach 5 million tones by 2000 A.D. With the GATT agreement, the subsidies on the phosphatic fertilizers are going to be reduced leading to major increase in its cost. The subsidy on P and K fertilizers in 1996-97 are a whopping Rs. 1,724 crores. As a result of the decontrol of phosphatic fertilizers the imbalances generated in the use of P and N fertilizers have become a major concern. The situation is further complicated by the fact that the major phosphatic ores of India are low in P_2O_5 content thus are not commercially viable for making phosphatic fertilizers (Agarawal & Satyanarayana, 1989). As a result, the phosphatic ores employed in fertilizer production are currently being imported in India resulting in a major burden on Indian economy. The fertilizer demand /supply balance to be met by imports is estimated to be 5,947,000 kg NPK nutrients by the year 2000 A.D. (Ahmed, 1995).

The production of chemical phosphatic fertilizers is a highly energy intensive process requiring energy worth 4 billion US $ per annum (Goldstein, 1993). In this process sulfuric acid is used to dissolve rock phosphate. This results in the release of undesirable ore contaminants which are potent water and air pollutant. The excess use of chemical fertilizers has also taken a considerable ecological toll. Additionally the fertilizer use is reaching the theoretical maximum beyond which further increase in crop yield cannot be achieved (Ahmed, 1995). Therefore it is essential that the chemical fertilizers are supplemented with biofertilizers. Recently there has been a lot of emphasis in increasing the biological activity around the roots- the rhizosphere. The term biofertilizers has made it's impact in recent years but the major emphasis has been towards nitrogen biofertilizers and a great dal of progress has been made in understanding Rhizobium-Legume symbiosis (Dilworth, 1974; Triplett & Sadowsky, 1992; Downie, 1994; Fischer & Long, 1992; Fischer, 1994; Michiels &

Vanderleyden, 1994; Spaink, 1995). As compared to biological nitrogen fixation, little success has been obtained in providing P by biological means although the efforts were in this direction were initiated as early as 1903 (Stalstrom, 1903).

1.4 CAN MICROORGANISMS PROVIDE AN ECOFRIENDLY ALTERNATIVE TO PHOSPHATIC FERTILIZERS?

Plants also seem to get benefited from the association of microorganisms under P deficient conditions. This association could result either in the better utilization of available P or solubilization of unavailable P sources. The Vesicular Arbuscular Mycorrhizae (VAM) belong to the former category and the later category include various bacteria and fungi isolated for their ability to solubilize insoluble calcium phosphate (Ca-P) complexes.

1.4.1 VESICULAR-ARBUSCULAR MYCORRHIZAE :

VAM fungi are known to be ubiquitous in agricultural soils. The association of VAM with plant roots has mainly been studied for its effect on P nutrition of the plant as it is known that the most obvious effect of this association is on P uptake by plant roots (Hayman, 1974; Mosse 1980). Apart from P, mycorrhizae have also been studied with respect to copper, zinc (Cooper & Tinker, 1978 ; Swaminathan & Verma, 1979) and sulfur (Cooper & Tinker, 1978; Rodes & Gerdemann, 1978) nutrition of plants.

Plants with mycorrhizal associations can take up P from sparingly soluble phosphate more readily than non-mycorrhizal plants (Mosse, 1980). This has been attributed to the ability of VAM to efficiently scavenge the low levels of soluble P. It has been proposed that the ability of mycorrhizal plants to absorb P at a greater rate than non-

mycorrhizal plants is due to the extension of VAM hyphae beyond the zone of P-depleted soil (Sanders & Tinker, 1973). Recently the P transporter from *Glomus vermiformis* has been cloned and it's expression was found to be localized to the external hyphae of the fungus (Harrison & Van Buuren, 1995). There are also reports of organic acid production by VAM (Jurinak *et al.*, 1986), which could solubilize the insoluble mineral phosphates. It has also been suggested that there could be further effect on the availability of iron phosphates (Cress *et al.*, 1986; Bolan *et al.*, 1987) but so far no alternative to the original mechanism of Sanders and Tinker has been accepted. The production of organic acids by VAM would certainly effect the availability of acid labile insoluble phosphate and the whole issue of VAM mediated increase in available P requires a fresh look. The ectomycorhizal fungi have also been shown to posses phosphatase activities and are capable of taking up P from inositol phosphates (Antibus *et al.*, 1991) which could further affect their ability to release P from soil organic matter. However, the use of VAM as phosphate biofertilizers is hindered by the inability to culture them *in vitro*, since they are obligate symbionts. There are also reports of dependence of VAM infection on the P status of the plant (Abbott *et al.*, 1984). It is known that the VAM are not able to colonize plant roots strongly under P sufficient conditions (Jasper *et al.*, 1979; Amjee *et al.*, 1989; Koide & Schreiner, 1992) and there also exists reports of growth depressions of plants colonized by VAM in the presence of available P (Buwalda & Goh, 1982 ; Son & Smith, 1988; Peng *et al.*, 1993).

1.4.2 PHOSPHATE SOLUBILIZING MICROORGANISMS (PSMs) AS POTENTIAL PHOSPHATE BIOFERTILIZERS :

The involvement of microorganisms in solubilization of inorganic phosphates was known as early as 1903 (Stalstrom, 1903). Since then there has been extensive studies on the solubilization of mineral phosphates by microorganisms. Phosphate

solubilizing microorganisms (PSMs) are ubiquitous, there numbers varying from soil to soil. In the soil the phosphate solubilizing (PS) bacteria and fungi constitute 0.5%-0.1% respectively of the general microbial population and bacteria generally out number fungi from 2 to 150 fold (Banik & Dey, 1982 ; Kucey, 1983; Kucey *et al.*, 1989). Microbial P solubilization was envisaged to play an important role in providing P to the plants in view of the cost and deleterious effects of super phosphate application (Subba Rao, 1982; Goldstein, 1986; Tandon, 1987; Kucey *et al.*, 1989; Gaur, 1990; Richardson, 1994).

There are various studies on microbial P solubilization in pure culture conditions (Sperber, 1958; Goswami & Sen, 1962; Das, 1963; Duff *et al.*, 1963; Gaur *et al.*, 1973; Khan & Bhatnagar, 1977; Agnihotri, 1970; Arora & Gaur, 1979; Thomas *et al.*, 1985; Gupta & Saxena, 1993; Halder *et al.*, 1990, 1993; Lapeyrie *et al.*, 1991; Nahas, 1996). Pure cultures of several bacteria, fungi, actinomycetes and yeast inoculated are capable of solubilizing P from various insoluble P sources. These results are compiled and reviewed (Tandon, 1987; Subba Rao, 1982; Kucey *et al.*, 1989; Richardson, 1994). The PSMs seem to be ubiquitous in soils and in fact, soils have been the major source of isolation of PSMs (Kucey *et al.*, 1989). Many of these bacteria were isolated from the rhizosphere of various plants and are known to be metabolically more active than those isolated from sources other than rhizosphere (Baya *et al.*, 1981; Katznelson & Bose, 1959).

The PS bacteria can loose their PS properties upon repeated subculturing, (Sperber, 1958a; Kucey, 1983) but no such loss has been observed in the case of PS fungi (Kucey, 1983). In general, fungal isolates exhibit greater PS activity than PS bacteria in both liquid and solid media (Gaur *et al.*, 1973, Banik & Dey, 1982; Kucey, 1983). The PS ability of PSMs is also dependent upon the nature of the nitrogen source used

in the media, with greater solubilization in the presence of ammonium salts as nitrogen source. This is attributed to the extrusion of protons to compensate for ammonium uptake. This leads to lowering of pH thus releasing P (Roos & Luckner, 1984). It has also been shown that the media components affect the PS ability probably due to different buffering strength (Cunningham & Kuiack, 1992).

1.4.3 WHAT IS THE MECHANISM OF P SOLUBILIZATION ?

Most of the PSMs have been isolated based on their ability to solubilize Ca-P complexes. The ability to solubilize Ca-P is attributed to the ability of the PSMs to reduce the pH of their surroundings either by the release of organic acids or protons. The organic acids secreted can either directly dissolve the mineral phosphate and/or chelate the cation associated with phosphate (Sperber 1958b ; Katznelson & Bose, 1959; Bajpai & Sundara Rao, 1971 a, b; Bardiya & Gaur, 1972, 1974; Moghimi *et al.*, 1978 c). The PSMs produce various types of organic acids like lactic, oxalic, tartaric, succinic, citric, gluconic, ketogluconic, glycolic, etc. (Sperber, 1958b; Louw & Webley, 1959; Duff *et al.*, 1963; Taha *et al.*, 1969; Banik & Dey, 1981a,b, 1982). These acids can bring down the pH of the media thereby solubilizing P from acid soluble Ca-P complexes. A strong correlation was found between the ability of the microorganisms to reduce the pH of the medium and to solubilize P from rock phosphate (Nahas, 1996). Additionally the PS activity of *Rhizobium* was reported to be abolished by addition of NaOH indicating that the PS activity of this strain was entirely due to its ability to reduce the pH of the media (Halder *et al.*, 1993). But acidification does not seem to be the only mechanism of solubilization, as the ability to reduce the pH did not correlate with the ability to solubilize mineral P (Chhonker & Subba Rao, 1967; Gaur *et al.*, 1973; Surange, 1985; Asea *et al.*, 1988; Kucey, 1988). The contribution of the chelating properties of the organic acids is also important as it has been shown that the addition of 0.05 M EDTA to the media had

the same solubilizing effect as inoculation with *Penicillium bilaii* (Kucey, 1988). Addition of EDTA is also known to increase the uptake of P, Al and Fe by Italian rye grass (Hartikainan, 1981). In contrast to microbial Ca-P soubilization, there are only few PSMs that can solubilize Fe-P and Al-P (Banik & Dey, 1983, Gaur & Gaind, 1983; Kucey *et al.*, 1989).

As compared to microbial nitrogen fixation, not much is known about the genetic basis of microbial P solubilization. Molecular genetic approaches have been used to understand the genetic basis of gluconic acid production from P solubilizng *Erwinia herbicola* and *Pseudomonas cepacia.* Cloning of the gene involved in PQQ biosynthesis from *Erwinia herbicola* resulted in production of gluconic acid by the Transformed *E. coli* thus giving it a mineral phosphate solubilizing (*mps*) phenotype (Goldstein & Liu, 1987; Liu *et al.*, 1992). Similarly a gene was cloned which could be putatively involved in PQQ transport (Babukhan *et al.*, 1995). Since CaP complexes can be solubilised by reduction in pH, incorporating any organic acid secreting ability into microorganisms should give it a *mps* phenotype. Such organisms would be able to solubilize P in soils having high content of CaP.

The PSMs showing promising P solubilization potential under laboratory conditions were inoculated with the plants to determine their effect on plant growth, uptake and crop yields.

1.4.4 ARE PSMs EFFECTIVE IN SOILS ?

Although many microorganisms show PS ability under laboratory conditions, it has been more difficult to prove solubilization of P under soil conditions. However, no generalizations can be made regarding the results because of the variability (Kucey *et al.*, 1989). There are various reports of growth enhancement and greater P content

in plants inoculated with PSMs (Kundu & Gaur, 1980 b; Raj *et al.*, 1981; Banik & Dey, 1982; Saber *et al.*, 1977; Khafallah *et al.*, 1982; Piccini & Azcon, 1987). However, no growth enhancement or P solubilization was observed in certain cases (Banik & Dey, 1982; Badr El-Din & Saber, 1983; Kavimandan & Gaur, 1971). Doubts have been raised on the ability of these PSMs to liberate P under soil conditions (Subba Rao, 1982; Kucey *et al.*, 1989). The screening of the PSMs is carried out in the laboratory using unbuffered condition with various calcium phosphate complexes. The Ca-P complexes can be easily solubilized at lower pH. This will result in isolation of microorganisms which can reduce the pH of the media, there by solubilizing Ca-P complexes. But, since the soils have a very good buffering capacity, this may not reflect the true PS potential of the isolates. Also, it was calculated that the amount of acids required to solubilize P from soil was much higher than that required for solubilization of Ca-P complexes and it was suggested that microorganisms cannot secrete acids at these concentrations (Nye & Tinker, 1977). Nevertheless, the nature of the acid secreted is more important than the amount (Sperber, 1958a; Chhonker & Subba Rao, 1967).

Since PS bacteria can increase plant growth by mechanisms other than P solubilization, there have been some doubt their PS activity (Brown, 1974; Tinker, 1980). The release of plant growth substances by these bacteria would result in a larger plant, with obviously more amount of P. The growth enhancement of plants, by the PSMs was attributed in some cases to the production of bioactive compounds (Barea *et al.*, 1975; Azcon *et al.*, 1976). To account for this it has been proposed that if the amount of a particular nutrient increases within the plant tissues, it can be taken as evidence the factor has increased the uptake of that particular nutrient (Jarrel & Beverly, 1988). Such an increase of P within the plant tissues upon inoculation with the PSMs, has been reported indicating that PSMs are effective in

certain cases (Taha *et al.*, 1969 ; Kundu & Gaur, 1980, 1984 ; Banik & Dey, 1981b ; Khalafallah *et al.*, 1982).

1.5 RATIONALE OF THE PRESENT INVESTIGATION :

As is clear from the above disscussion that there are no general rules regarding the effectiveness of PSMs in soil conditions and though various reasons have been suggested for the observed variability, there are not enough evidences to support these reasons. Most of the PSMs have been isolated based on their ability to solubilize Ca-P complexes (which are acid soluble) in media lacking any buffering component. However, the alkaline soils containing Ca-P complexes have high buffering capacities. Therefore, It is possible that the PSMs isolated using this screening procedure may not be able to release P form soil. Also not much is known about the genetic basis of organic acid secretion.

Thus, the objectives of this work were (i) to find out the effectiveness of PSMs isolated under unbuffered media conditions to release P from CaP rich vertisol soil, (ii) to find out the effect of buffering of the media on the P solubilizing property of the PSMs, (iii) to determine the properties of PSMs necessary to release P from alkaline soils, (iv) to isolate and characterize novel PSMs which could be effective in solubilizing P from alkaline soils, and (v) to clone *mps* genes which would result in higher production of organic acids.