CHAPTER 1 LITERATURE REVIEW - PHOSPHORUS FIXATION IN SOIL AND MOBILISATION BY BACTERIA

1.1 Introduction

The world environment is increasingly polluted each day from more intensive application of chemical fertilisers and pesticides for crop production. For example, in 2005-2006, world consumption of fertiliser nitrogen was over 90 million tonnes (http://www.fertilizer.org, 2006), while it is estimated that by 2020, nitrogen requirements for cereal production will be 160 million tonnes, considering the need for food for the world population (Dyson, 1996). The dual discoveries of symbiotic biological nitrogen (N) fixation in legumes and the Haber-Bosch process for industrial nitrogen fixation by the reduction of atmospheric nitrogen to ammonia in the early decades of the 20^{th} century have allowed food production to increase. Increasing use of N fertilisers demands a similar increase in phosphorus (P) fertiliser for agricultural production, but increasing application of chemical fertilisers can be detrimental to the environment. The Australian environment may also be polluted, with increasing greenhouse effects in a more vulnerable, poorly buffered area, compared to other parts of the world. Therefore, more advanced approaches are necessary to allow increased food production while keeping the environment sustainable for people and other living beings.

Despite soils having significant amounts of total phosphorus (P), many soils are deficient in P available to the plant to maintain adequate growth and crop production. Therefore, continued chemical fertiliser applications, such as superphosphate, are required to maintain high level productivity. Unfortunately, the recovery of P fertiliser by plants is only 10 to 20 per cent of the P applied in the year of application (McLaughlin *et al.*, 1988; Holford, 1997). The majority of the P applied becomes chemically fixed within the soil system. Global consumption of P-based fertilisers in 2005-2006 exceeded 36 million tonnes of P_2O_5 (http://www.fertilizer.org, 2006). Based on this rate of consumption, the world's known reserves of high-quality rock phosphate will be consumed within the next 80 years (Isherwood, 2000). In Australia, about 0.45 million tonnes of P nutrient is consumed each year from different sources of phosphatic fertiliser (FIFA, 2004). Improving the efficiency by which plants are able to access P from the soil through microbial associations would, therefore, be of considerable benefit, both economically and environmentally. In this literature review, therefore, the importance of P, the causes and mechanisms of P fixation and example

of microorganisms that can mobilise unavailable forms of P to forms available to the plants are discussed.

1.2 Background and importance of phosphorus

Phosphorus has been called 'the key of life' because it is directly involved in most essential life processes. Since it is a part of the nucleic acids DNA and RNA which carry the genetic code, it is an essential component of every living cell (Brady and Weil, 2002). It is found in a wide variety of biochemicals, including nucleotides, coenzymes, phosphoproteins, phospholipids, and sugar phosphates. For the physical framework of protoplasm and cell membranes, phospholipids such as lecithin and cephalin are essential components. Such phospholipids also occur as an essential part of chloroplast structure.

It plays a vital role in the energy transfer compounds needed to carry on life activities. Phosphorylated compounds act as 'energy currency' within plants (Kennedy, 1992). Energy acquired from photosynthesis and the metabolism of carbohydrates is temporarily stored in these phosphate compounds for subsequent use in growth and reproductive processes. The most common P energy currency is adenosine triphosphate (ATP). It is associated with the high-energy pyrophosphate bond between phosphate groups located at the terminal position in ATP (Figure 1.1).

ATP has two pyrophosphate bonds between its three phosphate molecules; therefore it carries the most energy. When the terminal phosphate molecule either ATP is released in normal metabolism a relatively large amount of free energy is liberated (57.1 kJ; Kennedy, 2001). ATP is the source of energy that powers most energy-requiring metabolic process in plants. The transfer reaction of the energy-rich phosphate molecules from ATP to substances in the plant is known as phosphorylation. In organic phosphorylation reactions, ATP is converted to ADP with a phosphate molecule being attached to the phosphorylated compound. ATP is regenerated from ADP and inorganic phosphate at sites of energy production, such as the oxidative reactions of respiration in mitochondria. It is known that every aspect of the process

of formation and function of the N_2-fixing nodule is limited by the availability of P (McDermott, 1999) so that legumes require more P.

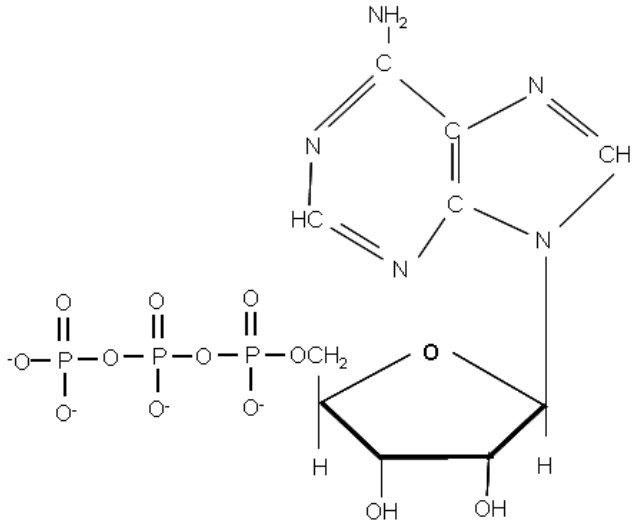

Figure 1.1 Structure of adenosine triphosphate (ATP) http://chm.bris.ac.uk, 2005)

An adequate supply of P early in the life of a plant is important for developing the primordia for its reproductive parts. This supply of P is provided by the seed. Phytate, composed of calcium and magnesium salts of phytic acid, is the principal storage form of P in seeds (Brady and Weil, 2002). Although P is second only to nitrogen in frequency of use as an essential fertiliser, in some cases P levels can be more limiting than nitrogen because certain microorganisms can make atmospheric nitrogen available to plants.

Given all these essential roles, it is not surprising that the quality of certain fruit, forage, vegetable and grain crops is considered to be improved and disease resistance increased when these crops have satisfactory P nutrition.

1.2.1 Phosphorus chemistry

1.2.1.1 Historical perspective of phosphorus

In 1669, Henning Brand of Hamburg, discovered phosphorus in the residue from distilling urine (Corbridge, 1978). This substance glowed in the dark and burst into

flame when exposed to the air. Subsequently it was named '*phosphorus*' meaning light-bearing.

About 1770 P was recognised as an essential ingredient of animal bones and teeth and in 1779 the first P-containing mineral, pyromorphite, was identified by Gahn (Corbridge, 1978). Significant advances were made in the science of plant nutrition, and the value of phosphates as fertilisers was realised during the first half of the 19th century. The first P-containing striking matches were invented by Derosne in 1812, and in 1842 Lawes and Murray, at Rothamsted Experiment Station in the U.K., took out patents for the manufacture of fertilisers from sulphuric acid and bones. By the end of the 19th century matches and fertilisers had become the most important commercial uses for P compounds (Corbridge, 1978). In the late 18th century, substantial phosphate mineral deposits had been found and commercial production of P compounds from these ores commenced in Europe about 1850. In 1888 Readman invented the electrical furnace method for the continuous production of the pure element.

Inorganic P compounds remain the most important for human use, with fertilisers constituting the largest single application (about 85 per cent). Synthetic detergents, introduced about 1950, are now second in magnitude of use and animal feedstuffs third. The organic compounds, commercially important since 1940, have had numerous applications in plastics, pesticides and pharmaceuticals, but at present utilise only about two per cent of the P manufactured (Corbridge, 1978). In 1929, Fiske and Subarrow first discovered the universal energy transfer compound, adenosine triphosphate (Figure 1.1), in muscle. The intimate involvement of P compounds in numerous biochemical reactions had been firmly demonstrated in the 1930s; by 1940 it had been clearly established that the highly polymerised phosphate esters, known as nucleic acids, were normal constituents of all cells. Additionally, it was realised that these compounds were the essential constituent of the chromosomes long recognised for their function in hereditary processes. The elucidation of the molecular structure of the P-rich nucleic acids by Crick and Watson in 1953, probably represents the most profound achievement in 20th century biology (Corbridge, 1978). In DNA, phosphate groups comprise an outer hydrophilic sheath freely interacting

with the aqueous phase while the base pairs coding for amino acids are buried in the relatively hydrophobic interior of the double helix.

1.2.1.2 Atomic properties and bonding

Phosphorus, symbol P, atomic number 15, and atomic weight 30.97, belongs to Group V of the periodic table of the elements. The elements of this group, namely nitrogen, P, arsenic, antimony and bismuth are sometimes known as pnictides. The chemistry of P generally resembles that of arsenic much more closely than that of nitrogen. The stable isotope ^{31}P has a nuclear spin of ½ and constitutes 100 per cent of the naturally abundant species. Six unstable isotopes are known (Corbridge, 1978).

The electronic structure of the P atom is $1s^2\ 2s^2\ 2p^6\ 3s^2\ 3p^3$, with three unpaired electrons in the outer 3p orbitals which are available for chemical bonding. Phosphorus can be formally trivalent or pentavalent, using only three or all five electrons in the outer M shell to form shared electron pairs with other atoms. In the vast majority of its compounds, the element forms three, four or five covalent linkages to other atoms and among these, the four-connected compounds are both the most numerous and technically the most important. In group V, nitrogen is the most electronegative element and is, like P, a non-metal. Nitrogen is sufficiently electronegative to form strong hydrogen bonds whereas those involving P are very weak, and they are not formed at all by As, Sb and Bi (Corbridge, 1978).

The diverse stereochemical configurations of 3, 4, 5 and 6-connected P compounds are illustrated by the halides (Figures 1.2a-1.2f).

The pyramidal structure of P trichloride, PCl_3 (1.2a), typifies trivalent P compounds, while tetrahedral P oxychloride $POCl_3$ (1.2b), and the pentachloride, PCl_5 (1.2e) typify the spatial arrangements adopted in pentavalent P compounds. The hexachlorophosphonium ion PCl_6^- is based on an octahedral bond configuration and represents a comparatively small group of compounds. Two-connected P atoms are found in the PH_2^- ion and in such molecules as $F_3C.P.CF_2$ and phosphorin C_5H_5P. One-connected atoms are represented by PCH, P_2 vapour and various unstable

spectroscopic molecules such as PH, PN and PO (Figures 1.3g-1.3l). These highly variable configurations illustrate the versatility of P in its formation of compounds and help explain its range of chemical behaviour in soil and biology.

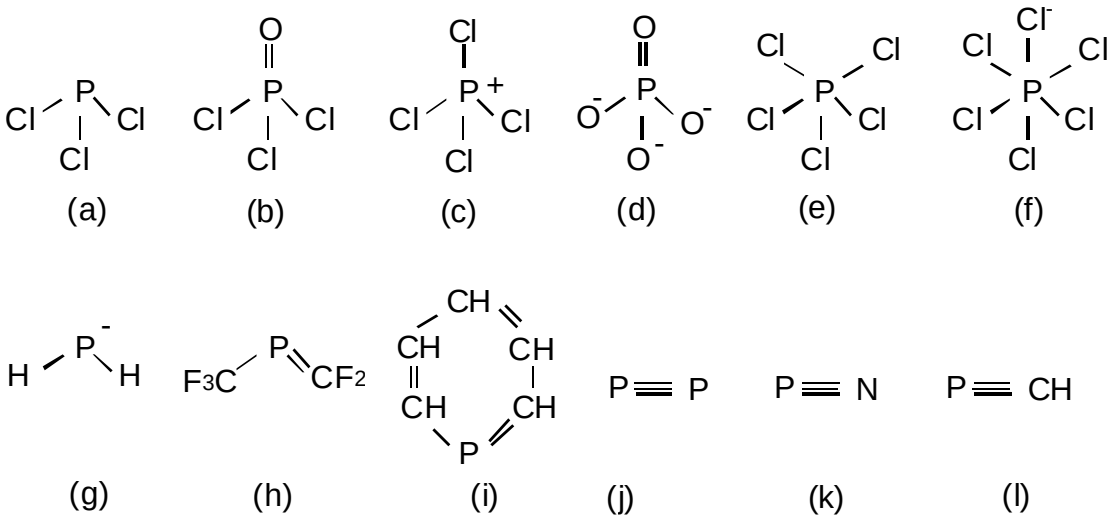

Figure 1.2 The common stereochemical configurations of 3, 4, 5 and 6-connected P compounds (Figures 1.3a-1.3f) and various unstable spectroscopic molecules (Figures 1.3g-1.3l) (Corbridge, 1978).

1.2.2 Soil phosphorus chemistry

1.2.2.1 Soil organic phosphorus

The organic and inorganic forms of soil P and the soil P cycle are shown in Figure 1.3 (Sims and Sharpley**,** 2005).

The levels of organic P in soils vary widely, ranging from zero to over 0.2 per cent soil mass. The organic fraction generally constitutes 20 to 80 per cent of the total P in surface soil horizons (Brady and Weil, 2002). McLaughlin *et al.* (1990) postulated that organic P in soil generally accounts for around 50 per cent of total soil P and up to 80 per cent of the total P for pasture soil. In Australian soils, inositol P has been shown to constitute up to 29 per cent (mean 18 per cent) of the total organic P (Williams and Anderson, 1968).

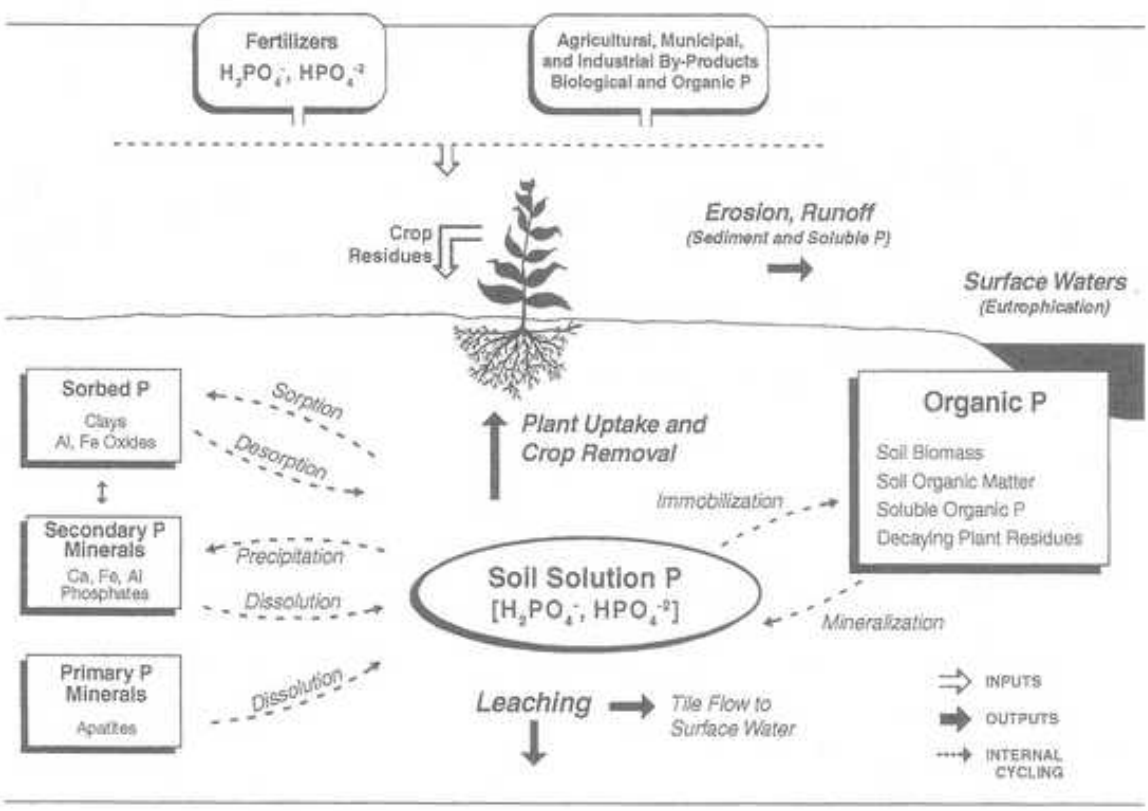

Figure 1.3 Soil-plant phosphorus cycle (from Sims and Sharpley, 2005).

The diagram shows that P fertiliser added to soils can be converted to ionic forms available to plants. Ionic P becomes unavailable to the plant after adsorption reactions with clay, Al, and Fe Oxides, and Ca, Fe and Al phosphates. Some available P becomes unavailable by immobilisation with the soil biomass. Available P is taken up by plants and is lost from the soil by crop removal (from Sims and Sharpley, 2005).

It is usually considered that plants derive their P only from inorganic sources and that organic P compounds must be mineralised before they are available to plants. Attention has been focused on the inorganic rather than the organic P in soil, so that knowledge of the specific nature of most of the organic bound P in soils is limited. Most naturally occurring organic forms of P are esters of orthophosphoric acid and numerous mono and di-esters have been characterised.

These organic P esters have been identified in five classes of compounds as: (a) inositol phosphates, phosphate esters of a sugar-like compound, inositol [$C_6H_6(OH)_6$]; (b) nucleic acids; (c) phospholipids; (d) nucleotides and (e) sugar phosphates. Black (1968) concluded that about two per cent of the total organic P in soil was present in nucleic acids, one per cent in phospholipids, and 35 per cent in inositol phosphates, with the remaining 62 per cent unidentified.

1.2.2.2 Inositol phosphates

The most abundant organic P compounds yet found in soils are the stable group of inositol hexaphosphates, six-membered carbon rings with a phosphate group on each carbon (Figure 1.4).

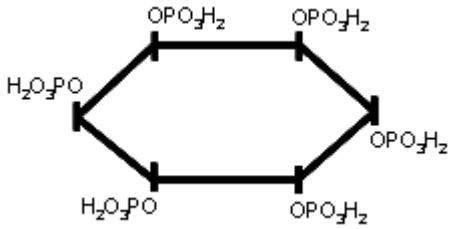

Figure 1.4 Structural configuration of inositol hexametaphosphate (Hesse, 1971).

All the phosphate groups are linked to the inositol as esters and have two replaceable hydrogen atoms; in soil these are most probably replaced by iron, aluminium, zinc, calcium and magnesium (Caldwell and Black, 1958).

Inositols are a homocyclic sugar-like compounds, $[C_6H_6(OH)_6]$, which can form a series of phosphate esters ranging from monophosphates to a hexaphosphate. Phytic acid (*myo*-inositol hexaphosphoric acid) is the most common ester of this group and forms phytates in soils. The inositol phosphates are released from organic materials in soil at a much slower rate than many other esters, but they are quickly stabilised and can accumulate in some soil so that they account for more than 50 per cent of the organic P and about 25 per cent of the total P (Tisdale *et al.*, 1985). Inositol hexaphosphate forms a number of very insoluble salts through reactions with iron and aluminium under acid conditions and with calcium in alkaline solutions. It also forms strong complexes with proteins and with some metal ions. In these forms, inositol hexaphosphate is more resistant to enzymic attack than are the more soluble ester salts.

Clay minerals such as montmorillonite and finely divided sesquioxides will strongly adsorb inositol hexaphosphate. Other *myo*-inositol phosphates are adsorbed, with the degree of adsorption decreasing with declining numbers of phosphate groups.

1.2.2.3 Nucleic acids

Both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) occur in all living organisms. Each consists of a chain of sugar units, either ribose or deoxyribose, joined by phosphate ester bridges. A nitrogenous base derived from either a purine or a pyrimidine is attached to each sugar molecule. It is assumed that nucleic acids are released into soil much more rapidly than inositol phosphate and that they are broken down quickly. It is difficult to isolate pure nucleic acid from soils; its total measurement has usually been based on the amounts of nucleotides or purine and pyrimidine derivatives liberated by hydrolysis of soil organic-matter fractions. More recently, improved techniques for isolation of DNA from soil have been developed, enabling the microbial biodiversity of soil to be analysed (Stackebrandt, 2003). The Polymerase Chain Reaction (PCR) is a technique that is used to make a large number of copies of a gene, necessary to have enough starting material for base sequencing. Currently PCR is a very important method for identifying gene base sequences of living cells (http://allserv.rug.ac.be, 2004).

1.2.2.4 Phospholipids and other esters

Soil phospholipids are esters of fatty acids containing P. Some of the most common phospholipids are derivatives of glycerol. Phosphatidylcholine (lecithin) and phosphyatidylethanolamine are the predominant phospholipids in soils. The rate of release of phospholipids from organic sources in soils is rapid (Tisdale *et al.*, 1985).

Much of the remainder of organic P in soils is believed to originate from microorganisms, especially from bacterial cell walls, which are known to contain a number of very stable esters (Tisdale *et al.*, 1985).

1.2.3 Inorganic phosphorus compounds

Most inorganic P compounds in soil fall into two groups: (a) calcium compounds and (b) iron and aluminium compounds. Calcium compounds are found in alkaline soil

where they are more stable and insoluble than iron and aluminium compounds. Calcium compounds become more soluble as soil pH decreases, tending to dissolve in acid soils. Of the common calcium phosphates, the apatite minerals are the least soluble, and are therefore the least available source of P. Some apatites such as fluorapatite ($Ca_5(PO_4)_3F$) are so stable that they persist even in acid soils. Phosphorus from the simpler monocalcium phosphate ($Ca(H_2PO_4)_2.H_2O$)), as found in superphosphate, and to a lesser extent dicalcium phosphates ($CaHPO_4.2H_2O$)), is available for plant uptake (Brady and Weil, 2002). Typical iron and aluminium hydroxy phosphate minerals are strengite ($FePO_4.2H_2O$) and variscite ($AlPO_4.2H_2O$), which are insoluble in strongly acid soils and become more soluble as soil pH rises (Brady and Weil, 2002). Therefore, these minerals are unstable in alkaline soils, but they are dominant in acid soil.

1.2.4 Phosphorus in the soil solution

The solubility of the inorganic forms of P is a more serious problem for P nutrition of plants than for any other macronutrient. Phosphorus is unavailable in high and low pH ranges of soil solution. The most favourable pH for P availability is near neutral to slightly acid. Phosphorus is absorbed by plants largely as the primary, secondary and tertiary orthophosphate ions ($H_2PO_4^-$, HPO_4^{2-} and PO_4^{3-}), which are present in the soil solution and the amount of each form depends on the pH of the soil solution (Troeh and Thompson, 1993). Most of the P absorbed by plants is in the monovalent orthophosphate form, $H_2PO_4^-$, which is predominant at pH values below 7.2 and is typical of most agricultural soils. The freely interconvertible HPO_4^{2-}, a form which is more dominant above pH 7.2, may be used by some plants. Another phosphate ion (tertiary orthophosphate PO_4^{3-}) occurs at pH values too high for it to be significant in plant nutrition. Even at a pH of 12 the HPO_4^{2-}, concentration is still relatively greater than that of PO_4^{3-} (Kardos, 1967). From this relationship it is obvious that all phosphate reaction systems will be fundamentally influenced by the hydrogen ion activity in the systems. Some plants may also absorb certain soluble organic phosphates but these are very minor amounts. For example, nucleic acid and phytate can be taken in by plants from sterile sand or solution cultures (Tisdale *et al.*, 1985).

Figure 1.5 shows available forms of P ions at different pH values (Troeh and Thompson, 1993).

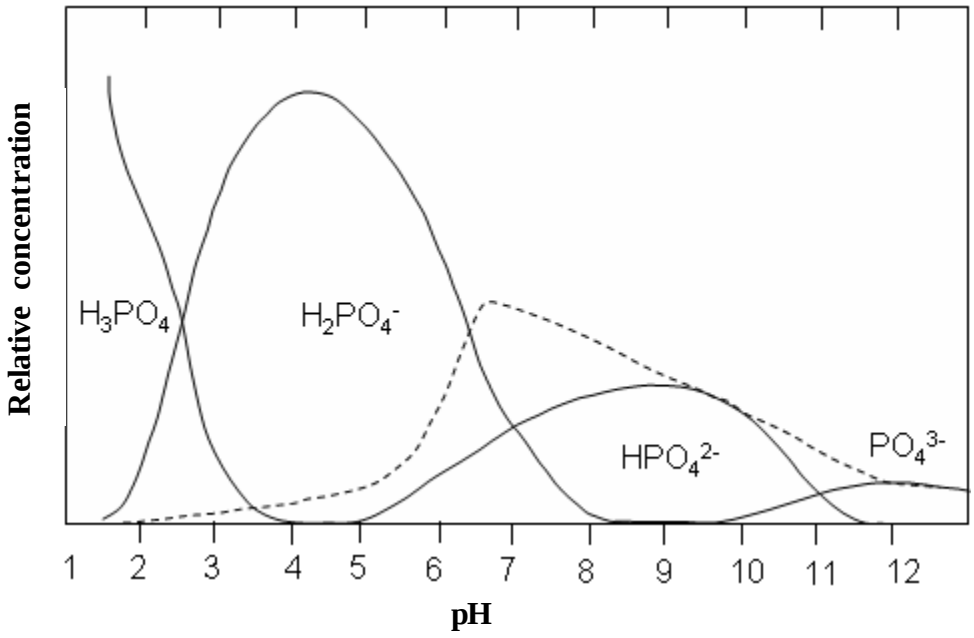

Figure 1.5 A schematic illustration of the relative proportions of phosphate ions in solution at different pH levels in a Ca-H_3PO_4 system.

The dashed lines shows the upper limit of available P in solution imposed by the solubility of calcium phosphates above pH 6.5 or iron and aluminium phosphate below pH 6.5 (Troeh and Thompson, 1993).

1.2.5 The phosphorus status of soil

For agriculture, the management of available P is second only in importance to the management of available nitrogen for the production of healthy plants and profitable yields. Inadequate supplies of soil P restrict plant growth and reduce the benefits from available nitrogen. Different figures for organic, inorganic and total P in soil have been reported. Hemwall, (1957) reported that the measured organic P pool in soils varies from 20 to 4000 kg ha^{-1} and is also of highly variable availability. Lindsay and Vlek (1977) stated that P in Australian soils usually ranges from 200 mg kg^{-1} to 5000 mg kg^{-1} with an average content of about 500 mg kg^{-1}. The total P content has been reported to be between 100 and 2500 kg ha^{-1} with an average value of about 1000 kg ha^{-1} in the surface 20 cm of soil (Tisdale *et al.*, 1985). In another report the total P content in average soils is described as about 500 mg kg^{-1} but only about 0.1 per cent of the total P is available to plants (Scheffer and Schachtschabel, 1992). It can be

seen, that the maximum figure for the organic P pool reported by Hemwall (1957) is much more than the total P pool as reported by Tisdale *et al.* (1985).

Apart from nitrogen, the level of P in soils, more than that of any other single nutrient, governs soil fertility (Norrish and Rosser, 1983). Although some soil profiles have a higher P pool in the surface soil as a result of adding fertiliser P, it is a relatively immobile element in the landscape and hence the fertility of a soil is inherited from its parent material. The other major soil nutrients such as N, S, Mg, Ca and K are more mobile and the parent material is not such a controlling factor. Lindsay and Vlek (1977) suggested that the P required by a wheat crop is only a small fraction of the total P in the top 15 cm of the soil. Soil tests (Bray 1) indicate that the mobile P level for optimum wheat production should be maintained above about 30 mg kg^{-1} in the top 15 cm of soil (http://www.ppi-far.org, 2002). Available P is dynamic and many soils cannot supply P at a sufficient rate for plant growth, despite the presence of massive amounts of unavailable forms of P. There is no simple relationship between the amount of P available and the total P content.

Only about 15 per cent of P applied as fertiliser to the soil is used by plants in the subsequent cropping season and the remaining 85 per cent forms different insoluble complexes in the soil. Although fertiliser added to the soil will increase the P content of the surface soil, under normal agricultural practice the annual increase in total P is slight (Norrish and Rosser, 1983). For example, about 10 kg ha^{-1} of P as superphosphate is typically applied in wheat production, and if this is distributed over a depth of about 15 cm then it represents an increase of 5 mg kg^{-1} soil. If larger amounts of P are added, the P content of the surface soil will increase in proportion. The proportion of ions of available P from that added could be increased if more could be mobilised.

1.2.6 Phosphorus fixation in soil

The process of soluble phosphate anions being bound with soil constituents so that they become insoluble and unavailable to plants is termed fixation. Several mechanisms of P fixation have been described. These include precipitation-

dissolution reactions, adsorption-desorption reactions, and immobilisation-mineralisation reactions. In the most recent quarter century, the research focus has switched to the relatively fast reactions by which P is either adsorbed by or desorbed from soil solids with charged surfaces (Tisdale *et al.*, 1985). The later investigators tend to conclude that adsorption-desorption reactions on soil constituents are of greater significance than precipitation-dissolution reactions of phosphorus minerals.

Adsorption is defined as the net accumulation of matter at the interface between a solid phase and an aqueous solution phase (Sposito, 1989). It differs from precipitation because it does not include the development of a three-dimensional molecular structure, even if such a structure grows on a surface. The matter that accumulates in two-dimensional molecular arrangements at an interface is the adsorbate and the solid surface on which it accumulates is the adsorbent. A molecule or an ion in the solution that potentially can be adsorbed is termed as adsorptive.

Tisdale *et al.* (1985) describes all these terms in a more active way. Adsorption means the removal of P from solution and its retention at soil surfaces. When P is held at the surface of a solid, it is said to be adsorbed. If the retained P penetrates more or less uniformly into the solid phase, it is considered to be absorbed or chemisorbed. Desorption is the release of adsorbed P into solution. A common term, 'fixation', is used collectively to describe both adsorption and precipitation reactions of P. Although many authors use the terms fixation and retention interchangeably, it has been argued that retention refers to that part of adsorbed P that can be extracted with dilute acids and this portion of P is relatively available to plants (Tisdale *et al.*, 1985). Conversely, the term fixation is reserved for the portion of soil P that is not extractable by dilute acids; this portion of P is not readily available to plants. The fixation of P on metal oxides via water and hydroxyl groups is shown in Figure 1.6. Generally, P fixation occurs in acid soil, clay mineral, and alkaline soil conditions. These three types of P fixation are described in the following section.

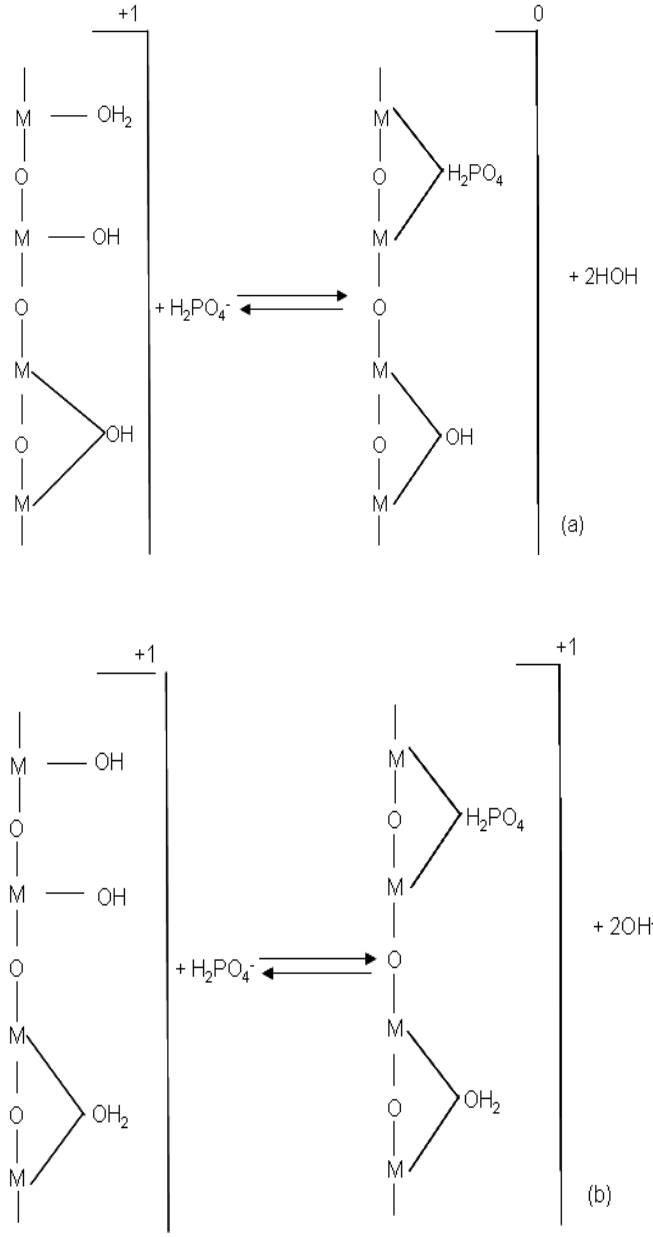

Figure 1.6 Displacement of (a) water and (b) hydroxyl groups from a metal oxide surface by phosphate (modified from Tisdale *et al*., 1985).

1.2.6.1 Phosphorus fixation in acid soil

Iron and aluminium oxides and hydroxides have been recognised as playing a significant role in P fixation in acid soils. The removal of the free iron oxide content of soil colloids reduced the magnitude of P fixation indicating that these compounds are partially responsible for P fixation (Toth, 1937). It has also been suggested that iron and aluminium sesquioxides play an important role in P fixation (Struthers and Sieling, 1950; Bradley and Sieling, 1953). More recently, it was demonstrated that

amorphous and poorly crystalline iron and aluminium minerals are commonly involved in P adsorption processes in soils (Pant *et al.*, 2002). Phosphate solubility and its reactions are influenced predominantly by the dissolution-precipitation activities of amorphous aluminium, iron and crystalline iron oxides (Zhang *et al.*, 2001). The reactions of phosphatic solutions with acid soils, with aluminium and iron hydrous oxides, and with silicate clays demonstrated that when crystalline products can be detected they are usually either simple aluminium/iron phosphates related to variscite, or alkali aluminium/iron phosphates related to taranakite (Haseman *et al.*, 1950; Kittrick and Jackson, 1955; Lindsay and Stephenson, 1959). The fixation reaction can occur between phosphate and iron, aluminium, and manganese hydrous oxide or between phosphate and silicate minerals. Many acidic soils contain high amounts of free iron and aluminium and of iron and aluminium hydrous oxide clays. The free iron, aluminium and the sesquioxide clays react rapidly with phosphate, forming a series of insoluble or poorly soluble hydroxyphosphates such as a monodentate complex (Figure 1.7) or bidentate complex (Figure 1.8).

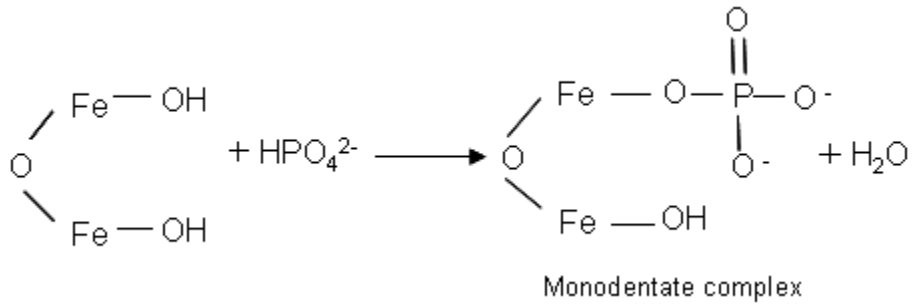

Figure 1.7 Structural configuration of phosphate fixation with iron (monodentate complex) in acidic soil conditions (modified from Tan, 1993).

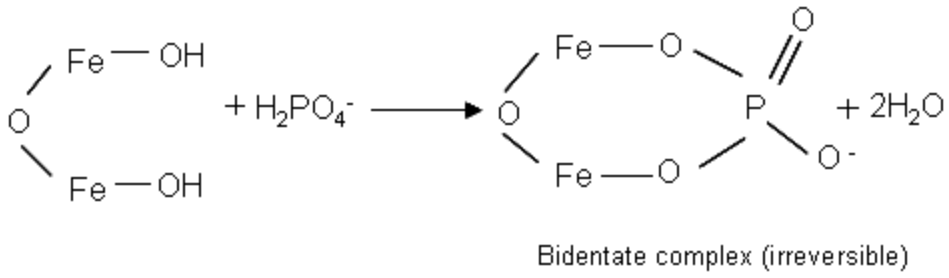

Figure 1.8 Structural configuration of phosphate fixation with iron (bidentate complex) in acidic soil conditions (modified from Tan, 1993).

Phosphate retention occurs mainly in acidic soil conditions while phosphate fixation by hydrous oxide clays occurs over a relatively wider pH range. The products formed

by both retention and fixation reactions are not pure aluminium or pure iron phosphate. Solubility criteria indicate that the immediate reaction products of applied phosphate in acid soils are moderately soluble but upon ageing, these intermediate reaction products are slowly transformed into variscite, which may coexist with gibbsite as a stable solid phase (Lindsay *et al.*, 1959). A similar reaction happens in the case of iron. The ultimate end product formed between aluminium hydroxides and phosphates is variscite ($AlPO_4.2H_2O$) and between iron and phosphate is strengite ($FePO_4.2H_2O$); these two products are the least soluble (or most stable) compounds at acid pH. A series of intergrades between variscite and strengite is usually present in soils and is called the variscite-strengite isomorphous series (Lindsay *et al.*, 1959). It is generally agreed that the formation of iron and aluminium phosphate ($FePO_4$ and $AlPO_4$) is most unlikely to account for much P fixation except where the pH is less than 4 (Hemwall, 1957). Potentiometric titration curves for iron and aluminium chlorides in the presence of varying amounts of phosphate demonstrate that in the pH range of acid soils, the compounds formed are $Fe(OH_2)_3(OH)_2H_2PO_4$ or $Al(OH_2)_3(OH)_2H_2PO_4$ rather than $FePO_4$ or $AlPO_4$ (Swenson *et al.*, 1949).

Another type of phosphate fixation occurring in acidic conditions causes a reaction between phosphate and silicate clays. Especially in the case of soil clay minerals exhibiting exposed hydroxyl groups, such as the kaolinitic groups, there is a strong affinity for phosphate ions. Phosphate ions react rapidly with octahedral aluminium by replacing the hydroxyl groups located on the surface plane of the mineral. Generally, clays (eg kaolinite) with low SiO_2/R_2O_3 (sesquioxide) ratios have higher phosphate-fixing capacity than clays (montmorillonite) with high SiO_2/R_2O_3 (sesquioxide) ratios (Tisdale *et al.*, 1985).

Thus P fixation in acid soils is primarily due to the formation of iron and aluminium compounds of the general formula $M(H_2O)_3(OH)_2H_2PO_4$. The iron- and aluminium-containing soil minerals, including the clay minerals, are the source of the iron and aluminium. The formation of these compounds is governed by the solubility product, the common ion, and salt effect principles. Under certain conditions, a precipitate is formed, whereas under other conditions the compounds are adsorbed (Hemwall,

1957). In a generalised manner, the fixation of P in acid soils can be visualised as follows:

$$[M(H_2O)_xA_y)^{+3\ to\ +1} + A + H_2PO_4^- - (M(H_2O)_xA_zH_2PO_4]$$

M_2O_3,$M(OH)_3$, Precipitated or
Clay Minerals and Exchange Sites Adsorbed

Here the symbol M stands for the cations of iron or aluminium and the symbol A stands for oxide or hydroxide.

1.2.6.2 Phosphorus fixation by clay minerals

The reactions of P with clay minerals causing P fixation in soil have also been studied. It is considered probable that aluminium associated with clay is largely responsible for this P fixing property (Hemwall, 1957). It was deduced that P fixation by clay minerals was clearly associated with the sesquioxide aluminium content of the clays not with the intact aluminosilicate clay minerals (Coleman, 1944). The amount of P fixed by clays was shown to be proportional to the amount of free aluminium oxides on the clays, and that fixation occurs only as long as this form of aluminium is present. Additional support for the hypothesis that free aluminium oxides are necessary for clay minerals to fix P was provided by the demonstration that montmorillonite will not fix P once all iron and aluminium oxides are removed from the clay (Ellis and Truog, 1955). It was shown that the presence of fluoride or ammonium tricarboxylic acid, both strong complexing agents for aluminium, inhibits the fixation of P by montmorillonite (Hemwall, 1957). Moreover, the X-ray diffraction evidence showed that fixed P is not sorbed between the lamellae of the clay minerals and does not interfere with the swelling properties of the clay.

An experimental procedure for developing a P retention characteristic of soil layers has been developed (Pant *et al.*, 2002). The P adsorption maxima were positively correlated with oxalate-extractable aluminium and citrate dithionite bicarbonate-extractable aluminium under anaerobic conditions, but there was no significant

correlation with either oxalate-extractable iron or citrate dithionite bicarbonate-extractable iron. The X-ray diffraction patterns indicated that smectite was the dominant mineral in the clay-size fraction. It is likely that the permanent negative charge of smectite limits its contribution to P adsorption. In addition, the equilibrium P concentration of the soils was significantly correlated to citrate dithionite bicarbonate-Al but not to citrate dithionite bicarbonate-Fe, suggesting that associated Al is the primary factor in elevating equilibrium P concentration in these experimental soils.

1.2.6.3 Phosphorus fixation in alkaline soil

Soil phosphate fixation is not limited to acidic soil conditions, but also occurs in alkaline soil. Phosphorus fixation in alkaline calcareous soil is usually attributed to the formation of insoluble phosphate compounds of calcium. The iron and aluminium compounds discussed in relation to fixation in acid soils are also responsible for some fixation in soils of higher pH (Hemwell, 1957). However, alkaline soils contain high amounts of soluble and exchangeable Ca^{2+} and, frequently, $CaCO_3$ (Tan, 1993; Brady and Weil, 2002). In these alkaline soil conditions, phosphate is reported to react with both the ionic and the carbonate form of Ca. The reactions can be illustrated as follows:

$$3Ca^{2+} + 2PO_4^{3-} \longrightarrow \underset{\text{(insoluble)}}{Ca_3(PO_4)_2}$$

$$\underset{\substack{\text{Monocalcium}\\ \text{phosphate (soluble)}}}{Ca(H_2PO_4)_2.H_2O} + 2CaCO_3 \longrightarrow \underset{\text{(insoluble)}}{Ca_3(PO_4)_2} + 2CO_2\uparrow + 3H_2O$$

Other forms of insoluble Ca phosphate such as hydroxyapatite [$Ca_5(PO_4)_3OH$], oxyapatite ($Ca_5(PO_4)_3O$), carbonated apatite ($Ca_5(PO_4)_3CO_3$) and Ca-fluoroapatite ($Ca_5(PO_4)_3F$) can also be formed by this type of reaction between calcium and phosphate in alkaline soil conditions (Brady and Weil, 2002). Calcium phosphate could adsorb additional phosphate, consequently creating even more complicated systems (Hemwall, 1957). In a different approach to the role of clay minerals in P fixation in alkaline soil, it was shown that P can also be fixed by the $Al(OH)_2^+$ in the clay fraction of alkaline soils, this fixation being partially a function of the other cations present (Wild, 1953). This kind of P fixation is a serious problem in arid

region soils; however, it can also become a significant in the acid tropical soils when the soil needs high applications of lime. In such conditions, applications of P fertilisers generally give low plant growth responses.

1.2.7 Factors affecting phosphorus fixation in soil

From the preceding discussion about mechanisms of P fixation reactions, some preliminary factors influencing P fixation are apparent. Because of the importance of retention and fixation in modifying the effectiveness of applied fertiliser P, these factors, and the extent to which they influence fixation, need further consideration. The various factors fall into the following groupings: (1) the nature and amount of soil components (2) pH, (3) other ions, (4) kinetics and (5) saturation of the adsorption complexes.

1.2.7.1 Nature and amount of soil components

Adsorption-desorption reactions are affected by the types of surfaces contacted by P in the soil solution.

Hydrous metal oxides of iron and aluminium: These substances, particularly the hydrous ferric oxide gel, have the capacity to adsorb very large amounts of P. Although these substances are present in all soils, they are most abundant in well-weathered soils. Aluminium and iron oxides and their hydrous oxides can occur as discrete particles in soils or as coatings of or associated with other soil particles. These substances can also exist as amorphous aluminium hydroxyl compounds between the layers of expandable aluminium silicates in clays.

Generally, it is accepted that in soils with significant contents of iron and aluminium oxide, the less crystalline the oxides are, the larger their P fixation capacity because of their greater surface areas. However, crystalline hydrous metal oxides are usually capable of retaining more P than clay layer silicates.

Type of clay: Phosphorus is retained to a greater extent by 1:1 (eg. kaolinite) than 2:1 (eg. montmorillonite) clays. The layers of the 1:1-type minerals are made up of one tetrahedral (silica) sheet and one octahedral (alumina) sheet, while 2:1 type minerals are characterised by an octahedral sheet sandwiched between two tetrahedral sheets (Brady and Weil, 2000). Soil high in kaolinitic clays, such as those found in areas of high rainfall and high temperature, can fix or retain larger quantities of added P than those containing the 2:1 type such as montmorillonite. The higher amount of P fixed by 1:1 clays is probably largely due to the higher amounts of hydrated oxides of iron and aluminium associated with kaolinitic clays. In addition, kaolinite develops pH-dependent charges on its edges which can enter into adsorption reactions with P. Clays such as kaolinite with a low SiO_2/R_2O_3 ratio (R represents the elements aluminium or iron in sesquioxide) can fix larger quantities of P than clays with a high ratio. Kaolinite, with a 1:1 silica/alumina lattice, has a larger number of exposed hydroxyl groups in the gibbsite layer that can be exchanged for P.

Clay content: Soils containing large quantities of clay can fix more P than those comprising smaller amounts of clay and more siliceous sand and silt. To some extent, the greater surface area exposed in high clay soils, the greater the tendency to retain P. Silica does not retain P.

Amorphous colloids: In young soils, such as the Andepts, texture is often meaningless because of the presence of large quantities of X-ray amorphous colloids. Phosphorus retention in these soils is closely related to the content of X-ray amorphous colloids and with surface area (Tisdale *et al.*, 1985). Volcanic ash is an important parent material of such soils and its weathering imparts a high P-adsorption capacity to soils.

Calcium carbonate: A minor portion of the P sorption capacity of soils is said to originate from calcium carbonate. Much of the adsorption attributed to it, however, may actually be due to hydrous ferric oxide impurities. The activity of P will be lower in those soils that have a high Ca^{2+} activity, a large amount of highly reactive calcium carbonate, and a large amount of calcium-saturated clay (Tisdale *et al.*, 1985).

1.2.7.2 Effects of pH on predominantly insoluble phosphorus changes

Soil pH has a profound influence on the amount of soluble P fixed and the manner in which it becomes fixed. Adsorption of P by iron and aluminium oxides declines with increasing pH. Raising the pH of clays above 4 can increase P adsorption because of the increase in hydration of Al^{3+}. Raising the pH may, however, reduce P adsorption where interlayer hydroxyaluminum polymers exist, as in many types of vermiculite.

Phosphorus availability in most soils is at a maximum in the pH range 6.0 to 6.5. At lower pH values, retention results largely from the reaction with iron and aluminium and their hydrous oxides. Above pH 7.0 the ions of calcium and magnesium, as well as the presence of carbonates of these metals in the soil, cause precipitation of the added P, and its availability again decreases (Figure 1.9) (1.2.8.3).

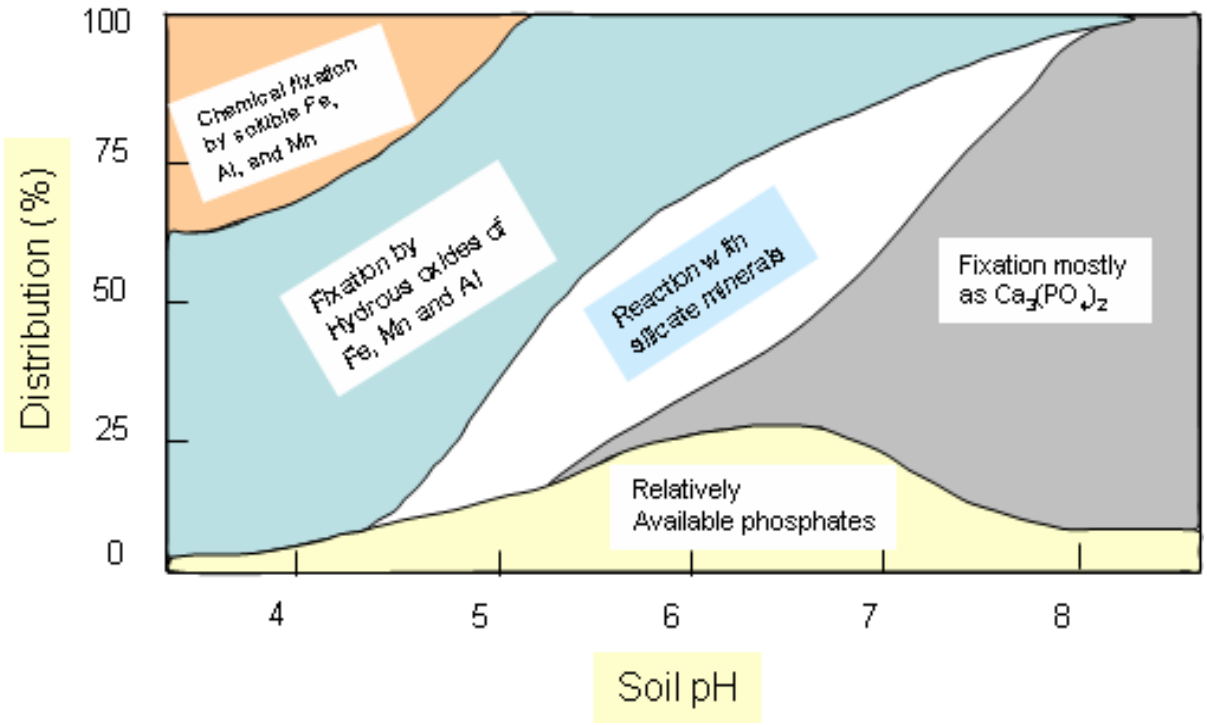

Figure 1.9 Phosphate availability and fixation as related to soil pH (from Brady and Weil, 2002).

Inorganic fixation of added phosphate at various soil pH values. Average conditions are postulated and it should not be presumed that any particular soil would have exactly this distribution. The actual proportion of the phosphorus remaining in an available form will depend upon contact with the soil, time for reaction, and other factors. Some of the added phosphorus may be changed to an organic form in which it would be temporarily unavailable (from Brady and Weil, 2002).

1.2.7.3 Ionic effects

Both cations and anions influence soil P adsorption. A brief description of the mechanism of cationic and anionic soil P fixation is given below.

Cationic effects: Adsorption of P by soils is influenced by the species and concentration of cations in the system. Divalent cations enhance P adsorption relative to monovalent cations. For example, clays saturated with Ca^{2+} can retain greater amounts of P than those saturated with sodium or other monovalent ions. Current explanations for this effect of Ca^{2+} involve making positively charged edge sites of crystalline clay minerals more accessible to the P anions (Tisdale *et al.*, 1985). This action of Ca^{2+} is possible at pH values slightly less than 6.5, but in soil more basic than this, dicalcium phosphate and other more basic calcium or magnesium phosphates would probably be directly precipitated from solution.

Anionic effects: Both inorganic and organic anions can compete in varying degrees with P for adsorption sites, resulting in some cases in a decrease in the adsorption of added P or desorption of retained P. Weakly held inorganic anions such as nitrate and chloride are of little consequence, whereas specifically adsorbed anions and acids such as hydroxyl, sulfate and molybdate, and silicic acid can be competitive. The strength of bonding of the anion with the adsorption surface determines the competitive ability of that anion. Phosphate is capable of forming a stronger bond at the surface than is sulfate (Tisdale *et al.*, 1985).

Other anionic effects: Organic anions from various sources, such as organic waste material and waste water treatment, can affect the P adsorption-desorption reactions in soil. The impact of organic anions on the reduction of P adsorption is related to their molecular structure and the pH of the system. It has been found that organic anions which form stable complexes with the iron and aluminium of soil components are particularly effective in reducing P adsorption. For example, citrate can form such complexes indicating that some organic anions are indirectly beneficial for P availability.

1.2.7.4 Saturation of the adsorption complex

The amounts of P adsorbed by soil are dependent on the extent of saturation of the adsorption complex or the number of available sites for reaction with the added P. Similarly, desorption of P is strongly influenced by the extent of saturation of the adsorption complex. Ease of desorption is greater at higher saturations because P is held less tightly with increasing surface coverage. These correspond to higher action steady states, requiring less energy to cause dissociation (Kennedy, 2001). There is a pronounced relationship between the amount of fixation of added fertiliser P and the R_2O_3/P_2O_5 ratio of the soil, where R represents the iron and aluminium content of soil. The ratio R_2O_3/P_2O_5 is a measure of the amount of P present in relation to the iron and aluminium oxide content of the soil. A high ratio indicates a small amount of P present or a low P saturation value. Under such conditions larger amounts of added P can be fixed than when the ratio is small.

Organic matter: It has been demonstrated that organic P compounds are mobile in soils to a greater depth than inorganic P. A range of organic P compounds moved four to six times deeper into a clay loam than did inorganic P (Tisdale *et al.*, 1985). It is well established that certain organic anions arising from the decomposition of organic matter can form stable complexes with iron and aluminium by chelating them, thus preventing their reaction with P. The anions that are most effective in replacing phosphate are citrate, oxalate, tartrate, malate and malonate, some of which may be produced as degradation products during organic matter decay. It was also found that allophonic surfaces adsorb P resulting in the formation of organic matter-P associations, and also the humus-P complexes make up the major portion of total-P in allophonic soils (Borie and Zunino, 1983).

Temperature: Temperature affects most physical processes and the rate of chemical reactions generally increases with a rise in temperature, doubling for each 10 degree Celsius increase. High temperature slightly increases the molar solubility of compounds such as apatite, hydroxyapatite, octacalcium phosphate ($Ca_8H_2(PO_4)_6.5H_2O$), variscite and strengite. Mineralisation of P from soil organic matter or crop residues is dependent on soil biological activity and increases in

temperature generally stimulate biological activity up to the optima for the predominant biological systems.

The dissolution of granules of water-soluble P and the resultant reactions with soil components to produce less soluble reaction products are hastened by higher temperatures raising their action and entropy (Kennedy, 2001). Most studies are in agreement that P retention increases at higher temperatures. The soils in warm regions of the world are generally much greater fixers of P than the soils of more temperate regions. These warmer climates also give rise to soils with higher content of the hydrous oxides of iron and aluminium.

Time of reaction: Phosphorus adsorption by soils and many soil components follows two distinct patterns: an initial rapid reaction followed by a slower reaction. The compounds precipitated during the reaction of P fertiliser salts in soils are metastable and usually change with time into more stable and less soluble compounds.

1.3 Adsorption-desorption behaviour of phosphorus in soil

1.3.1 Adsorption behaviour of phosphorus in different types of soils and equilibrium solutions

The buffering capacity of P has been defined as the relationship between change in adsorbed P (ΔP) and the activity of $H_2PO_4^-$ (Beckett and White, 1964). This relationship is similar to an adsorption isotherm. It differs in that the scale of the vertical axis is related to the initial P status rather than to zero. This relationship is usually found to be curved. The effects of soil P-buffering capacity on P desorption have been examined by different methods.

The effect of the soil P-buffering capacity on the uptake of P by soft brome (*Bromus mollis*) grown in 42 different surface soils has been examined (Barrow, 1967). The P remaining in the soil was extracted by sodium bicarbonate. The experiment was conducted on soft brome grass in a pot trial where the grass was grown for 183 days in a glasshouse in 1.6 kg of 42 different surface soils. The tops were clipped on five occasions. A strong relationship was found in P uptake between extractable P and the

P-buffering capacity. For a given value of extractable P, uptake was greatest on soils of lowest P-buffering capacity. It was concluded that the bicarbonate extraction procedure may overestimate the availability of P in soils of high P-buffering capacity. This probably occurs because at equal values of bicarbonate-soluble P, a soil of low P-buffering capacity has a higher solution concentration of phosphate. As 0.01 M $CaCl_2$ is a weak extractant (Brookes *et al.*, 1984), it could give a useful variation among the treatments in soil with high P-buffering capacity. In a study of this kind the use of 0.01 M $CaCl_2$ solution as an extractant could reveal more useful information.

The affinity for P-adsorption decreases as the amount of adsorption increases (Barrow, 1978). This effect is inherent in the process of P adsorption and occurs because specific adsorption of anions increases the negative charge on the adsorbing surface.

It is also reported that the major soil P characteristic controlled by soil composition is the P-buffering capacity (Holford, 1977). Thirty-nine soils from northern NSW were used to examine the effects of P-buffering capacity on (a) the extraction of labile P by four soil tests, (b) the relationships between the four soil tests, and (c) the critical level of each soil test required for near-maximum yield of wheat under field conditions (Holford, 1980). It was reported that the larger the negative effect of buffer capacity on extraction of labile P by a soil test, the higher is the correlation between the soil test and plant yield response to P. Increasing buffer capacity raises the ability of soils to retain P from whatever sources. Phosphate deficiency tended to be greater on strongly buffered than on weakly buffered soils. It was also found that the acidic ammonium fluoride extractant of Bray and Kurtz was the most sensitive to P-buffering in this respect (Holford, 1980), while the alkaline sodium bicarbonate extractant of Olsen *et al.* (1954) was less sensitive and the modified sodium bicarbonate test of Colwell least sensitive to P-buffering (Holford, 1980). It was reported that $NaHCO_3$-extractable P was much more closely correlated with total P uptake from a mixed group of soils than the quantity of labile P, but less correlated in the uniform group (Holford, 1988). Sodium bicarbonate is known to extract varying quantities of non-labile P, by hydrolysis of iron and aluminium phosphates, especially from acidic and neutral soils (Holford, 1988).

The effect of temperature and flooding duration on phosphate adsorption in an acid sulphate soil from Vietnam has been studied (Quang and Dufey, 1995). Acid sulphate soils were flooded for up to 56 days at 20^{O}C and 30^{O}C. Some samples were air dried at 30^{O}C for 30 days. A low redox potential (Eh< 0) and pH greater than 6 were rapidly reached in soil flooded at 30^{O}C, whereas less drastic reducing condition (Eh = 0.2 V) and pH 4-5 occurred at 20^{O}C. Phosphate adsorption increased during flooding and the increase was two-fold at 20^{O}C and 10-fold at 30^{O}C. Despite the phosphate adsorption index decreasing in the soil that was air dried after flooding at 30^{O}C, it was still two to three times greater than before flooding. These results were compared to the changes in oxalate-extractable iron, i.e. poorly crystalline or amorphous Fe-oxihydroxides. The increase of P adsorption per unit increase of oxalate-Fe was seven to eight-fold larger at 30^{O}C that at 20^{O}C.

An experiment was conducted using surface soil (0-20 cm depth) from experimental plots receiving different amounts of P over six years of a trial (Abedin and Saleque, 1998). After collection soil samples were shaken with 0.01 M $CaCl_2$ solution containing P from 0 to 75 µg/mL for 24 h at a soil to solution ratio of 1:10. The P isotherm data for all the soils at equilibrium P concentration were found to fit well to Langmuir, Freundlich and Temkin equations (R^2 = 0.979). The relationship between applied P and solution P for all the soils could be described by the equation $1/C = a + b\ln P_a$ which explained about 99 per cent of the variation. Here, C is solution P, P_a is added P, a and b are intercept and slope, respectively. When P was applied to soils that had received P for several years for each crop, and soils that had not received any applied P for several years, the soil that had received P seemed to adsorb less added P than the soils that had not received applied P. Soil that had received P for several years in each crop showed consistently lower P-buffering capacity, lower maximum adsorption capacity and higher energy of adsorption than soil that received no applied P. However, the differences were not statistically significant (Abedin and Saleque, 1998).

In an experiment on P retention and release of anions and organic carbon by two Andisols, it was found that Andisols can adsorb large amounts of P rapidly, and then release it slowly, yet the retention and release affecting plant growth are poorly

understood (Beck *et al.*, 1999). Ligand exchange of organic compounds from Al-humic complexes by P and/or Si release due to the breakdown of allophonic microstructures to provide adsorption sites might account for the retention of P. Phosphate ($H_2PO_4^-$, HPO_4^{2-}) adsorption and concurrent anion desorption were obtained by passing a 1g P L^{-1} (32 mM KH_2PO_4 in 1 mM $CaCl_2$) solution through soil columns. The general pattern and changes in pH of the eluent coincided with changes in the patterns of release of organic C and Si and the rate of P retention. Release of silica was correlated with less than 6 per cent of the P adsorbed and had only a minor role in P retention. Release of organic C by the two Andisols, was correlated on a molar basis for 40 per cent and 83 per cent, respectively, of the P adsorbed. Direct measurements of the pH of the eluent and release of anions and organic C concurrent with P retention can contribute to rapid assessment of the controlling mechanisms of P retention which indirectly confirm the hypothesis of ligand exchange of solution P with organic complexes held on allophonic surfaces. No relationship was found between organic C release and the fast or the slow P retention phase. An abrupt change in the pH of the eluent was correlated with a change from fast to slow retention of P.

The P adsorption capacity of acidic soils in Ireland has been estimated (Maguire *et al.*, 2001). The relationship between P adsorption capacity and oxalate-extracted aluminium and iron, and P was determined for 37 soil samples from Northern Ireland with relatively large clay and organic matter contents. Adsorption of P, measured over 252 days, was strongly correlated with the amounts of oxalate-extracted aluminium and oxalate-extracted iron, but there was also a negative correlation with oxalate-extracted P. When P adsorption capacity was calculated as the sum of the measured adsorption after 252 days and oxalate-extracted P, the multiple regression of P adsorption capacity on oxalate-extracted aluminium and oxalate-extracted iron gave the equation P adsorption capacity = 36.6 + 0.61 aluminium (oxalate-extracted) + 0.31 iron (oxalate -extracted) with a coefficient of determination (R^2) of 0.92. A significantly larger regression coefficient of P adsorption on oxalate-extracted aluminium than on oxalate-extracted iron was found. Values of P adsorption measured over 252 days were on average 2.75 (range 2.0-3.8) times greater than an

overnight index of P adsorption including a rapid initial stage followed by a slow process of extra adsorption.

An experiment was conducted for measuring adsorption strength of dissolved P from undisturbed soil cores (Akhtar *et al.*, 2003). Soil from the upper two horizons of each series (Arkport, Hudson, Honeoye, Genesee, Lackawanna) was passed through a 2 mm sieve, and 10 g samples were equilibrated in triplicate with 10 mL aqueous solutions containing 0-1800 mg P L^{-1}. For matching with field condition, mechanical shaking was avoided and the suspension was gently hand-mixed and left for 24 h to attain adsorption equilibrium at room temperature (Chen *et al.*, 1996). After filtering through 0.45-µm porosity filters, the filtrate was analysed. The amount of P added but not recovered in solution was assumed to be adsorbed. The fitted Langmuir isotherm adsorption equations for surface and subsurface soil horizons fitted the data well with an $R^2 \geq 0.90$ except Arkport where $R^2 \approx 0.80$. The Genesee and Lackawanna soils had the greatest affinity for adsorbing P, with Lackawanna showing slightly greater affinities at low concentrations of P.

The effect of dithionite and oxalate-extractable iron and aluminium on P adsorption in a Savanna Alfisol was considered by examining pedons from cultivated and uncultivated sites (Agbenin, 2003). A soil pedon is described as the smallest three-dimensional unit at the surface of the earth that is considered as soil (http://www.pedosphere.com, 2006). Twelve pedons from four cultivated fields and three pedons from an uncultivated natural site were examined for profile distribution of dithionite and oxalate-extractable iron and aluminium. Sixty samples, consisting of surface and subsurface samples, were chosen from the pedons to determine P adsorption. Generally dithionite-extractable iron and aluminium and oxalate-extractable aluminium increased with soil depth, while oxalate-extractable iron decreased from the surface up to 20 cm depth, and thereafter remained constant with depth. Phosphate adsorbed ranged from 103 mg kg^{-1} in the surface soils to 460 mg kg^{-1} in the subsurface soils representing from six to 29 per cent of applied P. Phosphate adsorption was linearly related to dithionite-extractable iron ($R^2 = 0.71$), dithionite-extractable aluminium ($R^2 = 0.69$) and oxalate-extractable aluminium ($R^2 = 0.52$), and was unrelated to oxalate-extractable iron. The coefficients of the multiple regression model indicated that a unit change in dithionite-extractable aluminium

concentration changed the P adsorbed by 74 mg kg^{-1} as compared with 21 mg kg^{-1} by dithionite-extractable iron. Dithionite-extractable aluminium is Al^{3+} substituted isomorphically for Fe^{3+} in crystalline iron oxides in soils; the degree of this substitution appeared to have a profound effect on P adsorption and fertility of Savanna Alfisols.

The magnitude and controls of P leaching and the risk of colloid-facilitated transport of P from sandy soils were assessed (Siemens *et al.*, 2004). The concentration of soluble reactive P in drainage water and groundwater was monitored from 0.9 to 35 m depth. Total P concentrations, P saturation, and P adsorption isotherms of soil samples were determined. The concentration of dispersible soil P and colloidal P in drainage water and ground-water were investigated. The concentration of soluble reactive P in drainage water and ground-water were close to background concentrations (>20 µg P L^{-1}). Experimentally determined equilibrium concentrations and the degree of P saturation of soil were good predictors of mobile P concentration in drainage water. The concentration of soluble reactive P in drainage water is controlled by rapid adsorption in the sandy soils. Colloidal P was transported in P-rich subsoil when there was a large flow of water and after nitrate had been flushed from the soil profile and total solute concentrations were small.

1.3.2 Phosphorus desorption by bacteria and phosphorus content in bacterial cells

Many bacteria are known to accumulate excess inorganic P in the form of polyphosphate (Harold, 1966; Kukaev and Vagabov, 1983). Numerous investigations have been carried out to enhance the ability of bacteria to accumulate polyphosphate, since this is essential in biological P removal from wastewater (Deinema *et al*., 1980). With genetic manipulation, the P content of *E. coli* cells reached a maximum of 16 per cent on a dry weight basis (with 49 per cent of the total as inorganic P), approximately 10-fold more than that of the control strain. Over 60 per cent of cellular P was stored in the form of polyphosphate in the engineered *E. coli* strain. More importantly, *E. coli* recombinants were capable of removing inorganic P regardless of whether they were starved for inorganic P. It was found that *E. coli* recombinants released polyphosphate into the medium once they accumulated high

levels of polyphosphate (Hardoyo *et al.*, 1994). Engineered *E. coli* strains did not release inorganic P and continued to take up inorganic P even after releasing polyphosphate.

The bacterial P content in soil varies widely according to estimates based on vegetation, land characteristics and laboratory analysis. It was predicted from microbial biomass estimates and from published values of microbial P contents that the soil biomass of cool native grassland could contain more P than the vegetation (Halm *et al.*, 1972). Estimates determined by simulation studies suggested that soil biomass P uptake was three to five times as great as plant P uptake in semi-arid grassland. Estimates of soil P based on laboratory analysis may be unreliable because concentrations of P in laboratory-grown microorganisms can vary widely, depending on the conditions under which they are grown (Van Veen and Paul, 1979).

It has been reported that the P content in soil biomass, such as fungi and bacteria differs according to the stage of their growth. The P content was measured in 14 representative species of soil fungi, grown on several concentrations of glucose and 10 species of bacteria all grown on 10 g glucose L^{-1} (Anderson, 1980). It was found that in fungi, the mean P concentration ranged from 4.8% at 1.0 g glucose L^{-1} to 3.1 % P at 10 g glucose L^{-1}. Bacteria (grown at 10 g glucose L^{-1}) contained a mean of 2.4% P (dry wt basis). The P concentration reported in the soil biomass was 3.3% (1.4-4.7%) (Perrott and Sarathchandra, 1982; Brookes *et al.*,1984).

The amount of P held in soil microorganisms (biomass P) is calculated from the difference between the amount of inorganic P extracted by 0.5 M $NaHCO_3$ (pH 8.5) from fresh soil fumigated with $CHCl_3$ (to lyse bacteria) and the amount extracted from unfumigated soil (Brookes *et al.*, 1982). This method underestimates microbial P because of the incomplete release of P from the microbial cells during fumigation, microorganisms resistant to fumigation, and subsequent inorganic P adsorption on to the mineral soil surface (Brookes *et al.*, 1982; Hedley and Stewart, 1982; McLaughlin *et al.*, 1986).

To take account of the fact that the microbial P is not totally extractable a correction factor is needed to estimate total P held in the microbial biomass. This factor varies with soil conditions, due to either changes in microbial communities or P adsorption (Brookes *et al.*, 1982; Hedley and Stewart, 1982; McLaughlin *et al.*, 1986). Using three different soils it was determined that inorganic P correction factors ranged from 0.32 to 0.38 and total P correction factors ranged from 0.44 to 0.49 (Brookes *et al.*, 1982). In other studies similar total P correction factors were reported as 0.32 to 0.47 (Hedley and Stewart, 1982) and 0.33 to 0.57 (McLaughlin *et al.*, 1986). It was suggested that the factor must be calculated for each soil because of different microbial populations present in different soils (McLaughlin *et al.*, 1986). It can be concluded that an inorganic correction factor of 0.40 provides a good estimate for soil microbial P (Brookes *et al.*, 1982; Hedley and Stewart, 1982).

The flushes (increases following $CHCl_3$-fumigation) of total P and inorganic P determined by the Olsen extraction method provided little useful information for estimating the amount of microbial biomass P in the soils (Wu *et al.*, 2000). Using the Bray-1 extractant at a soil:solution ratio of 1:4, and analysing inorganic P instead of total P, improved the reproducibility (statistical significance and coefficient of variance (CV)) of the P flush in the soils. The recovery of cultured bacterial and fungal biomass P decreased from 86 per cent at pH 4.6 to 13 per cent at pH 3.6. This shows that correcting for the incomplete recovery of biomass P using added inorganic P requires consideration of the pH of the soils and that although microbial biomass P in soil is generally estimated using the inorganic P flush and a conversion factor of 0.4, for reliable estimates, correction factor values should be determined independently for each soil.

The correction factor was reported to be dependent on the soil water potential, the soil horizon and the extractant used (Bliss *et al.*, 2004). The range in correction factor at different water potentials using 3 mM oxalate was found to be 0.31 to 0.67 in the A horizon, 0.48 to 0.91 in the E horizon, and 0.22 to 0.45 in the Bh horizon. The highest correction factors tended to be at water potentials near saturation and under the driest condition. Estimating a literature value of correction factor using $NaHCO_3$ as an extractant, instead of measuring correction factor directly, caused an overestimate of 7 to 63 per cent in the A horizon, 63 to 160 per cent in the E horizon and 7 to 32 per

cent in the Bh horizon. It was suggested that the best estimate of microbial P required that any correction factors be evaluated for specific soil conditions.

1.3.3 Materials used for microbial lysis and phosphorus extraction

Several materials have been used for lysing microorganisms and different types of extractant have been employed to determined P held in the biomass. Chloroform ($CHCl_3$) is widely used in lysing soil biomass by dissolving lipid membranes (Coles *et al.*, 1978; Brookes *et al.*, 1982; Hedley and Stewart, 1982). Ethylene oxide and isopropanol can also be useful (Hedley and Stewart, 1982). Since $CHCl_3$ readily evaporates from the treated soil, it is considered as the most suitable microbial lysing agent. Evaporation of the $CHCl_3$ also prevents extraction of soil phospholipids, which could produce an overestimate of microbial P (Hedley and Stewart, 1982).

Much less P was extracted from fumigated or non-fumigated soil by KCl or $CaCl_2$ solution than by 0.5 M $NaHCO_3$ or by modified Morgan's reagent (0.5 M CH_3COOH + 0.75 M CH_3COONa, pH 4.8) (Tinsley and Pizer, 1946). In a calcareous arable soil, much more P was extracted from unfumigated soil by Morgan's reagent than by $NaHCO_3$ because the acidic Morgan's reagent possibly dissolved $CaCO_3$ thus releasing any adsorbed P, while $CaCO_3$ was not attacked by the alkaline $NaHCO_3$ (Hedley and Stewart, 1982).

1.3.4 Effect of equilibration time on phosphorus recovery

Although the amount of extracted P from a soil depends on the extracting reagent, the shaking time also has influences P extraction from soil-microbe systems.

The P that is readily extractable from different microorganisms is variable. For example, approximately 50 per cent of bacterial P was nonextractable with $CHCl_3$-$NaHCO_3$ extractant whereas an average of only 22 per cent of fungal P was nonextractable (Hedley and Stewart, 1982).

Time influences the degree of P extraction. A 16 h 0.5 M $NaHCO_3$ extraction removed approximately 20 per cent more labile soil P than a 30 min $NaHCO_3$ extraction (Bowman *et al.*, 1978; Hedley and Stewart, 1982). A 16 h 0.5 M $NaHCO_3$ extraction recovered 20 per cent more microbial P from $CHCl_3$-treated samples than a 30 min 0.5m $NaHCO_3$ extraction. A 16 h 0.5 M $NaHCO_3$ extraction without $CHCl_3$ treatment increased microbial P extraction by 8-17 per cent.

Measuring total P rather than inorganic P in the $NaHCO_3$ extract and extracting the soil for 16 h rather than 30 min are important modifications for extraction (Hedley and Stewart, 1982). The measurable recovery of added microbial P from the soil was 25 to 34 per cent for fungal and 24 to 33 per cent for bacterial-P in a 30-min $NaHCO_3$ extraction and 35 to 39 per cent for fungal and 36 to 38 per cent for bacterial P in a 16 h extraction.

1.3.5 Bacterial influence on phosphorus desorption and method of phosphorus determination

Microbial influences on P desorption have been examined and it has been established that recovery of both bacterial-P and fungal-P was slightly reduced in higher P adsorbing soil (Hedley *et al.*, 1982). This suggests that, over a range of soils, recovery of microbial-P varies inversely with P adsorption capacity (Hedley and Stewart, 1982).

Using two different methods (Malachite green and ascorbic acid methods) for chloroform-lysed soil microbial biomass P analysis, it was found that the P concentrations measured using the Olsen method with malachite green and ascorbic acid were significantly correlated (Jeannotte *et al.*, 2004).

1.4 Soil-root rhizosphere system

For acquisition of mineral nutrients with low mobility in the soil, factors such as plant root growth and root surface area are important. Within a particular genotype, the root surface area may be modified in response to both external and internal factors. One of the factors regulating changes in shoot and root growth is the stronger competition by the roots for photosynthates when the supply of mineral nutrients is limited. In

considering microbial activity in the rhizosphere it is important to know about the plant root, the rhizosphere, and secretion from components of the rhizosphere.

Although solubilisation of P compounds by microbes is very common under laboratory conditions, results in the field have been highly variable (Gyaneshwar *et al.*, 2002). This variability is a potential constraint for the large scale use of phosphate mobilising microorganisms in agriculture. Many reasons have been suggested for this variability but none of them have been extensively investigated. For example genetic engineering has been proposed for the development of more effective phosphate solubilising microorganisms but little work has been done.

1.4.1 What is the rhizosphere?

The word rhizosphere is derived from the Greek 'rhiza', a root, and 'sphere' (Lynch, 1990). Although the word "rhizosphere" was introduced by Hiltner in 1904 (Lynch, 1990), it has been modified and redefined more specifically as endorhizosphere (the root itself where microorganisms colonise), ectorhizosphere (the area surrounding the roots) and rhizoplane (the root surface). The simplest well known definition of rhizosphere is the volume of soil influenced by the root (Vale *et al.*, 2005). The extent of the rhizosphere may vary with soil type, plant species, age and many other factors (Curl and Truelove, 1986), but it is usually assumed to extend from the root surface (rhizoplane) out into the soil for up to a few millimetres, or possibly a few centimetres in extreme cases in some desert and sand dune plants. Generally the root surface is not a well-defined entity and its nature may change during the life of the root. Furthermore, the cortex may be invaded by harmless bacteria and fungi and cells of the cortex and epidermis may die or be damaged while the root as a whole remains alive and healthy. The rhizosphere can therefore be considered as a microbial continuum stretching out into the soil from the root endodermis (Old and Nicholson, 1978). A schematic representation of major physiological factors associated with plant roots and soil microorganisms that influence the availability of soil P in the rhizosphere (Richardson, 2001) is presented in Figure 1.10. Microorganisms may directly affect P solubilisation and mineralisation, impact on root structure and

function, or immobilise readily available sources of P thereby rendering them unavailable to plants.

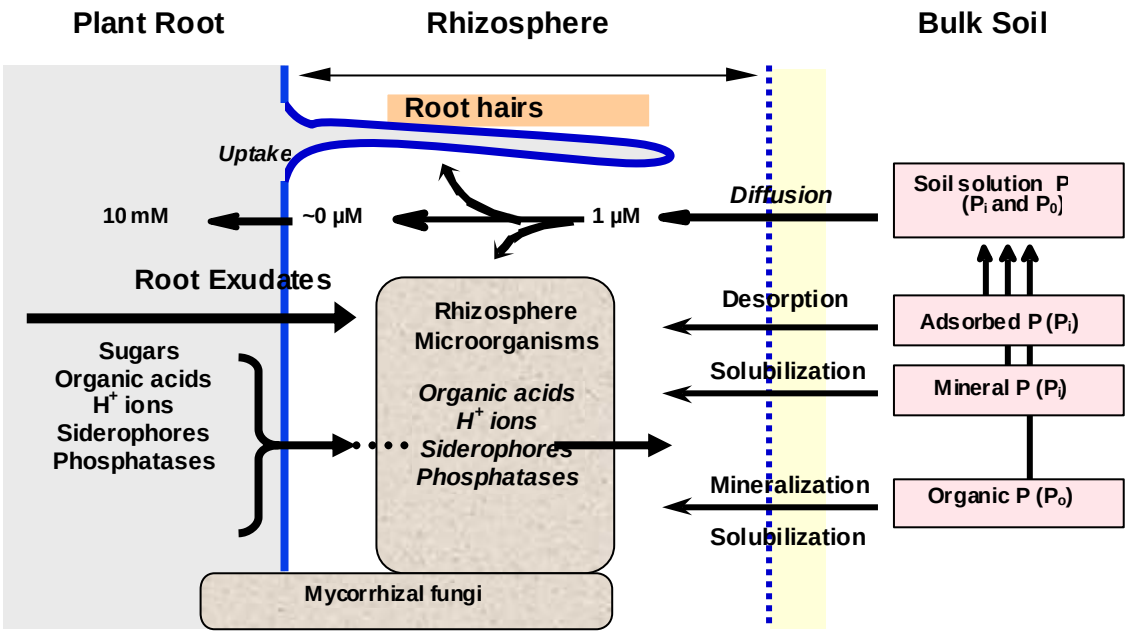

Figure 1.10 Major physiological factors associated with plant roots and soil microorganisms that influence the availability of soil P in the rhizosphere (after Richardson, 2001).

The diagram shows that phosphatases from rhizosphere microorganisms are required for the mineralisation of organic P and plant root exudates (sugars, H^+ ions, organic acids) are required for the solubilisation of inorganic P.

1.4.2 What is the rhizoplane?

The 'rhizoplane' can be defined as the root surface colonised by microorganisms (Lynch, 1990). The rhizoplane is shaded from sunlight by bulk soil and it is therefore maintained under relatively stable conditions with respect to humidity and temperature (Hashidoko, 2005). Hence, the rhizoplane is described as resembling an environment like a tropical forest where the elemental concentrations are constant but there is highly active circulation on a micro scale (Hashidoko, 2005).

1.4.3 Organic acid/ anions release under phosphorus deficiency

The insolubility of most P salts and the high adsorption capacity of many soils for limited P supply can be a major constraint to plant growth (Marschner, 1995). There

is now evidence that suggests that some plants can directly modify the rhizosphere in order to gain access to previously unavailable soil P reserves. This can include the manipulation of root hair length/density, the provision of additional C for mycorrhizal exploitation of non-rhizosphere soil, the release of phosphatases to release organically bound soil P, and the release of organic anions and H^+ to solubilise inorganic P. It has now been demonstrated that some dicotyledonous plant roots, and specially non-mycorrhizal plants such as *Lupinus albus* and *Brassica napus*, are capable of releasing large amounts of organic anions into the rhizosphere in response to P deficiency. Other dicotyledonous (e.g. *Sisymbrium officinale*) and graminaceous (wheat, maize) plants do not appear to do this (Laheurte and Berthelin, 1988; Hoffland *et al.*, 1992; Gerke, 1994; Johnson *et al.*, 1996*a*, *b*; Imas *et al.*, 1997). It is also well established that P deficiency significantly increases the leakiness of the root plasma membrane to solutes. This indicates that for some exudation studies the observed increases in organic anion release may be an indirect root response of minimal importance (Ratnayake *et al.*, 1978).

Malate and citrate appear to be the primary organic anion components released by roots under P deficiency. In *Brassica napus* the 4-fold increase in organic anions exudation is largely associated with the root apex, while smaller amounts are also released from mature root regions (Hoffland *et al.*, 1992). In a different response to low P levels, lupin and other Australian native species such as Banksia develop short-branched, tertiary lateral roots (proteoid or 'cluster' roots) (Dinkelaker *et al.*, 1995). These roots are directly responsible for the 13 to 40-fold increase in the citrate and malate excretion which constitutes greater than 90 per cent of the root exudate under P deficiency and which commences three days after proteoid root development (Dinkelaker *et al.*, 1989; Gardner *et al.*, 1983; Grierson, 1992; Johnson *et al.*, 1996 a, b). The organic anion exudation under P deficiency constitutes a drain of five to 25 per cent of the plant's photosynthetically fixed C. This does not; however, appear to significantly affect dry matter production (Gardner *et al.*, 1983; Johnson *et al.*, 1996*a*). In lupins, it appears that C is mainly supplied in the form of phloem-translocated sugars (70 per cent) whilst some is also supplied in the form of organic C produced in the root (30 per cent) (Johnson *et al.*, 1996*a*, *b*). The phloem-translocated sugars are subsequently converted to organic anions via the enzymes PEP

carboxylase, malate dehydrogenase and citrate synthase at the site of release (Hoffland *et al.*, 1992; Johnson *et al.*, 1994; 1996*a, b*). The transport mechanisms controlling organic anion release and the number and regulation of genes determining this P deficiency trait have still to be identified.

The benefits derived from having organic anions in the rhizosphere are twofold; they compete with phosphate groups for certain binding sites in the soil, and they form stronger complexes with Al^{3+}, Fe^{3+} and Ca^{2+} than phosphate does. Phosphorus can be liberated from Ca-P minerals as the organic anion complex with Ca (Jones and Darrah, 1994) or block the adsorption of P to other charged sites (Lunstrom *et al.*, 1995). Ligand exchange can also occur in which the P bound to iron or aluminium oxyhydroxides is replaced by the organic anion (Lan *et al.*, 1995).

1.4.4 Do organic acids cause acidification of the rhizosphere?

Changes in the mineral nutrition of plants may affect the rhizosphere in various ways (Marschner *et al.*, 1986). For example the source of nitrogen (NH_4-N versus NO_3-N), and iron and P deficiency may result in changes in rhizosphere pH. These pH changes can be readily demonstrated by infiltration of the soil with agar containing a pH indicator. The rhizosphere pH may be as much as 2 units higher or lower than the pH of the bulk soil. In response to iron deficiency most plant species in their apical root zones increase the rate of net H^+ excretion (acidification), the reducing capacity, the rate of Fe^{3+} reduction and iron uptake. It was also reported that in the proteoid roots of white lupin (*Lupinus albus* L.) sparingly soluble iron and aluminium phosphate are mobilised by the exudation of chelating substances (probably citrate), net excretion of H^+ and greater reducing capacity. In mixed culture with white lupin, P uptake per unit root length of wheat (*Triticum aestivum* L.) plants from a soil low in available P is increased, indicating that wheat can take up P mobilised in the proteoid root zones of lupin.

Organic acids can acidify the rhizosphere (Marschner, 1995) although H^+ release and organic acid anion release are two biochemically separate but spatially coordinated transport events (Jones, 1998). The cytosolic pH of roots ranges from 7.1-7.4

(Marschner, 1995) and at this pH, it can be predicted from chemical equilibria models that the primary organic acid species in the cytosol will be the fully dissociated anionic forms of malate^{2-} and citrate^{3-} (Figure 1.11).

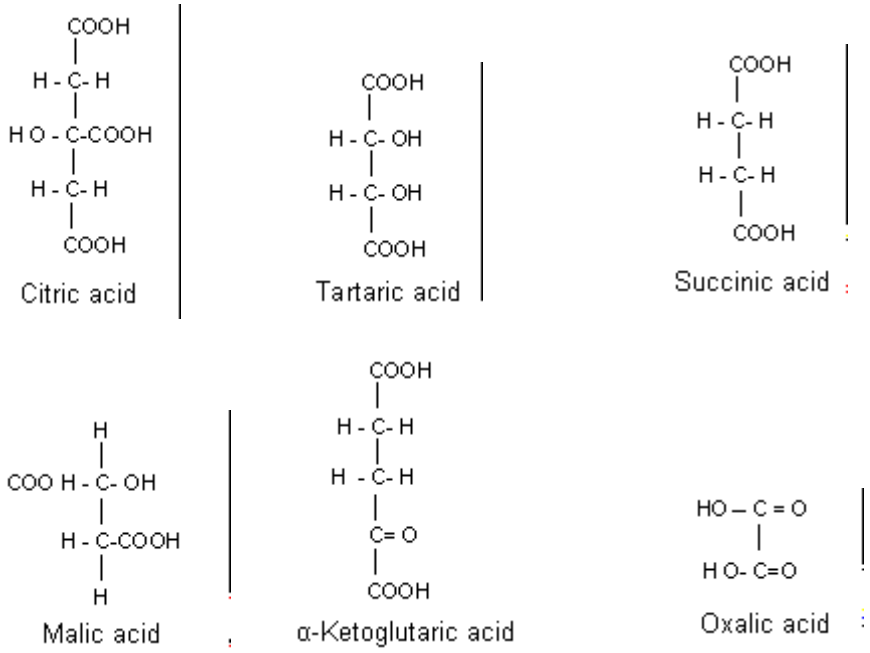

Figure 1.11 Structures of some undissociated organic acids

During passage of the organic acid anions across the membrane into the apoplast, a counter ion is required to maintain electrical neutrality. Under aluminium rhizotoxic conditions, no stimulation of H^+ excretion and no change in rhizosphere pH is observed when malate is being released, indicating that H^+ is not the counter ion transferred under these conditions (Kochian, 1995). This is possibly functional, as a lowering of the external pH would increase Al^{3+}solubility in the rhizosphere and increase aluminium rhizotoxicty. In the case of malate release from wheat roots, it appears that K^+ is the counter cation which accompanies malate across the membrane with a K:malate stochiometry of 2:1 (Ryan *et al.*, 1995). For other organic anion release situations the counter ion has yet to be identified. To confirm that the H^+ counter ion is involved in these processes, it will be necessary to separate direct H^+-organic anion coupled transport events from uncoupled transport events.

1.4.5 Aluminium toxicity and its remedy in the rhizosphere

Organic anions play an important role in both internal and external detoxification of aluminium (Ma, 2004). Many aluminium-resistant species and cultivars respond to aluminium stress by secreting specific organic acid anions (citrate, oxalate, malate) from their roots (Ma and Hiradate, 2000; Ma *et al.*, 2001) and a good correlation between organic anion secretion and aluminium resistance has been established in some species such as wheat and barley (Ryan *et al.*, 1995; Zhao *et al.*, 2003). The effect of aluminium on organic anion metabolism has been examined but there has been no consensus regarding the effect of aluminium on internal organic anion contents or citrate synthesis activity. A detailed study was conducted with two *Triticale* lines differing in Al resistance by correlating root elongation and organic anion secretion with the internal content of organic anions and enzyme activity (Hayes and Ma, 2003). It was found that the metabolism of organic anions is poorly correlated with the aluminium induced secretion of organic anions, indicating that the Al-dependent efflux of organic anions from the root of Triticale is not closely regulated by their internal levels in the roots or by the capacity of the root cells to synthesize malate and citrate.

The process leading to the accumulation of aluminium in buckwheat has been investigated. The roots took up aluminium passively in the form of ionic aluminium (Al^{3+}) due to a large inwardly directed electrochemical gradient for this ion (Ma and Hiradate, 2000). Following uptake, aluminium was immediately chelated with the internal oxalate in the root cells, forming a stable, non-phytotoxic complex of Al-oxalate at a 1:3 ratio (Ma *et al.*, 1997). When aluminium was translocated from the roots to the leaves, Al-oxalate (1:3) was converted to Al-citrate (1:1) in the xylem. When Al-citrate moved from the xylem to the leaf cells, it was re-converted to Al-oxalate (1:3) (Ma and Hiradate, 2000). This Al-oxalate was then sequestered in the vacuoles in the leaves (Shen *et al.*, 2002). The sequestration of this complex in the vacuoles plays an important role in internal detoxification of aluminium. The form of aluminium in the buckwheat leaves varied with leaf position, but mainly depending on aluminium concentrations (Zheng *et al.*, 2003).

1.5 Microbial effects on phosphorus mobilisation

From the early 20th century studies have been carried out on many aspects of microbially mediated increases in plant-available P including mycorrhizal fungi, P-mineralising or solubilising microorganisms and dissolution of P by the oxidation products of sulphur (Kucey *et al.*, 1989). Gerretsen (1948) first showed that a pure culture of soil bacteria could increase the P nutrition of plants through increased solubility of Ca-phosphates. In the 1950s soils of the USSR and several eastern European countries were inoculated with bacteria which were claimed to increase the plant availability of native and applied soil P. Several species of microorganisms were reported to be effective in this respect. The term 'phosphobacterin' was used in the former USSR for a phosphate-dissolving bacterial culture (Tisdale *et al.*, 1985) and *Bacillus megaterium* var. *phosphobacterin* was one strain of that biofertiliser (Cooper, 1959; Tisdale *et al.*, 1985). The yield increase from the use of phosphobacterin was about 10 per cent for sugar beets, potatoes, cereals, vegetables, some grasses and legumes.

The use of phosphate-solubilising bacteria as inoculants simultaneously increased P uptake by the plant and crop yield (Rodríguez and Fraga, 1999). Some powerful phosphate solubilisers such as *Pseudomonas, Bacillus* and *Rhizobium* can dissolve appreciable amounts of unavailable soil P to supply a form available to the plant. The ability of phosphate-solubilising rhizobacteria to enhance P uptake and growth in both laboratory and field trials has been demonstrated using strains of *Pseudomonas putida* and *Pseudomonas fluorescens* that increased root and shoot elongation in canola, lettuce and tomato (Hall *et al.*, 1996; Glick *et al.*, 1997). Increases in crop yield in potato, radishes, rice, sugar beet, tomato, lettuce, apple, citrus, beans, ornamental plants and wheat have been reported (Suslow, 1982; Kloepper *et al.*, 1988; Lemanceau, 1992; Kloepper, 1994). It was reported that wheat yield increased up to 30 per cent with *Azotobacter* inoculation and up to 43 per cent with *Bacillus* inoculants (Kloepper *et al.*, 1989). A 10 to 20 per cent yield increase in wheat in a field trial using a combination of *Bacillus megaterium* and *Azotobacter chroococcum* has been demonstrated *(*Brown, 1974). More recently, in Vietnam, a more than 10 per cent yield increase in rice production was obtained in a field trial in 65 different

farmers' fields treated with an application of biofertiliser in comparison with a control plot, where there were N-fixing and P-mobilising bacteria and a companion bacterial strain was used to act as a protection of desirable bacteria (Nguyen *et al.*, 2002; Nguyen *et al.*, 2003). It was not possible in any of these studies to attribute the yield increases to P-mobilisation although increased P uptake was shown. The ability of many bacteria to act as plant growth promoting rhizobacteria (PGPR), may be a factor in these experiments.

It is well known that the mycorrhizal fungi are an important part of the soil microbial system. Endomycorrhizae, which include vesicular-arbuscular (VA) mycorrhizae, and ectomycorrhizae are significant for their potential economic importance (Gerdemann, 1968). The relative benefit from mycorrhizal infection is suggested to decrease as P availability increases (Ross, 1971), partially due to the negative effect of P on the levels of mycorrhizal infection (Hayman *et al.*, 1975). Effectively the fungi provide significantly increased root surface areas for P uptake at the soil interface with the plant. The influence of mycorrhizae on plant P uptake is considered as obvious and well established (Gianinazzi-Pearson and Gianinazzi, 1985). The prevalence of mycorrhizal fungi associations with plants is very common under natural soil conditions (Smith and Read, 1997). Generally, mycorrhizal plants contain higher concentrations of P than similar but non-mycorrhizal plants which is due to inorganic phosphate uptake by mycorrhizal plants (Marschner and Dell, 1994; Smith and Read, 1997).

It has been shown that *Aspergillus foetidus* significantly increased the availability of P in soil treated with rock phosphate and triple super phosphate (Salih *et al.*, 1989). Some naturally occurring soil fungi such as *Penicillium bilaii* can increase phosphate uptake of crops by solubilising P (Kucey, 1988; Salih *et al.*, 1989). This fungus can be co-cultured with *Rhizobium meliloti* in a common delivery system for both organisms (Nikolaev *et al.*, 1994) to fix nitrogen and to mobilise unavailable soil P.

1.5.1 Phosphate mobilising ability of bacteria

Bacterial phosphate mobilisation has been studied in *in vitro* and *in vivo* experiments. In the laboratory P mobilisation by bacteria is very common, but in the field variable results have been obtained.

1.5.1.1 Phosphorus solubilising ability of bacteria under laboratory conditions

The first detection of phosphate dissolving microorganisms in soils used an agar plate method (Sackett *et al.*, 1908). It was demonstrated that soil microorganisms could dissolve tricalcium phosphate, dicalcium phosphate and calcium carbonate, by their ability to make clear zones around colonies in media containing these insoluble substances. Specific organisms were not identified in the early studies.

More than 50 per cent of the bacteria isolated from oat plants produced haloes around their colonies on dilution plates. This pleomorphic group had the highest proportion of isolates which could produce clear zones on agar media containing calcium carbonate, dicalcium phosphate, tricalcium phosphate and freshly precipated hydroxyapatite or basic slag (Louw and Webley, 1959). The results indicated that, of 133 isolates, the proportions that could dissolve various calcium salts were 100%, 91.7%, 39.15%, 63.2% and 64.7%, respectively for calcium carbonate, dicalcium phosphate, tricalcium phosphate, hydroxy-apatite and basic slag. None of these isolates showed dissolving ability on agar media containing gafsa rock phosphate, variscite, strengite or tranakite. Proprietary gafsa rock phosphate is not considered insoluble; it is soluble in soils of pH 6.5. The natural acidity of soil releases P from gafsa rock to give a sustained release of available nutrient throughout the growing season (http://www.carrs-fertiliser.co.uk, 2006). It was found that 82 per cent of the isolates were able to release phosphate from gafsa rock phosphate but none of the isolates released phosphate from variscite, strengite, or tranakite. These results show that reliance on the agar plate method alone for determining P dissolving microorganisms may exclude some organisms that are potentially P mobilisers. The majority of these isolates produced mainly lactate, but a few also produced an acid with chromatographic properties similar to 2-keto-gluconate. Under certain conditions

2-keto-gluconate had seven times the chelating power of lactate towards calcium (Mehltretter *et al.*, 1953).

Phosphate–solubilising microorganisms and total bacterial and fungal populations in several soils were determined by serial dilution and plate counting techniques (Kucey, 1983). Available P constituted an average of one per cent of the total P present in the soils. Phosphate-solubilising bacteria and fungi made up 0.5 and 0.1 per cent respectively, of the total bacterial and fungal populations in any soil type. A highly significant correlation was found between the numbers of total and P-solubilising fungi and the levels of total P in the soil. As a group, the fungi were superior to bacteria in solubilising both freshly precipitated calcium phosphate and Idaho rock phosphate. Fungi also retained this ability over many subculturing transfers. A high percentage of the bacterial isolates lost their solubilising ability when sub-cultured. A significant correlation was found between an organism's ability to solubilise freshly precipitated calcium phosphate in agar plates and Idaho rock phosphate in solution culture. It was found that 112 isolates possessed P-solubilising ability on potato-dextrose-yeast extract-agar (PDYA) plates. Of these, approximately 60 per cent were fungal (68) and 40 per cent were bacterial (44). Most fungal isolates were identified as *Penicillium* and *Aspergillus* species through microscopic examination. As a group, the fungi solubilised an average of ten times more P than the bacteria. The fungal isolates expressed phosphate solubulising ability early in colony growth and maintained it over the entire growth period. Bacterial colonies were slower growing and their phosphate solubilising ability was less constantly expressed. Some isolates showed a clear zone on an agar plate early in growth, but later failed to keep the clear zone beyond their colony edge.

Bacteria isolated from proteoid roots and non-proteoid lateral roots of *Telopea speciosissima* seedlings, were able to solubilise calcium phosphate through acidifying the medium while grown in agar culture in the presence of ammonium salts and sucrose (Wenzel *et al.*, 1994). Bromocresol purple dye (0.2 mM) was used in the agar plate as an indicator of pH change; below pH 4.5 the medium became yellow. A yellow zone 2-3 cm in diameter developed within a few hours of inoculation. After 1-2 weeks the entire plate was yellow with pH less than 4.5 with a large clear region

around each bacterial growth zone. A surface pH electrode was used for measuring the pH of the surface of the agar. None of these isolates caused any detectable solubilisation of the $FePO_4$ or $AlPO_4$ under any of the conditions used. No phosphate solubilisation was detected on phosphate solubilising media containing glucose and NO_3^-. However, some bacteria produced a small zone of phosphate solubilisation during the first day after inoculation when pH decreased. This effect was transient and within 24 h the pH indicator became purple again, starting adjacent to the inoculum and spreading progressively outwards as the entire plate gradually became more alkaline.

An experiment was carried out in a growth chamber to explore the ability of phosphate-solubilising rhizobacteria to enhance the growth and P uptake of canola (*Brassica napus* L., cv. Legend) (de Freitas *et al.,* 1997). The 111 rhizobacteria isolated from the rhizosphere of field grown plants were selected to represent distinct types based on differences in colony morphology including colony form, elevation, opacity and pigment production. The rhizobacteria were identified using whole-cell fatty acid methyl ester profiles. Nine bacteria were shown to be effective plant growth-promoting rhizobacteria (PGPR), capable of P-solubilision *in vitro*. The best P-solubilisers were two *Bacillus brevis* strains, *B. megaterium*, *B. polymyxa*, *B. sphaericus*, *B. thuringiensis*, and *Xanthomonas maltophilia* (PGPR strain R85). Bacteria screened from bulk soil and rhizosphere of soybean (*Glycine max* L.), that can increase early soybean growth in nonsterile soil (PGPR traits) have been examined (Cattelan *et al.*, 1999).

A comparative experiment to find the best medium for isolating P-solubilising bacteria has been conducted (Nautiyal, 1999) using two media (Table 1.1). It was found that the National Botanical Research Institute's phosphate growth medium (NBRIP) consistently demonstrated about 3-fold higher efficiency compared to the Pikovskaya medium (Pikovskaya, 1948) for P solubilisation ability. The results indicated that the identification of P-solubilising bacteria based solely on the formation of visible haloes on agar plates is not a reliable technique, as many isolates that did not show any clear zone on agar plates solubilised inorganic phosphates in liquid media. Screening using the NBRIP phosphate growth medium broth assay for

the identification of the most efficient phosphate solubilisers is likely to identify more P-mobilising strains than the agar plate method.

Table 1.1 Nutrient composition of the Pikovskaya medium and the National Botanical Research Institute's phosphate growth medium (NBRIP).

Nutrients	Pikovskaya L^{-1}	NBRIP L^{-1}
Glucose	10 g	10
$Ca_3(PO_4)_2$	5	5
$(NH_4)_2SO_4$	0.5	0.1
$MgSO_4.7H_2O$	0.1	-
KCl	0.2	0.2
NaCl	0.2	-
Yeast Extract	0.5	-
$MnSO_4.H_2O$	0.002	-
$FePO_4.7H_2O$	0.002	-
$MgCl_2.6H_2O$	-	0.25

Four unidentified P-solubilising bacteria have been isolated from the rhizosphere of chickpea grown in alkaline soils (Nautiyal *et al.*, 2000). All four strains demonstrated diverse levels of P solubilisation activity under *in vitro* conditions in the presence of various carbon and nitrogen sources. One strain was capable of solubilising P in the presence of 10 per cent NaCl, at pH 12, and/or 45^{O}C. The strains showed varying levels of phosphate solubilisation when the effects of different sources of nitrogen were examined during growth. The P solubilisation by the four isolates was monitored up to five days in NBRIP medium at 30^{O}C in the presence of 0 per cent salt (NaCl) at pH 7. The level of P solubilised by all strains increased linearly, until the second, third, second and the third day respectively. Maximum solubilisation of phosphate was achieved after three days of incubation. All four strains produced acid and lowered the pH of the growth medium from neutral to 3 to 3.5 after one day. Thereafter, the pH of the medium remained stable for up to five days. The decrease in pH indicates the production of acids, which were considered to be responsible for P solubilisation.

An efficient protocol was developed for qualitative screening of phosphate solubilising bacteria, based upon visual observation (Mehta and Nautiyal, 2001). Results indicated that, by using a formulation containing bromophenol blue, it was possible to rapidly screen on a qualitative basis for the phosphate solubilising

bacteria. Qualitative analysis of the P solubilised by various groups correlated well with grouping based on quantitative analysis of bacteria isolated from soil; the effect of carbon, nitrogen, salt and P solubilisation-defective transposon mutants. This simple protocol reduced time significantly compared to quantitative analysis methods that involve time-consuming biochemical procedures.

Vermicompost was inoculated with nitrogen fixing *Azotobactor chroococcum* strains, *Azospirillum lipoferum* and the P solubilising *Pseudomonas striata* and the nitrogen and available P content of the vermicompost was determined (Kumar and Singh, 2001). It was found that inoculation by N-fixing bacteria increased the N and P content of the vermicompost, but inoculation of vermicompost enriched with rock phosphate by *P. striata* increased only the available P content.

Although solubilisation of P compounds by microorganisms is very common under laboratory conditions, results in the field are highly variable (Gyaneshwar *et al.*, 2002). The conditions employed to isolate P-solubilising microorganisms do not reflect soil conditions in the field and P-solubilising microorganisms capable of effectively releasing P from soil are not as highly abundant as was suggested in earlier studies. It was also found that the mineral P-solubilising ability of microorganisms could be linked to specific genes, and that these genes can be found even in non-P-solubilising bacteria.

Bacteria from the rhizosphere of three different varieties of rice, Siam Unus, Siam Ubi and Siam Puntal in the Balandean district, South Kalimantan, Indonesia have been isolated and identified (Purnomo *et al.*, 2004). Isolated bacteria were identified as *Burkholderia* sp. and were able to mobilise P from aluminium phosphate ($AlPO_4$) as well as tricalcium phosphate $Ca_3(PO_4)_2$. Using Al-P, all of the isolates worked almost equally well, whereas using Ca-P, there was a variation in their P-solubilising ability.

Several bacterial strains were isolated from the rhizosphere of various crops in Korea that could mobilise insoluble P from $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ *in vitro* using the Pikovskaya medium (Chung *et al.*, 2005). These bacteria were identified as

Enterobacter sp., *Pantoea* sp. and *Klebsiella* sp. all grouped under one family, Enterobacteriaceae. Using 5 g L^{-1} concentrations of different sources of P, *in vitro* using Pikovskaya medium, *Pantoea* sp., *Enterobacter cloacae* and *Klebsiella* sp. (based on 16S rDNA) could mobilise 96.2, 121 and 107 µg P mL^{-1} from $Ca_3(PO_4)_2$; 8.00, 3.7 and 7.2 µg P mL^{-1} from $AlPO_4$ and 18.8, 10.2 and 49.1 µg P mL^{-1} from $FePO_4$, respectively. In this study, the amount of insoluble P included in the medium was very high.

A salt- and pH-tolerant bacterium able to mobilise insoluble P, was isolated from soybean rhizosphere in Korean soil (Son *et al.*, 2006). The bacterium was identified as *Pantoea agglomerans* and could solubilise P from different sources of unavailable P in an optimal medium. The optimal medium used was 3 per cent (w/v) glucose, 0.1 per cent (w/v) NH_4NO_3, 0.02 per cent (w/v) $MgSO_4.7H_2O$, and 0.06 per cent (w/v) $CaCl_2.2H_2O$. The initial pH was adjusted to 7.5 and the medium was incubated at 30°C. It was found that the solubilisation of insoluble P was associated with a drop in the pH of the culture medium. *Pantoea agglomerans* R-42 showed resistance to different environmental stresses such as 5-45°C temperature, one to five per cent salt concentration and 3-11 pH range. This strain solubilised the highest P from $CaHPO_4$ (1367 mg L^{-1}), hydroxyapatite (1357 mg L^{-1}) and $Ca_3(PO_4)_2$ (1312 mg L^{-1}). It also produced soluble P from $FePO_4$ (28 mg L^{-1}) and $AlPO_4$ (19 mg L_{-1}) in the medium.

1.5.1.2 Phosphorus mobilisation by bacteria in plant experiments

Bacterial isolates from the roots of wheat rhizoplane were more active in oxidising glucose and alanine than cultures isolated from rhizosphere and non-rhizosphere soils (Katznelson and Bose, 1958). Generally greater microbial activity was found with alanine than with glucose. More than one third of the cultures were capable of dissolving insoluble P in the form of $CaHPO_4$. The P-solubilising organisms from the rhizoplane were also the most active in oxidising glucose and alanine and those from the rhizosphere soil were intermediate in this respect. By far the majority of these phosphate dissolving bacteria were in the nutritional group requiring unidentified substances contained in yeast and soil extracts for optimal growth. The greater metabolic activity of bacteria from the rhizoplane and in the rhizosphere, may be

interpreted as indicating that microorganisms can cause greater phosphate turnover at the root-soil surface. It was suggested that although these bacteria were not preferentially stimulated in the root zone, their large numbers and their greater metabolic activity may contribute significantly to the phosphate economy of the plant.

The effects of isolated P-mobilising bacteria, *Bacillus megaterium, B. polymyxa, B. sphaericus, B. thuringiensis and Xanthomonas maltophila* on growth and P-uptake of canola plants in a P-deficient soil amended with rock phosphate has been reported (de Freitas *et al.*, 1997). Although there was significantly increased plant height or pod yields in the presence of in P-solubilising rhizobacteria there was no increase in P-uptake from rock phosphate. The most effective inoculant was a *B. thuringiensis* isolate that significantly increased the number and weight of pods and seed yield, even without rock phosphate. Inoculation with *Xanthomonas malitohilia* resulted in increased plant height and pods yield**.** These results indicate the potential use of these P-solubilising rhizobacteria as inoculants for canola, but also indicate that P-solubilisation was not the main mechanism responsible for positive growth response and that plant growth promotion by other mechanisms could be more important.

Microorganisms can increase the growth of plants by means of P solubilisation in barley and chickpea (Peix *et al.*, 2001). *Mesorhizobium mediterraneum* was used with chickpea and barley plants in a soil with and without the addition of phosphates in a growth chamber. In barley and chickpea grown in soil treated with insoluble phosphate (calcium phosphate) and inoculated with *Mesorhizobium mediterraneum* (strain PECA21), the P content of the plants was significantly increased by 100 and 125 per cent, respectively. Also, the dry matter, nitrogen, potassium, calcium and magnesium content in both plants were significantly increased in the inoculated soil with insoluble phosphate.

The effect of the application of P solubilising bacteria, *Bacillus megaterium* var. *phosphaticum*, at 10 kg ha^{-1} of lignite-based culture of sugar cane has been examined (Sundara *et al.*, 2002). The changes of soil-available P, and growth, juice quality and yield of sugarcane were determined with and without varying amount of P fertiliser. The application of P-solubilising bacteria increased their population in the rhizosphere

and the consequent plant available P status of the soil. It enhanced tillering, stalk population and stalk weight, which eventually led to a cane yield increase of 12.6 per cent over the control. Application of P-solubilising bacteria reduced the required P dosage by 25 per cent. Additionally, it was demonstrated that 50 per cent of the more costly superphosphate could be replaced by rock phosphate, when applied with P-solubilising bacteria. The influence of these P-solubilising bacteria was greatest when rock phosphate constituted a part of the P fertiliser applied.

A pot experiment was conducted to examine the compatibility of fly ash with P-solubilising bacteria and their combined effect on soybean productivity (Sunita and Guar, 2002). Fly ash was applied at 20, 40, 60 and 80 t ha^{-1} with N and P fertiliser singly as well as with an efficient phosphate solubiliser *Pseudomonas striata*. The applications of fly ash at 40 t ha^{-1} along with *P. striata* inoculation improved the bean yield and P uptake by grain and also increased available P in the soil. There was no detrimental effect on the population of inoculated bacteria.

Solubilisation of rock phosphate on addition of pyrite (FeS_2) to soil was studied coupled with inoculation with *Thiobacilli* (Kapoor, 2004). Addition of pyrite to an alkaline soil resulted in a decrease in pH. Inoculation with a mixed culture of *Thiobacillus thiooxidans* and *T. ferrooxidans* increased the rate of pyrite oxidation as depicted by decreases in pH and an increase in the sulphate content of the soil indicating oxidation of reduced sulphur. Addition of insoluble phosphates such as Mussoorie and Jhamarkotra rock phosphate in soil with pyrite and inoculation with a mixed culture inoculum led to a increase in P availability to mungbean as depicted by an increase in dry matter production and P uptake.

1.5.2 Factors affecting phosphate mobilisation by bacteria

As a consequence of the wide range of chemical forms of insoluble phosphate, there are many factors which influence P solubilisation from these compounds by bacteria.

1.5.2.1 Effects of carbon and nitrogen sources on phosphate solubilisation

Phosphate solubilisation activity of four unidentified isolates was evaluated in liquid cultures in the presence of nine carbon and four nitrogen sources replacing the standard glucose and $(NH_4)_2SO_4$, respectively (Nautiyal *et al.*, 2000). The four strains demonstrated diverse levels of phosphate solubilisation activity. One isolate proved to be the most efficient strain considering its capability to solubilise P utilising a wide range of carbon and nitrogen sources. The other three isolates preferred xylose, lactose and xylose plus glucose respectively, as their best carbon sources for phosphate solubilisation.

This experiment showed that for these isolates ammonium and nitrate sources were equally effective for phosphate solubilisation (Nautiyal *et al.*, 2000), which is contrary to previous results that phosphate solubilisation by bacteria varied when ammonium and different sources of nitrates were used as the source of N (Halder *et al.*, 1991; Abd-Alla, 1994).

1.5.2.2 Effects of high salt, high pH and high temperature on phosphate solubilisation

The strains isolated by Nautiyal *et al.* (2000) seemed well adapted to the environments from which they had been isolated. For example, strains isolated from alkaline soils had the potential to solubilise phosphate at high salt, high pH and high temperature. Of the four strains isolated, one proved to be capable of solubilising P in the presence of 10 per cent NaCl at pH 12 and at $45^{O}C$ (Nautiyal *et al.*, 2000). This may be the first report on phosphate solubilising bacteria under such extreme conditions. Generally, phosphate solubilisation was accompanied by a decrease in the pH of the medium. All four strains demonstrated a significant decline in phosphate solubilisation when the final pH of the medium was about 8.0 and above. However, a decrease in the medium pH was not always correlated with phosphate solubilisation activity, indicating that acid production is not the only reason for phosphate release into the medium.

1.5.2.3 Effect of calcium supplements on phosphate solubilisation

The effects of increasing amounts of various calcium salts containing 0 to 5 mg mL^{-1} Ca ($CaCl_2$, $CaSO_4$, $CaCO_3$ and $Ca(OH)_2$) on phosphate solubilisation has been determined (Nautiyal *et al.*, 2000). An increase of up to 122 per cent in the phosphate solubilisation ability of four isolates was observed in the presence of calcium salts. Inhibition of P solubilisation in the presence of the calcium salts $CaCO_3$ and $Ca(OH)_2$ at 2.5 and 5.0 mg mL^{-1} was observed as these Ca salts were associated with a higher pH than the control. Contrary to the growth of strains in $CaCl_2$ and $CaSO_4$, the growth was poor in the presence of $CaCO_3$ and $Ca(OH)_2$. The presence of $CaCO_3$, due to its P-buffering nature, may have provided resistance to the decrease in pH normally caused by the bacterial production of acid. At 5.0 mg mL^{-1} of $CaCO_3$, the final pH for all four strains was close to neutral and the level of P solubilisation was negligible suggesting that acid is important for phosphate solubilisation. However, acid production was not always directly correlated with phosphate solubilisation activity.

1.5.2.4 Effects of different types of microorganisms on phosphorus mobilisation

There is evidence that different phosphate solubilising microorganisms mobilise different forms of unavailable soil phosphate. For example, *Pseudomonas* sp. and *P. aurantiogriseum* are more effective where Ca-phosphates are concerned and *Aspergillus niger* and *P. simplicissimum* are more effective where Al-phosphates are concerned (Illmer and Schinner, 1995).

1.5.3 Possible bacterial mechanisms for solubilising phosphorus for plant growth

A major mechanism of mineral phosphate solubilisation is proposed to occur by the action of organic acids synthesised by soil microorganisms (Duff and Webley, 1959; Salih *et al.*, 1989; Halder *et al.*, 1990). Microorganisms secrete organic anions and if H^+ is also secreted the surrounding area becomes acidic. Consequently, soluble inorganic P may be released from mineral phosphate by substitution of Ca^{2+} by protons (Goldstein, 1994). There is, however, no linear correlation between pH and the amount of solubilised phosphate (Thomas, 1985; Asea *et al.*, 1988; Ehrlich, 1990).

The production by microorganisms of substances that are able to chelate metallic ions (Fe, Al or Mn) has long been considered a factor in P-mobilisation (Sperber, 1958). Such chelating substances are likely to include organic anions. In reports of the relationship between organic acids/anions and P mobilisation, it is not always clear whether acidification or chelation is the cause of P mobilisation.

The mobilisation of P has been reported to be greatest with citrate, followed by oxalate while malate and tartrate were moderately effective at mobilising P (Nagarajah *et al.*, 1970). Of the wide range of organic anions, produced by microorganisms, gluconate has been frequently reported as an agent of mineral phosphate solubilisation. Strains producing 2-keto-gluconate include several unidentified soil bacteria (Duff and Webley, 1959; Duff *et al.*, 1969), *Bacillus firmus* (Banik and Dey, 1982), *Rhizobium leguminosarum* (Halder *et al.*, 1990) and *Rhizobium meliloti* (Halder and Chakrabartty, 1993). *Bacillus liqueniformis* and *Bacillus amyloliquefaciens* were found to produce a mixture of lactate, isovalerate, isobutyrate, and acetate. Other organic anions, such as glycolate, oxalate, malonate, and succinate have also been identified as products of some phosphate solubilisers (Banik and Dey, 1982; Illmer and Schinner, 1992; Illmer and Schinner, 1995).

1.5.3.1 Phosphorus solubilisation by changing the rhizosphere pH using bacteria

In acid soil, sparingly soluble phosphate, such as iron or aluminium phosphate, can be partly mobilised by the lowering of the rhizosphere pH, as well as desorption from sesquioxide surfaces by anion (ligand) exchange (Gerke, 1994). Bolan *et al.* (1997) reported that organic anions form stable complexes with iron and aluminium, allowing the phosphate to be less adsorbed to the soil particles and thus more available for plant uptake by roots (Otani, *et al.*, 1996). In alkaline soils, where P is present as calcium phosphate minerals, organic acids caused dissolution of these minerals, by lowering the pH and by chelation of Ca by the organic acid anions (Bolan *et al.*, 1997).

A pH of from 3.4 to 4.6 was reported to be low enough for significant solubilisation of the calcium phosphate mineral, but not for the solubilisation of Fe^{3+} and Al^{3+} phosphate minerals (Stumm and Morgan, 1995). A pH of 2.7 was low enough for the

solubilisation of colloidal Al-P but a pH of from 2.0 to 2.2 was not low enough for the solubilisation of $AlPO_4$ (crystalline), colloidal Fe-P or $FePO_4.4H_2O$ (crystalline). Phosphorus solubilisation was higher from the Ca phosphate minerals than from the aluminium and Fe^{3+} phosphate minerals which was probably due to the higher solubility of the Ca phosphate minerals at the pH of *Penicillium radicum* cultures (Stumm and Morgan, 1995).

It has been suggested that microorganisms which decrease the medium pH during growth are efficient P solubilisers (Halder *et al.*, 1991; Kpomblekou-A and Tabatabi, 1994). The four strains isolated by Nautiyal *et al.* (2000) produced acid and lowered the pH of the growth medium from neutral to 3 to 3.5 after one day. Thereafter, the pH of the medium remained stable for up to five days. The decrease in pH clearly indicates the net production of acids, considered to be responsible for the P solubilisation. Bacteria from proteoid roots and non-proteoid lateral roots of *Telopea speciosissima* have been used in field and laboratory experiments (Wenzel *et al.*, 1994). It was suggested that acidification may have been caused by secretion of H^+ which could solubilise phosphate by exchange with Ca^{2+}. The secretion of H^+ in exchange for NH_4^+ ions is a well documented phenomenon in both bacteria and roots (Raven and Smith, 1976).

Changes in pH resulting from microbial activity are highly localised. Production of acids at micro-sites in the rhizosphere can have significant effects on local pH at the rhizosphere. It is not necessary to change the pH of the bulk soil for a useful P-solubilising effect.

High-affinity phosphate transporters are generally accepted as entry points for phosphate in the roots. The physiological, genetic, molecular and biochemical analysis of phosphate starvation response mechanisms highlight the ability of plants to adapt and thrive under phosphate limiting conditions (Raghothama and Karthikeyan, 2005). Enhanced ability to acquire phosphate appears to be regulated at the level of transcription of high affinity phosphate transporters. These transporters are encoded by a small family of genes with characteristic tissue and organ associated expression patterns. Many of them are strongly induced during phosphate deficiency thus providing plants with enhanced ability to acquire and transfer phosphate. In

addition, altered root morphology and mycorrhizal symbiosis further enhance the ability of plants to acquire phosphate.

1.5.3.2 Phosphorus solubilisation by chelation

Chelation is a mechanism by which formation of complexes of the cations associated with insoluble phosphate with organic anions can result in P solubilisation. The capability of organic anions to chelate Al^{3+} is correlated with the relative positions of hydroxyl and carboxylic groups on their main carbon chains (Hue *et al.*, 1986). Many effective chelators of Al^{3+} have hydroxyl groups adjacent to carboxylic groups (α-hydroxyl anion structures), positions which favour the formation of 5-bond ring structures with Al^{3+}. For example, gluconate has a α-hydroxyl anion structure and is able to chelate Al^{3+} and to lesser extent, Ca^{2+} and Fe^{3+}. The stability constant (K) for the formation of these gluconate complexes is highest for Al^{3+} (K = 96.5, Motekatitis and Martell, 1984), lower for Ca^{2+} (K = 16.2, Cannan and Kibrick, 1938) and the lowest for Fe^{3+} (K = 3.2×10^{-6}, Pecsok and Sandra, 1955).

It has been reported that different types of anions, such as citrate are responsible for dissolving insoluble phosphate (Cunningham and Kuiack, 1992). Citrate is known to be responsible for solubilisation of Al-compounds as well as having high complex–forming abilities with aluminium (Fulton *et al.*, 1989). Oxalate can also form a complex with aluminium (Lapeyrie *et al.*, 1991). Other anions including gluconate (Eckhardt, 1979), lactate and succinate (Kucey, 1983), α-ketogluconate (Kucey, 1988), and other organic anions (Berthelin *et al.*, 1991; Yadav and Singh, 1991) have also been reported as enhancing P mobilisation from insoluble P complexes.

Organic anions with only one carboxyl group (lactate, formate and acetate) have very little metal-complexing ability. Malate, citrate and oxalate have a high affinity for trivalent metals such as Al^{3+} and Fe^{3+} and these metals are mobilised most readily by organic acids in most soils (Pohlman and McColl, 1986; Jones and Kochian, 1996). However, this does not always imply efficient complexation at all soil solution pHs (Mench and Martin, 1991). Parker *et al.* (1995) reported that complexation of iron by malate, citrate and oxalate is highly dependent upon soil solution pH with little or no

complexation at high soil pH. Unlike citrate and malate, oxalate has a tendency to precipitate in the presence of Ca^{2+}.

The position and type of functional groups within each carboxylic compound seemed to be the dominant factor that influenced the amounts of P released (Kpomblekou-A and Tabatabai, 1994). For example, both citrate and cis-aconitate have α-, β-, and γ-carboxyl groups, and citrate has, in addition, a β-hydroxyl group. The amounts of P released with citrate (about 500 mg P kg^{-1} rock phosphate) were almost twice the amount released with *cis*-aconitate acid (254 mg P kg^{-1} rock phosphate) from the same sample. This suggests an active participation of the hydroxyl group in the P release process. The effect of the hydroxyl group can be seen within the carboxylate anion series. For example, succinate has no hydroxyl groups and released 145 mg P from Kodjari rock phosphate, whereas malate, with one hydroxyl group, released 240 mg P, and tartrate, with two hydroxyl groups released 290 mg P kg^{-1} from the same rock phosphate. Oxalate was the most effective in releasing P from kodjari rock phosphate (about 400 mg P kg^{-1} rock phosphate), even greater than that released with H_2SO_4 (about 374 mg P kg^{-1}). For the carboxylate anions, the closeness of the two carboxylate groups is a very important factor to be considered; the more the carboxylate groups are separated from each other, the more difficult the chelation process (Razzaghe-Karimi and Robert, 1975). This explains why oxalate (two carboxylate groups next to each other) was more effective than succinate, which has the two carboxylate groups separated by two -CH_2 groups.

The main P-solubilising effect of the organic anion, citrate, is considered to be through chelation of metal ions (especially Fe, Mn, and Al) that would otherwise immobilise P, or through formation of soluble anion-metal-P complexes, or both (Kirk *et al.*, 1999). Another possible mechanism is the displacement of P by anions on soil adsorption sites. Displacement will only be important where the amounts of organic anion adsorbed are comparable to the amounts of P desorbed. Unless very large amounts of organic anion are excreted, this would require that the anion be strongly sorbed on the soil; it would therefore diffuse only very slowly through the soil and the effect would be confined to a narrow zone around the root. It has been

assumed that chelation is likely to be one of the most important mechanisms, at least in highly weathered soils containing large amounts of metal oxides (Kirk *et al.*, 1999).

It was found that the amount of colloidal Al-phosphate solubilised by gluconate was higher than that solubilised by HCl at the same pH, indicating that chelation of Al^{3+} by gluconate had occurred (Whitelaw *et al.*, 1999). It was also reported that formation of the Al-gluconate complex led to enhanced solubility of P by removing Al^{3+} from the equilibrium reaction between colloidal Al-phosphate, Al^{3+} and $H_2PO_4^-$ (Cumming and Weinstein, 1990). The chelation of aluminium by gluconate made aluminium less toxic to *P. radicum* (Whitelaw *et al.*, 1999). It was found that although a combination of low pH (pH 2.7) and high P solubilisation from collodial Al-phosphate in the ammonium culture resulted in a high free aluminium concentration, the fungal biomass was not significantly lower than that in a $Ca_3(PO_4)_2$ ammonium culture (pH 4.1). It was reported that the solubilisation of $Ca_3(PO_4)_2$ or $CaHPO_4$ with gluconic acid plus HCl, was not higher than solubilisation by HCl alone indicating that at low pH Ca^{2+} was not complexed by gluconate (Whitelaw *et al.*, 1999). Similarly, it was found that abiotic P solubilisation of two Ca phosphate minerals (hydroxylapatite and $CaHPO_4.2H_2O$) by gluconic acid was due to lowered pH alone and not due to chelation of Ca^{2+} by gluconate (Illmer and Schinner, 1995), unlike the situation with aluminium ions (Whitelaw *et al.*, 1999).

The effect of chelation on the solubilisation of phosphate from tricalcium phosphate was studied by adding EDTA salt up to 10 mg mL^{-1} to four different unidentified phosphate solubilising bacterial isolates (Nautiyal *et al.*, 2000). Results indicated that the addition of 0.5 mg mL^{-1} EDTA caused maximal increases of 76, 28, 48 and 15% phosphate solubilisation with these four isolates compared to controls. It was shown that this could be due to chelation of Ca^{2+} produced during tricalcium phosphate dissolution. Further increase in the EDTA concentration caused a linear decrease in the solubilisation of phosphate. This result suggests that these bacterial strain isolated from alkaline soils have evolved to solubilise phosphate in an environmental ecosystem which contains high salt, and has a high pH and/or high temperature.

1.5.3.3 Organic acids

The role of organic acids and anions in lowering pH and complexing metal ions has been discussed in relation to P mobilisation from insoluble phosphate. The mobilisation of P is greatest with citrate followed by oxalate, while malate and tartrate are moderately effective. Acetate, succinate and lactate are the least effective at mobilising P (Nagarajah *et al.*, 1970). Malonate, the major component of root exudates of pigeon pea, dissolves $AlPO_4$ better than $FePO_4$, while piscidic acid, present in pigeon pea root exudates in nine-fold lower concentration than malonic acid, chelates iron better than aluminium and therefore solubilises $FePO_4$ better than $AlPO_4$ (Ae *et al.*, 1990; Otani *et al.*, 1996).

Phosphate dissolution rates can be greatly accelerated in soil in the presence of organic anions such as malate, citrate and oxalate, leading to 10- to 1000-fold higher soil solution P concentrations depending on soil type and organic anions (Fox *et al.*, 1990; Gerke, 1994; Jones and Darrah, 1994; Earl *et al.*, 1979). The efficiency of inorganic P dissociation by the organic anions is in decreasing order, citrate> oxalate> malate> acetate with P release dependent on the ability of the anion to complex aluminium (Lan *et al.*, 1995). However, organic acid induced P release depends on many factors, including pH and soil mineralogy (Fox *et al.*, 1990; Jones and Darrah, 1994; Bolan *et al.*, 1994; Lan *et al.*, 1995).

There are at least two mechanisms by which P release can occur (Jones, 1998). The first involves direct ligand exchange, whereby citrate directly replaces P on ligand exchange surfaces (e.g. on crystalline $Al(OH)_3$ or $Fe(OH)_3$). The second could involve the complexation of metal ions from the solid, which constitutes the exchange matrix holding the P (e.g. Ca^{+2}, in rock phosphate, or Fe^{3+} in $Fe(OH)_3$). Metal ions are often released from soil concomitantly with P release, which implies that the second pathway may be dominant although both are probably operating simultaneously. The desorption/release of P is extremely soil dependent, with high concentrations of organic anions (>100 µM for citrate, > 1 mM for oxalate, malate and tartrate) required to mobilise significant quantities of P to the soil solution (Jones and Darrah, 1994; Lan *et al.*, 1995; Earl *et al.*, 1979).

It has been reported that in some soils, no P appears to be mobilised upon the addition of organic acids (Jones and Darrah, 1994; Lan *et al.*, 1995). Many studies of this kind have used unnaturally high concentrations of organic acids (>1 mM) which is 100-fold higher that of a typical soil solution (Jones and Darrah, 1994; Lan *et al.*, 1995).

1.5.3.4 Mobilisation of phosphorus by bacterial ammonium assimilation

Several potential mechanisms for phosphate solubilisation such as the modification of pH by the secretion of organic acids or protons have been discussed (Wenzel *et al.*, 1994). Secretion of H^+ from roots into the medium in response to uptake of NH_4^+ and of OH^- (or HCO_3^-) for NO_3^- uptake are well documented, and the decrease in pH of the medium as a result N-nutrition with NH_4^+ has been correlated with solubilisation of inorganic phosphate (Raven and Smith, 1976; Jarvis and Robson, 1983; Marschner *et al.*, 1986; Haynes, 1990; Marschner, 1991; Kennedy, 1992). It was considered that a transient initial acidification of the medium in the presence of NO_3^- ions was probably the result of a high initial cation exchange with the K^+ or Ca^{2+} in the medium, but the ultimate pH increase was probably due to the extrusion of OH^- or HCO^{3-} during uptake of NO_3^-.

Two microorganisms (*Penicillium aurantiogresem* and *Pseudomonas sp.*) were used to examine solubilisation of inorganic phosphate (hydroxylapatite and brushite) (Illmer and Schinner 1995). Direct contact between microorganisms and calcium phosphates was not necessary for effective P solubilisation. For these two organisms, it was suggested that phosphate solubilisation was not caused by the release of organic acids but that phosphate solubilisation without acid production resulted from the release of protons accompanying respiration or NH_4^+ assimilation.

The mobilisation of unavailable P may be stimulated by protons originating from NH_4^+ assimilation (Taha *et al.*, 1969; Banik and Dey, 1983; Kucey, 1983; Dighton and Boddy, 1989; Parks *et al.*, 1990) or by respiratory H_2CO_3 production (Jurinak *et al.*, 1986), although H^+ from respiration cannot be entirely responsible for P solubilisation. Respiratory acidification, however, may play a role in initating P

mobilisation. Proton excretion accompanying NH_4^+ assimilation, may account for P solubilisation by local acidification as a result of net cation uptake by plants or microorganisms (Kennedy, 1992).

1.6 Effects of plant growth promoting rhizobacteria (PGPR)

1.6.1 What are endophytes?

The term "endo" is drived from the greek word "endon" meaning "within"; "phyte" from the Greek "phyton", meaning "plant"; literally, an endophyte is an organism that lives inside the plant (http://www.dekker.com, 2002). Endophytes can be isolated from surface-disinfected plant tissue or the inner parts of plants (Hallman *et al.*, 1997). Endophytes make symbiotic relationships with the host plants. Furthermore, they colonize an ecological niche similar to that of phytopathogens, which might be potentially useful as biocontrol agents (Hallman *et al.*, 1997). Endophytic microorganisms have the capacity to control pathogens (Duijff *et al.*, 1997; Krishnamurthy and Gnanamanickam, 1997; Sharma and Nowak, 1998), insects (Petrini *et al.*, 1989; Azevedo *et al.*, 2000) and nematodes (Hallman *et al.*, 1998). In some cases, they can also accelerate seedling emergence and promote plant establishment under adverse conditions (Chanway, 1997) and enhance plant growth and development (Bent and Chanway, 1998; Pillay and Nowak, 1997). For endophytes, the advantages may lie in their capability to preserve a protected population in the plant tissue, which may serve as a permanent source for external colonisation (Kovtunovych *et al.*, 1999).

1.6.2 Examples of some PGPR effects

Rhizosphere bacteria that exert beneficial effects on plant growth and development are referred to as plant growth promoting rhizobacteria (PGPR) (Zahir *et al.*, 2004). The PGPR can affect plant growth by production and release of secondary metabolites (plant growth regulators/phytohormones/biologically active substances), lessening or preventing deleterious effects of phytopathogenic organism in the rhizosphere and/or facilitating the availability and uptake of certain nutrient from the root environment.

Some phosphate mobilising bacteria also have PGPR effects as phytohormone produces.

Five bacterial strains and one fungus which produced growth-promoting substances as well as acting as P phosphate solubilisers have been isolated (Sattar and Guar, 1987). Phosphate-dissolving bacteria, *Bacillus polymyxa, B. pulvifaciens* (R1), *B. pulvifaciens* (R5), *Pseudomonas striata* and the fungus *Aspergillus auramori,* produced auxins and gibberellins to an appreciable extent that enhanced plant growth.

In recent years, the suggested use of PGPR to promote plant growth has increased dramatically in various parts of the world. It has been reported that inoculation with PGPR can result in increased germination, seedling emergence and modify growth and yield of various cereal and non–cereal crops by secreting auxins (indole acetic acids), gibberellins or other hormones (Kloepper and Schroth, 1978; Suslow, 1982; Kloepper *et al.,* 1986; Hassouna, 1990; Xia *et al.*, 1990; Chen *et al.*, 1994; Javed and Arshad, 1997; Van *et al.*, 2000; Dobbelaere *et al.*, 2001).

Phosphorus solubilising bacteria that produced IAA-like hormones as determined by *in vitro* assays have been isolated (de Freitas *et al.*, 1997). Some of these isolates influenced plant growth development through hormone production. For example, *Bacilli* inoculants known to be P solubilisers increased the growth rate but not the P nutrition or N_2 fixation of canola, suggesting that plant responses to these bacteria could be associated with plant growth hormones, rather than direct P solubilisation action.

1.6.3 Mechanism of PGPR effects

Several mechanisms have been postulated to explain PGPR effects on the growth and development of inoculated plants. The mechanisms can be broadly categorised as producing both direct and indirect growth stimulation. Direct growth promotion occurs when a rhizobacterium produces metabolites (e.g. phytohormones) or improves nutrient availability directly promoting plant growth (Kloepper *et al.*, 1989). Bacteria producing antibiotics, siderophores and hydrogen cyanide (HCN), decreasing

the activity of pathogens or deleterious microorganisms and thereby increasing plant growth, provide examples of indirect growth promotion. It has been argued, however, (Kloepper, 1993) that there is often no clear separation between direct growth promotion and biological disease control promoting indirect growth. Rather, growth promotion and biological control of disease should be viewed as 'two sides of a coin' giving the same overall effect. Generally, evaluation of bacterial strains for growth promotion is done *in vitro* in the absence of pathogens. PGPR selected initially for growth promotion in the absence of pathogens may demonstrate biological control of disease when challenged with the pathogen.

1.7 The aims of this research project

Phosphorus is in short supply in many agricultural soils as a result of adsorption to soil minerals. This problem has been difficult to resolve and it has not been possible to manage P nutrition from soil using microorganisms for P mobilisation to assist plant growth. Gerretsen (1948) first showed that pure cultures of soil P-mobilising bacteria could increase the P nutrition to plants but despite extensive studies little real practical development has occurred. The advent of new techniques and approaches could include an understanding of the ecology of microorganisms in the soil environment, soil chemical behaviour responding to effects of bacterial supplementation and even the possibility of direct manipulation of organisms through gene technology. This project specifically focuses on the interaction of soil chemical properties with microbial impact in the soil environment. Therefore the synchronization of the physico-chemical properties of soil with microbial impact is a major objective of this research project.

The overall aim of this thesis is to test the hypothesis that it is possible to develop a potentially effective bacterial P- mobilising biofertiliser.

In order to develop such an effective P-mobilising biofertiliser, the following subsidiary aims were included

- To isolate P-mobilising bacteria from rhizosphere soil and to evaluate the ability of these isolates to mobilise insoluble P *in vitro*.

- To evaluate the performance of these isolates in glasshouse experiments with wheat to identify their possible effectiveness at the field level for P mobilisation.

- To test whether P-mobilising bacteria have a PGPR effect, in a glasshouse experiment using wheat to identify their PGPR effects, in addition to P-mobilising ability.

- To investigate the effect of physico-chemical properties of some Australian soils on P adsorption and to find the mechanism for P desorption.