EFFECTIVENESS OF MICROBIAL SOLUBILIZATION OF PHOSPHATE IN
ENHANCING PLANT PHOSPHATE UPTAKE IN TROPICAL SOILS AND
ASSESSMENT OF THE MECHANISMS OF SOLUBILIZATION

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DEDICATION

To my wife Klaudia

and

my daughters Sara, Sofia, and Susana
ACKNOWLEDGEMENTS

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ABSTRACT

A series of experiments were carried out in order to evaluate the effectiveness of a phosphate-solubilizing fungus (PSF), identified as *Mortierella* sp., to enhance plant phosphate (Pi) uptake and growth. The fungus was isolated from the rhizoplane of *Leucaena leucocephala* grown in a Hawaiian Andisol. An *in-vitro* test was developed to test the capacity of this fungus in dissolving rock phosphate (RP) by means of oxalic acid production in the presence of different types of soil. This PSF could desorb sorbed-Pi from soil mineral surfaces. The presence of high Pi sorbing soil minerals impaired the PSF effectiveness to enhance the supply of soluble Pi. The capacity of the fungus to solubilize P was N-source dependent, being enhanced to a greater extent by ammonium than by nitrate. This PSF was aluminum tolerant and moderately tolerant of low solution pH and its capacity to reduce solution pH and absorb Pi was not affected by these factors.

Under greenhouse conditions was determined the PSF effectiveness to enhance Pi uptake and growth of mycorrhized (*Glomus aggregatum* and *G. fistulosum*) leucaena grown in highly weathered Oxisols. The PSF effectiveness was higher if moderate amounts of RP (P: 150-600 mg/kg) were added to the Oxisol. When RP was not added, the effect of PSF inoculation was not significant. With high amounts of RP (P: 1200 and 2400 mg/kg), the effect of the fungus on leucaena was either nil or negative. Moreover, in the absence of mycorrhizal association the effect of PSF inoculation was not significant.
In very high Pi sorbing soils (Andisols), Mortierella sp. was not able to increase leucaena growth and Pi uptake, even in the presence of the mycorrhizal association and moderate amounts of RP (P: 300 mg/kg).

In contrast, Mortierella sp. alone was able to increase plant growth and Pi uptake of mycorrhiza-free leucaena grown in a low Pi-sorbing Mollisol without RP addition. In summary, four major factors can control the effectiveness of PSM to increase soil solution Pi, plant P uptake and growth: soil Pi sorbing capacity, mycorrhizal association, calcium phosphate content of soils, and the use of ammonium as nitrogen source.
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Problem

Phosphorus (P) deficiency is one of the most serious factors limiting plant growth in tropical soils. In these soils soluble orthophosphate ions (Pi) are converted to plant-unavailable forms. This phenomenon is called Pi fixation and consists of the adsorption of Pi on soil solid surfaces and/or the precipitation of Pi by Al and Fe ions. Phosphate fixation is particularly strong in highly weathered soils and in soils derived from volcanic parent materials.

Application of high rates of Pi fertilizers, particularly rock phosphates (RP), has been used to satisfy external Pi requirement of crops. However, the low solubility of RP and the high investment of capital required limit the adoption of this alternative in many developing countries of the tropics.

Fortunately, there are many soil microorganisms that can solubilize unavailable P forms, including RP. These microorganisms are termed phosphate-solubilizing-microorganisms (PSM). Inoculation of soils with PSM has increased plant Pi uptake and growth of diverse plants grown in soils of the temperate-zone that commonly exhibit low Pi fixation capacity. In contrast, the effectiveness of PSM to enhance soil Pi availability, Pi uptake, and plant growth in high Pi fixing soils has been little studied.
Justification

It is desirable to find methods for increasing soil P availability, dissolution of raw materials (e.g., RP) or added P fertilizers. The identification of mechanisms and soil factors that regulate the effectiveness of microbial P solubilization could enhance plant Pi uptake in soils with high Pi fixation capacity. A clear understanding of the factors could lead to reduce the amount of Pi fertilizer required to satisfy external Pi requirements of plants. However, the effectiveness of PSM to enhance plant Pi uptake is uncertain in the highly weathered and volcanic ash soils of the tropics since Pi fixation is more intense than in temperate soils. It is likely that most of the Pi released by PSM will be refixed again in these soils. Dual inoculation with mycorrhizal fungi and PSM may be more effective to enhance plant P uptake in high Pi fixing soils than inoculation with PSM alone.

Hypothesis

The effectiveness of PSM to dissolve RP, to desorb adsorbed Pi on soil surfaces, and to enhance plant P uptake may depend on soil mineralogy, extent of saturation of P sorbing sites, nitrogen form available for PSM, presence of mycorrhizal association, amount of added RP, and the amount of calcium phosphate in the soil. In that way:
• Phosphate solubilizing microorganism (PSM) may be present in high Pi sorbing soils.

• Effectiveness of PSM in dissolving RP may be influenced by the nitrogen (N) form present in the growth medium.

• The effectiveness of PSM in increasing soluble P, via RP dissolution, may be controlled by soil Pi sorption capacity.

• PSM can desorb Pi adsorbed on the surface of soil mineral but their effectiveness will depend on the Pi sorption capacity and the extent of saturation of Pi sorption sites in the soil.

• PSM may exhibit tolerance to acidity and aluminium (Al) toxicity.

• Enhancement of plant Pi uptake by PSM may be controlled by soil Pi sorption and mycorrhizal association.

• In highly weathered soils, the effectiveness of PSM to increase Pi uptake and growth of mycorrhizal plants may be enhanced by the addition of RP.

• In low Pi sorbing soils (e.g., Mollisols), PSF inoculation alone can increase plant P uptake of non-mycorrhizal plants, but not in very high P sorbing (e.g., Andisols). In Andisols, PSF can be effective in the presence of mycorrhizal association.

Objectives

• To screen effective PSM for RP solubilization activity in soils of the tropics.
• To develop an in-vitro method to accurately evaluate the ability of microorganisms to solubilize RP.

• To evaluate the tolerance of Mortierella sp. to acidity and Al toxicity.

• To determine the effectiveness of a PSF to increase Pi availability by dissolving RP in the presence of soil minerals and soils differing in their Pi sorbing capacity.

• To determine the effectiveness of Mortierella sp. to increase soluble Pi by desorbing Pi from the surfaces of soil minerals and soils differing in their capacity to sorb Pi.

• To assess the effects of nitrogen form on RP solubilization activity of Mortierella sp. to dissolve RP.

• To determine the synergistic effect of a PSF (Mortierella sp.) and a mycorrhizal fungus to enhance plant Pi uptake and growth of Leucaena leucocephala (Lam.) in soils differing in their P fixing capacity and with graded amounts of RP.
Phosphate (Pi) fixation is a serious problem in agricultural soils, particularly in highly weathered soils and those formed from volcanic ash (Trolve et al., 2003; Sanchez, 1976; Sanchez and Uehara, 1980). Sanchez and Logan (1992) estimated that the soils that exhibit high Pi fixation capacity occupy 1018 million ha in the tropics. In tropical America there are 659 million ha affected, 210 in Africa, and 199 in Asia. The term Pi-fixation is used to describe reactions that remove bioavailable Pi from the soil solution into the soil solid phase (Barber, 1995). There are two types of reactions: (i) Pi sorption on the surface of soil minerals, and (ii) Pi precipitation by cations such as Al$^{3+}$ and Fe$^{3+}$ in the soil solution (Havlin et al., 1999).

Phosphate sorption is particularly strong on iron and aluminum hydrous-oxides (crystalline or non-crystalline) that predominate in the highly weathered soils of humid regions and acid savannas (Mattlingly, 1975). Jones (1981) characterized the Pi sorption by 11 Puerto Rican soils and found that the surface area of Goethite was a primary factor accounting for Pi sorption, with Gibbsite and Hematite contributing little to Pi sorption. Jackman et al. (1997) observed
similar results for Hawaiian soils. Thus, soil Pi sorption was satisfactorily predicted by soil mineralogical composition.

In soils derived from volcanic parent materials, humus-Al/Fe complexes, Allophanes, Ferrihydrite, and Goethite are the soil minerals responsible for the strong Pi sorption observed (Parfitt, 1989; Schwertmann and Herbillon, 1992; Jackman et al., 1997; Shoji et al., 1993). On the other hand, in calcareous soils, Pi is sorbed on the surface of calcium carbonate (Mattingly, 1975).

In acidic soils Pi precipitation occurs with active forms of aluminum \([\text{Al}^{3+}, \text{Al(OH)}^2+, \text{Al(OH)}_2^+\]) and iron \([\text{Fe}^{3+}]\), while in neutral and alkaline soils it occurs mostly with calcium \([\text{Ca}^{2+}]\) (Bohn et al., 1985). The extent of dominance of these cations depends mainly on the degree of soil weathering and soil pH. Phosphate ions precipitate to form initially amorphous (non-crystalline) compounds, becoming much more stable as crystalline forms are formed over time (Brady and Weil, 1999). Amorphous minerals are slightly more soluble than their crystalline forms because they have smaller particle size, and consequently greater surface area. For instance, the crystalline mineral variscite \(\text{AlPO}_4.2\text{H}_2\text{O}\) has a surface area of 1.54 m\(^2\)/g (Taylor and Gurney, 1964) and its solubility product \((K_{sp})\) is \(10^{-30.5}\) (Bache, 1963). On the other hand, its amorphous aluminum-phosphate counterpart has a surface area of 10.5 m\(^2\)/g (Juo and Ellis, 1968) and its \(K_{sp}\) is \(10^{-28.1}\) (Veith and Sposito, 1977). In alkaline soils, Pi compounds are similarly transformed to more insoluble forms. Initially Pi ions precipitate to calcium-monohydrogen-phosphate \((K_{sp} = 10^{-4.5})\) (Stumm and
Morgan, 1995), which is then converted to calcium-orthophosphate \( (K_{sp} = 10^{-24}) \), and finally to apatite \( (K_{sp} = 10^{-55.8}) \) (Snoeyink and Jenkins, 1980).

Fox and Kamprath (1970) showed that the degree of P fixation varies among soils, with Andisols, Oxisols, and Ultisols (USDA soil taxonomy) having a high P fixation capacity (Boul et al., 1997). These soils are usually acidic and generally have high amounts of exchangeable Al and clay minerals that can sorb high amounts of Pi (Sanchez and Uehara, 1980). Juo and Fox (1977) proposed several categories for soil Pi sorption capacity in tropical soils as measured by Pi sorption isotherms and the usual mineralogy of each category (Table 2.1).

Table 2.1. Categories of soil Pi sorption as measured by Pi sorption isotherms (method of Fox and Kamprath, 1970) and usual mineralogy in each category.

<table>
<thead>
<tr>
<th>Category</th>
<th>( P_{0.2} ) (P: mg/kg)*</th>
<th>Usual mineralogy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very low (VL)</td>
<td>&lt;10</td>
<td>Quartz, organic materials</td>
</tr>
<tr>
<td>Low (L)</td>
<td>10-100</td>
<td>2:1 clays, quartz, and 1:1 clays</td>
</tr>
<tr>
<td>Medium (M)</td>
<td>100-500</td>
<td>1:1 clays with oxides</td>
</tr>
<tr>
<td>High (H)</td>
<td>500-1000</td>
<td>Oxides, moderately weathered ash</td>
</tr>
<tr>
<td>Very high (VH)</td>
<td>&gt;1000</td>
<td>Desilicated amorphous materials</td>
</tr>
</tbody>
</table>

* Amount of P required to achieve a soil solution P of 0.2 mg/L.

Soil PI diffusion

The immediate PI source for plants is the soil solution, which usually contains a low PI concentration \( (P: 0.001-0.01 \text{ mg/L}) \) (Fox, 1979; Barber, 1995). When PI is removed from the soil solution by roots and/or by PI fixation reactions, a gradient of PI concentration is created between the solid phase and the soil solution around the roots. Sorbed PI on the soil solid surfaces must desorb in order to replenish PI in the soil solution (Do Carmo Harta and Torrent, 2007). Hence, PI diffuses from the solid phase, where it is more concentrated, to the soil.
solution around the root surface where its concentration is continuously being depleted. However, the rate of Pi diffusion is quite slow (10^{-12}-10^{-15} \text{ m}^2/\text{s}) (Schachtman et al., 1998) which limits the Pi supply and creates a depletion zone of Pi around the roots of approximately of 1-2 mm (Barber, 1995). Phosphate ions beyond the zone of depletion cannot be accessed by the root surface (Barber, 1995). Phosphate ions that slowly diffuse into the soil solution originate mainly from Pi weakly sorbed on soil colloids and from those freshly precipitated (Sanchez, 1976; Stevenson, 1986; Lindsay, 2001).

Soil Pi supply depends on the Pi buffering capacity of soils (Pypers et al., 2006), which can be estimated from the relationship between the concentration of Pi in soil solution (intensity factor, I) and the quantity of labile Pi in the solid phase (quantity factor, Q) (Barber, 1995; Holford, 1997). The change \( \delta I \) in the intensity factor induced by changes in the quantity factor defines the soil Pi buffer capacity (PBC), which is mathematically expressed as:

\[
\text{PBC} = \delta Q + \delta I \quad [1]
\]

If a given change in Q produces a small change in I, the soil Pi buffer capacity will be high. Conversely, if a change in Q produces a large change in I, the soil Pi buffer capacity will be low. A high soil Pi buffer capacity indicates that the soil has the ability to maintain a concentration of Pi in soil solution nearly constant or, at least, with small variations, indicating fast kinetics of Pi release from the solid phase into the soil solution.
Phosphate fertilizer management

Sanchez and Uehara (1980) discussed different strategies to increase soil Pi availability of acidic tropical soils with high Pi fixation capacity. One strategy consists of applying a high dose of soluble Pi fertilizers (500-1000 mg/kg), followed by small amounts of annual application. Although a great part of the added Pi is fixed, it may be released over several years, thus generating a residual effect. These Pi fertilization rates are not added by most farmers in developing countries due to the high cost of Pi fertilizers. The proportion of the added Pi taken up by the first crop is quite low, ranging from 5 to 10%. It means that 90-95% of the added Pi fertilizer is fixed in the soils in chemical forms that slowly release Pi for plants (Engelstad and Terman, 1980).

Rock phosphates (RP) are highly recommended for acid soils with a high Pi fixation capacity, because other more soluble Pi forms are quickly fixed and are more expensive (Yusdar et al., 2007; Randhawa et al., 2006; Msolla et al., 2005). However, the greater the reactivity of a RP the greater its desirability because less reactive minerals are very insoluble (Shrivastava et al., 2007; Ojo et al., 2007; Chien and Hammond, 1978; Hammond and Leon, 1992). There is an increasing interest in tropical countries to use organic amendments and RP concurrently. The results suggest that the effectiveness of RP to increase plant growth and crop yield could be increased by mixing it with barnyard manures, compost, and green manures (Msolla et al., 2007; Yusdar et al., 2007; Shrivastava et al., 2007). Added manures can also facilitate desorption of sorbed Pi from soil particles (Redding et al., 2006).
Some treatments on RP such as ‘fine grinding’, thermal alteration, and fusion with silica, sodium or magnesium carbonate have been used (Sanchez and Uehara, 1980). Since RP are more soluble in acidic conditions, their acidulation with strong acids has been employed to produce more soluble fertilizers such as superphosphates (Young and Davies, 1980). Partial acidulation has also been employed; however, it increases the cost of production and the final price (Havlin et al., 1999). The direct application of non-acidulated RP is recommended for acid soils but not for neutral and alkaline soils. However, several authors have used RP successfully in alkaline soils with concurrent inoculation of P solubilizing microorganisms (PSM). These microorganisms can release Pi rapidly, increasing plant Pi uptake (Kucey and Leggett, 1989; Whitelaw, 2000).

Bar-Yosef et al. (1999) tested Pseudomonas cepacia for P solubilizing activity on a RP and noted that it produced superphosphate. These researchers found that this bacterium produced gluconic acid and 2-ketogluconic acid using glucose as the sole carbonaceous substrate. Once these acids were dissociated in solution, the protons reacted with the RP and released Pi into the solution that then was re-precipitated with Ca\(^{2+}\) to form superphosphate fertilizers.

Some authors have proposed the use of mycorrhizal fungi to increase efficiency in plant Pi uptake (Mosse, 1981). For instance, Manjunath et al. (1989) studied the effectiveness of Glomus aggregatum to enhance plant Pi uptake of Leucaena leucocephala grown in an Oxisol fertilized with RP (P: 340-5440 mg/kg). Plant dry weight and shoot P concentration did not increase significantly in uninoculated soils. In contrast, when the soil was inoculated with Glomus
aggregatum there was a significant increase in plant dry weight and tissue P concentration. However, in order to obtain adequate growth of mycorrhizal plants, it was necessary to apply a high P level, at least 2720 mg/kg. Despite the benefits of mycorrhizal inoculation, it is clear that it is necessary to apply a high rate of RP. This imposes economic limitations on use the mycorrhizal association as a strategy to manage Pi-deficient soils. It is clear that arbuscular mycorrhizal fungi (AMF) absorb only Pi from the soil solution, as plant roots do, and there is no evidence of their ability to solubilize insoluble soil P minerals (Bolan, 1991). The use of Pi solubilizing microorganisms may increase the amount of available Pi in soil solution and, consequently, enhance the effectiveness of AMF to increase plant Pi uptake.

Mycorrhizal Pi uptake

Plants exhibit different strategies to grow in Pi deficient soils: (I) changes in root morphology such as the production of an elongated root system with fine roots and abundance of root hairs; (II) release of phosphatase enzymes that release Pi from organic compounds, and (III) production and release of organic acids that solubilize Pi compounds (Radersma and Grierson, 2004; McCully, 1999; Hetrick, 1991). Plants with less root plasticity need to form symbiotic associations with mycorrhizal fungi that colonize the cortical tissue of roots (Smith et al., 2003; Smith, 2002; Sylvia, 1999) if they are to grow normally in Pi-deficient soils. The plant supplies carbonaceous compounds to the fungus, while the fungus provides nutrients, particularly diffusion-limited ones such as Pi, Cu\(^{2+}\),
and Zn^{2+} (Lynch and Ho, 2005; Hamel, 2004; Habte and Manjunath, 1991; Barber, 1995; Marschner, 1995). It is clear that arbuscular mycorrhizal fungi can only take up soluble Pi, from the same Pi pool that is available for roots (Cardoso et al., 2006; Bolan, 1991).

Roots can absorb available Pi from distances not exceeding a few mm away from their surface while mycorrhizal hyphae can extend to several cm from the root surface, exploring a greater volume of soil (Jeffries et al., 2003; Hattingh et al., 1973; Mosse, 1981). Forty-seven days after mycorrhizal inoculation of *Trifolium subterraneum*, Jacobsen et al. (1992) found mycorrhizal hyphae spreading from the root surface to 11 cm with the proportion between mycorrhizal hyphae and root length of 1-10 m/cm of infected root. Barber (1995) reported a lower value of 0.8 m/cm of root.

Mycorrhizal hyphae have a higher affinity for absorbing Pi than roots. Schachtman et al. (1998) reported that the hyphae of *Gigaspora margarita* had an affinity constant for Pi ($K_m$) of $2.5 \mu M$ (P: 0.077 mg/L), while many plants usually exhibited a $K_m$ of 6-44 $\mu M$ (P: 0.19-1.36 mg/L), particularly those highly dependent on the mycorrhizal association (Nye and Tinker, 1977; Barber, 1995). In addition, Barber (1995) affirmed that due to the small radius of the mycorrhizal hypha (1-3 $\mu m$) there is no Pi depletion zone around the hypha and this allows the mycorrhizal hypha to take up Pi more effectively due to a higher and more constant Pi concentration. However, Li et al. (1991) found a very narrow Pi depletion zone around the mycorrhizal hypha. In contrast, roots with a greater radius (150 $\mu m$) generate a zone of depletion of at least 1 mm, resulting in low Pi
concentration around the root surface. Smith and Read (1997) reported P influx in mycorrhizal roots of 3 to 5-fold higher than non-mycorrhizal roots (10^{-11} \text{ mol/m s}).

Plant species exhibit different degrees of mycorrhizal dependency (MD) to produce maximum growth at a given level of soil fertility (Gerdermann, 1975; Plenchette et al., 1983). Habte and Manjunath (1991) found that the MD of several plant species was determined by root characteristics of the host species (root length, root density, root surface area, incidence and length of hair roots), which determine their capacity to explore and absorb Pi from the soil solution. Since MD was significantly affected by soil solution Pi concentration, they proposed that MD should be estimated at different levels of available Pi, particularly at the soil solution P of 0.02 mg/L.

Role of organic acids/anions on P solubilization

Many organic acids are effective in solubilizing soil P compounds (Hue, 1991) and other soil minerals (Calvaruso et al., 2006; Welch et al., 2002). These acids or anions are produced by roots (Corrales et al., 2007; Radersma and Grierson, 2004; Kirk et al., 1999; Marschner, 1995) and by microbial activity during decomposition of organic matter or induced by Pi-deficiency stress (Jones et al., 2003; Fransson et al., 2004; Bohn et al., 1985). Bolan et al. (1994) found organic acids or anions in high concentrations in poultry manure, lesser amounts in the rhizosphere soil, very little in the bulk soil, and trace amounts in leaf litter. Le Bayon et al. (2006) found that there was a relatively high production of
organic anions (citrate, fumarate, and malate) in the rhizosphere of Lupinus albus under Pi-starvation.

Bolan et al. (1994) studied the influence of monocarboxylic (acetic, formic, and lactic), dicarboxylic (malic, tartaric, and oxalic), and tricarboxylic (citric) acids/anions on the solubilization and sorption of Pi on an Andisol (Hydric Dystrandept) and an Alfisol (Typic Fragiaqualf) of New Zealand. The addition of these organic acids significantly decreased Pi sorption on Allophane surface. The effectiveness of such acids followed the order, tricarboxylic > dicarboxylic > monocarboxylic. This was explained by the formation of complexes between Al with the conjugated anion of each organic acid and by their respective stability constant (Log $K_a$) (Hue et al., 1986) (Table 2.2). The addition of organic acids or anions also favored the solubilization of North Carolina rock phosphate (NCRP) and monohydrogen calcium phosphate (MCP), increased dry matter yield of ryegrass (Lolium rigidium) and enhanced plant Pi uptake (Bolan et al., 1994).

Hue (1991) obtained similar results on the availability of soil Pi when he added organic acids/anions on two Andisols, an Oxisol, an Ultisol, and a Vertisol of Hawai’i. The effectiveness of the acids to reduce Pi sorption from a soluble source ($\text{KH}_2\text{PO}_4$) was higher with malic acid (monohydroxy dicarboxylic), followed by protocatechuic acid (dihydroxy monocarboxylic), and acetic acid (monocarboxylic). The effect was higher when the acid was applied first and Pi last. The dry weight of lettuce was significantly higher when the soils received organic acids or anions plus Pi compared to those that received only Pi. The magnitude of this effect was much higher in the two Andisols and in the Oxisol
whereby plant dry weight was 5-15 fold higher than control plants, whereas in the Vertisol the increase was only 1.3-1.72 fold. Such effects were associated with differences in clay mineralogy that determines the soil Pi sorption capacity. Hue (1991) concluded that the efficiency of Pi fertilizers might be enhanced if they are added with organic acids or anions or, more practically with green manures or animal wastes. Results of more recent studies have shown that RP can be more effective if it is mixed with manures, composts, and crop litter in decomposition (Reddy, 2007; Bah et al., 2006; Singh et al., 2006).

The phenomenon of Pi desorption by organic anions is widely accepted by soil scientists. Recently, Sato and Comerford (2006) used organic anions to model the Pi desorption from a Brazilian soil. They conclude that the organic anions can increase soluble Pi by two process: (i) the desorption of P sorbed onto soil surface (ligand exchange) and (ii) the dissolution of soil P compounds (ligand dissolution) (e.g., calcium phosphates).

Table 2.2. Stability constant of organic anions with aluminum (Log \( K_{Al} \)) and calcium (Log \( K_{Ca} \)) and their effects on soil Pi sorption and solubilization of two Pi fertilizers.

<table>
<thead>
<tr>
<th>Organic Acid</th>
<th>Log ( K_{Al} )</th>
<th>Log ( K_{Ca} )</th>
<th>Sorbed P (mmol/kg soil)</th>
<th>Dissolved MCP(^a) (%)</th>
<th>Dissolved NCPR (^b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (control)</td>
<td>-</td>
<td>-</td>
<td>52</td>
<td>2.35</td>
<td>1.28</td>
</tr>
<tr>
<td>Formic</td>
<td>1.36</td>
<td>1.43(^b)</td>
<td>47</td>
<td>12.27</td>
<td>11.86</td>
</tr>
<tr>
<td>Acetic</td>
<td>1.60</td>
<td>1.18(^b)</td>
<td>45</td>
<td>10.35</td>
<td>12.57</td>
</tr>
<tr>
<td>Lactic</td>
<td>2.41</td>
<td>1.63(^c)</td>
<td>46</td>
<td>8.71</td>
<td>13.92</td>
</tr>
<tr>
<td>Malic</td>
<td>5.40</td>
<td>2.25(^d)</td>
<td>37</td>
<td>32.65</td>
<td>31.98</td>
</tr>
<tr>
<td>Tartaric</td>
<td>5.62</td>
<td>2.80(^e)</td>
<td>35</td>
<td>30.49</td>
<td>32.31</td>
</tr>
<tr>
<td>Oxalic</td>
<td>6.16</td>
<td>3.44(^d)</td>
<td>32</td>
<td>36.44</td>
<td>34.02</td>
</tr>
<tr>
<td>Citric</td>
<td>7.98</td>
<td>4.87(^b)</td>
<td>18</td>
<td>83.78</td>
<td>86.41</td>
</tr>
</tbody>
</table>

Source: Bolan et al. (1994); \(^a\) taken from Hue et al. (1986); \(^b\) taken from MINTEQA2 (a model for equilibrium speciation in geochemical environments, accessed at [www.epa.gov](http://www.epa.gov) on March 15, 2008); \(^c\) taken from Bazin et al. (1995); \(^d\) taken from Finlayson et al. (1972). \(^e\) Monohydrogen calcium phosphate. \(^f\) NCPR= North Carolina rock phosphate.
Since phosphate solubilizing microorganisms (PSM) can release the same organic acids studied by Hue (1991) and Bolan et al. (1994), presumably these microorganisms can reduce the activity of Al ions in the rhizosphere, decrease Pi sorption, and enhance plant Pi uptake. Miyasaka et al. (1991) found that the Al tolerance of plants is associated with the ability of roots to release organic acids/anions (citrate and oxalate in particular) into the rhizosphere. Since some soil microorganisms are able to produce organic acids/anions it is possible that such microorganisms could help plants to grow in soils with toxic levels of Al\(^{3+}\). In a series of experiments De la Fuente et al. (1999) isolated a gene that encodes for citrate synthetase overproduction in the TCA cycle of a strain of *Pseudomonas aeruginosa*, a known PSM. This gene was then transferred to tobacco cells of Al-intolerant plants. Transgenic plants were able to produce high amounts of citric acid and citrate, its conjugated anion, and to grow in solutions with high concentration of Al. The process was successfully replicated with papaya plants.

Although these experiments were aimed at enhancing Al tolerance of these plants, they also involved mechanisms proposed for the microbial solubilization of soil Pi. However, Delhaize et al. (2001) reported that they have were not able to repeat the results obtained by De la Fuente and collaborators (1999), and they suggest that the expression of *P. aeruginosa* genes are either sensitive to environmental conditions or that the observed Al tolerance and improved Pi nutrition were due to other factors.
The role of organic acid/anion production in the rhizosphere on Pi desorption and Pi solubilization has been accepted generally. However, experimental results indicated that its effectiveness to increase plant Pi uptake depends on plant species, age, physiological state, and soil mineralogy (Trolove et al., 2003). The concentration of these organic compounds is relatively low in most soils because they can be precipitated with free ions (e.g., Al³⁺, Fe³⁺, Ca²⁺) or sorbed on soil clay reactive surfaces. Also, their persistence in rhizosphere soil is very low because they can be used as carbon sources by soil microorganisms (Jones et al., 2003). Competent rhizosphere microorganisms that are capable of production of organic acids or anions can play an important role in the management of Pi deficient and high Pi-sorbing soils.

Phosphate Solubilizing Microorganisms (PSM)

Many soil microorganisms can solubilize inorganic soil P compounds, reversing the process of Pi fixation (Gyaneshwar et al., 2002; Rao, 1992). Soil bacteria of the genus Pseudomonas, Enterobacter, and Bacillus are particularly active as Pi solubilizers (Canbolat et al., 2006; Pandey et al., 2006; Xavier and Germida, 2003; Barea et al., 1975; Kim et al., 1998). Soil fungi especially those of the genus Penicillium and Aspergillus also have been demonstrated to be effective PSM (Reddy et al., 2002; Whitelaw, 2000). Although Pi solubilizing bacteria have received greater attention, Kucey (1983) indicated that Pi solubilizing fungi are more effective in solubilizing P compounds. While subcultures of P solubilizing bacteria can lose their ability to solubilize P,
subcultures of Pi solubilizing fungi can maintain this ability. Kucey and Leggett (1989) found in Mollisols of Canada that 0.5 % and 0.1 % of the total population of bacteria and fungi, respectively, exhibited the ability to solubilize insoluble Pi compounds.

**Mechanisms of microbial P solubilization**

Several mechanisms have been proposed to explain the microbial solubilization of P compounds. The mechanisms consist of: (i) release of inorganic and organic acids produced during organic residue decomposition (Hameeda et al., 2006; Bar-Yosef et al., 1999); (ii) excretion of protons due to \( \text{NH}_4^+ \) assimilation by microorganisms (Kucey, 1983; Roos and Luckner, 1984; Abd-Alla, 1994; Illmer et al., 1995a; Asea et al., 1988; Whitelaw, 2000); (iii) formation of complexes between organic acids/anions with cations (\( \text{Al}^{3+}, \text{Fe}^{3+}, \text{Ca}^{2+} \)) (Welch et al., 2002). *Nitrosomonas* and *Thiobacillus* species can also dissolve Pi compounds by producing nitric and sulfuric acid (Azam and Memon, 1996). Equally, P compounds may be solubilized by carbonic acid formed as a result of organic matter decomposition (Memon, 1996). Increase in soil P availability may be caused by several reactions involving microorganisms that produce organic acids and humic substances (Stevenson, 1986). Presumably, these substances can replace or compete with Pi ions for sorption sites. In addition, Lopez-Bucio et al. (2007) reported that *Bacillus megatherium* (a known PSM) promoted plant growth and stimulated root branching of *Arabidopsis thaliana*. 
Kim et al. (1997) found that the production of acidity was a major mechanism in the solubilization of hydroxyapatite by Enterobacter agglomerans under in vitro conditions. For comparison, Kim and coworkers employed citric acid, oxalic acid, lactic acid, and HCl at the same pH produced by E. agglomerans. They found that at pH 4.0-4.1 (and a shaking time of 48-50 hours) there were no significant differences among P solubilization produced by this bacterium and that produced by citric acid, oxalic, and HCl. However, lactic acid exhibited a lower (P<0.05) capacity for solubilizing hydroxyapatite.

Illmer et al. (1995b) reached the same conclusion studying AlPO₄ solubilization by several PSM. For instance, Aspergillus niger produced organic acids but other PSM species did not produce detectable amounts of the organic acids. Under in vitro conditions, the pH of the growth medium decreased as a result of acid production by PSM. Osorio and Habte (2001) found an inverse relation between culture medium pH and P released from RP by PSM isolated from Hawaiian soils. The microbial solubilization of RP was associated with the ability of microorganisms to depress the pH of the growth medium.

Some of the organic acids (or their respective anions) commonly associated with microbial solubilization of Pi are gluconic acid (Di-Simine et al., 1998; Bar-Yosef et al., 1999), oxalic acid, citric acid (Kim et al., 1997; Kucey and Leggett, 1989), lactic acid, tartaric acid, and aspartic acid (Venkateswardu et al., 1984). These acids are products of microbial metabolism, in some cases by oxidative respiration or by fermentation of carbonaceous substrates (e.g., glucose) (Troleve et al. 2003; Jones et al., 2003; Gyaneshwar et al., 2002; Atlas and
Bartha, 1997; Prescott et al., 1999; Mathews et al., 1999). The reactions of P solubilization are believed to occur in the rhizosphere where carbonaceous compounds are released and where solubilized Pi may be taken up by the root or mycorrhizal system. Whipps (1984) showed that in wheat plants up to 33-40 % of the total carbon fixed by photosynthesis could be excreted into the rhizosphere; Amos and Walters (2006) estimated a value of 29% for maize. Many rhizosphere microorganisms are heterotrophs and might use these carbonaceous substrates to produce organic acids. Recently, Hameeda et al. (2006) found that the type of carbon source affected the effectiveness of RP-solubilizing bacteria. For Serratia marcescens and Pseudomonas sp. the more favorable carbon source for RP solubilization followed the order, glucose > galactose > xylose > mannose = maltose > cellobiose > arabinose. No solubilization of RP was detected with the last carbon source of this series. The above bacteria were capable of solubilizing RP using different kinds of composted crop residues (rice, pigeon pea, and a grass). Reyes et al. (2006) also compared the effect of the carbon source on RP solubilization and found that Penicillium sp. and Azotobacter sp. were more effective if the medium contained sucrose than dextrose.

When PSM were inoculated in neutral or alkaline soils, the production of acids decreased rhizosphere pH, favoring the solubility of soil native calcium-phosphate and added RP (Kim et al., 1998a). These results have commonly been found in temperate-zone soils of Europe and North America (Kucey, 1983, 1987, 1988; Kucey and Leggett, 1989) and other countries, e.g., Egypt (Omar, 1998) where calcareous soils are abundant. Note in the following reactions
that if $H^+$ activity increases, the calcium-phosphates are solubilized as indicated. Moreover, if $Ca^{2+}$ is chelated by organic anions the dissolution of both solids is favored. Welch et al. (2002) found that organic acid/anions produced by microorganisms were capable of dissolving apatite by forming a complex with Ca either in solution and/or directly at the mineral surface.

\[
\text{(Dicalcium phosphate) } CaHPO_4 + H^+ \leftrightarrow H_2PO_4^- + Ca^{2+} \quad K=10^{0.3} \quad [2]
\]
\[
\text{(Hydroxyapatite) } Ca_9(PO_4)_3OH + 7H^+ \leftrightarrow 3H_2PO_4^- + 5Ca^{2+} + H_2O \quad K=10^{14.6} \quad [3]
\]
\[
\text{(Fluorapatite) } Ca_8(PO_4)_2F + 6H^+ \leftrightarrow 3H_2PO_4^- + 5Ca^{2+} + F^- \quad K=10^{0.2} \quad [4]
\]

On the other hand, in highly weathered acidic soils $P$ solubility is controlled by other compounds, mainly Variscite ($AlPO_4.2H_2O$) and Strengite ($FePO_4.2H_2O$) (Bohn et al., 1985). In this case, the decrease in soil pH may not increase the dissolution of Strengite and Variscite (Lindsay, 2001) and $P$ is not released. This happens because Gibbsite [$Al(OH)_3$] and Kaolinite ([$Al_2Si_2O_5(OH)_4$]) can control the solubility of $Al$ in these soils, while Goethite ($FeOOH$), Hematite ($Fe_2O_3$) and soil-$Fe(OH)_3$ control the solubility of $Fe$. Reductions in the soil pH would release more $Al$ and $Fe$ ions, which would precipitate $P$ ions. Thus, if soil pH decreases $P$ solubility will decrease as shown in reactions [5] to [9] (Lindsay, 2001).

\[
AlPO_4.2H_2O + H_2O \leftrightarrow H_2PO_4^- + Al(OH)_3 + H^+ \quad K=10^{-10.5}[5]
\]
\[
AlPO_4.2H_2O + SiO_2 (quartz) + 0.5H_2O \leftrightarrow H_2PO_4^- + 0.5Al_2Si_2O_5(OH)_4 + H^+ \quad K=10^{-9.2}[6]
\]
\[
FePO_4.2H_2O + H_2O \leftrightarrow H_2PO_4^- + Fe(OH)_3 + H^+ \quad K=10^{-8.6}[7]
\]
\[
FePO_4.2H_2O \leftrightarrow H_2PO_4^- + FeOOH + H^+ \quad K=10^{-8.8}[8]
\]
\[
FePO_4.2H_2O \leftrightarrow H_2PO_4^- + 0.5 H_2O + 0.5Fe_2O_3 + H^+ \quad K=10^{-6.6}[9]
\]

The microbial solubilization of soil $P$ seems to be associated with the presence of calcium-phosphates. In fact, most of the research on microbial
solubilization has been done with solubilizers of RP (mixture of hydroxy- and fluor-apatites) (Kim et al., 1998b; Osorio and Habte, 2001) or tricalcium phosphate (Ca$_3$PO$_4$) (Pikovskaia, 1948; Sperber, 1957, 1958; Louw and Webley, 1959; Agnihorti, 1970; Paul and Rao, 1971; Banik and Dey, 1981abc). However, researchers on PSM no longer accept isolation of PSM using culture medium with Ca$_3$PO$_4$ because it can supply free Pi. Some authors reported that in vitro microbial solubilization not only occurred with calcium-phosphate but also with Al and Fe phosphates. However, the solubilization was higher with calcium phosphates (Rose, 1957; Banik and Dey, 1983; Illmer et al., 1995a).

Solubilization of Al and Fe phosphates can be easily observed under in vitro conditions where no soil minerals interfere with Pi solubility [10,11]. Illmer et al. (1995b) found that A. niger, Penicillium simplicissium, Pseudomonas aurantiogriseum, and Pseudomonas sp. were effective in solubilizing AlPO$_4$ under in vitro conditions via organic acid production or proton excretion due to NH$_4^+$ assimilation. Aluminum and Fe ions in solution may be chelated by organic anions (e.g., oxalate and citrate) (Bolan et al., 1994), favoring the dissolution of Al and Fe phosphates. Whether organic acids released by PSM can solubilize Pi from Al- and Fe-phosphate under acidic soil conditions must be studied.

$$\text{AlPO}_4.2\text{H}_2\text{O} + 2\text{H}^+ \leftrightarrow \text{Al}^{3+} + 2\text{H}_2\text{PO}_4^- + 2\text{H}_2\text{O} \quad K=10^{-2.5} \quad [10]$$

$$\text{FePO}_4.2\text{H}_2\text{O} + 2\text{H}^+ \leftrightarrow \text{Fe}^{3+} + 2\text{H}_2\text{PO}_4^- + 2\text{H}_2\text{O} \quad K=10^{-6.8} \quad [11]$$

On the other hand, organic anions produced by PSM also can compete with phosphate for Pi sorption sites on the surface of soil minerals. He and Zhu (1997,
1998) suggested that Pi sorbed on the surfaces of some minerals was displaced when a culture medium was inoculated with soil samples containing microorganisms (unidentified) that presumably excreted organic acids.

Effects of PSM on plant Pi uptake

During the 1950-60's, inoculation with *Bacillus megatherium* var. *phosphaticum* (phosphobacterin) in Russian soils (mainly Mollisols) was the best-known use of PSM (Stevenson, 1986; Kucey and Leggett, 1989). The mechanisms of Pi solubilization were not fully understood, but the mineralization of organic P was proposed as the major mechanism. Trials carried out in many locations demonstrated little consistency in plant response; apparently other factors such as liming and/or organic material addition affected the effectiveness of phosphobacterin. The lack of response to phosphobacterin in many locations, pointed to a possible intensified organic matter decomposition, and the poor understanding of the mechanisms of P solubilization carried out by this microorganism discouraged its use. Since then, the research on microbial solubilization of P was oriented toward the study of the dissolution of inorganic P compounds (Kucey and Leggett, 1989).

Inoculation with PSM has produced positive results on crop yield, plant growth, and Pi uptake of several plant species (Kucey and Legget, 1989) (Table 2.3). For instance, some effective Pi solubilizing fungi are *Aspergillus niger* (Rosendahl, 1942; Omar, 1998) and *Penicillium bilaji* (Kucey, 1983, 1987, 1988; Asea *et al.*, 1988; Kucey and Legget, 1989; Gleddie, 1993). Kucey and Leggett
(1989) and Whitelaw (2000) reviewed the literature in the subject matter and showed several reports of increase in plant growth and P uptake.

Salih et al. (1989) inoculated a calcareous soil (Typic Torrifluvent, pH 8.2) with three PSM: *Penicillium* sp. and two *Aspergillus* sp. (1 and 2) (Table 2.3). They observed a slight increase in the soil available Pi. When the soil was fertilized with RP, *Penicillium* sp. increased plant Pi uptake by 17%. *Aspergillus* sp.-1 and sp.-2 increased plant Pi uptake by 13% and 18%, respectively. When the soil received triple-superphosphate, the increases in plant Pi uptake were lower, the corresponding values being 8, 3.5, and 3.6%, respectively.

Kucey (1988) inoculated a Mollisol (pH 7.7) of Canada with *Penicillium biloji,* the soil was either fertilized or unfertilized with RP (Table 2.3). Wheat Pi uptake increased only by 4% with RP alone, 14% with the PSM inoculation alone, and 12% with a combination of RP and *P. biloji.* In a similar experiment on a Mollisol (pH 8), Asea et al. (1988) found that the addition of RP increased plant Pi uptake by only 2%; *P. biloji* inoculation alone significantly increased it by 26%, and the combination of both RP and *P. biloji* by 28% (Table 2.3). The increase was even higher (35%) when an AMF was co-inoculated with *P. biloji* in presence of RP.

Several authors have reported that soil microorganisms can increase soil P availability (Table 2.4). Although in some soils this increase does not have practical implications, it has been important in other soils (Marschner et al., 2006). For instance, the increases in soil P availability reported by Goenadi (1995) and Goenadi et al. (1995) in two acidic Ultisols fertilized with RP were significant.
Table 2.3. Effect of PSM inoculation on plant Pi uptake of mycorrhiza-free and mycorrhized plants grown on temperate soils

<table>
<thead>
<tr>
<th>Soil type/plant</th>
<th>P added</th>
<th>PSM</th>
<th>Increase of plant P uptake due to PSM inoculation (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>- AMF</td>
<td>+ AMF</td>
</tr>
<tr>
<td>Mollisol pH 7.7</td>
<td>None</td>
<td><em>Penicillium bila</em></td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>Plant: wheat</td>
<td>RP</td>
<td><em>Penicillium bila</em></td>
<td>47</td>
<td></td>
</tr>
<tr>
<td>Mollisol pH 8.0</td>
<td>None</td>
<td><em>Penicillium bila</em></td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td><em>Penicillium bila</em></td>
<td>62</td>
<td></td>
</tr>
<tr>
<td>Mollisol pH &gt;7.0</td>
<td>None</td>
<td><em>Penicillium bila</em></td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Plant: wheat</td>
<td>MAP</td>
<td><em>Penicillium bila</em></td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>Calcareous</td>
<td>RP</td>
<td><em>Penicillium sp.</em></td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>TypicTorffluent pH 8.2</td>
<td>RP</td>
<td><em>Aspergillus foetidus</em></td>
<td>19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TSP</td>
<td><em>Penicillium sp.</em></td>
<td>8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TSP</td>
<td><em>Aspergillus foetidus</em></td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Mollisol pH 7.6</td>
<td>None</td>
<td><em>Penicillium bila</em></td>
<td>39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TSP</td>
<td></td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TSP</td>
<td></td>
<td>18</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TSP</td>
<td></td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Hapludoll pH 6.2</td>
<td>None</td>
<td><em>Aspergillus awamori</em></td>
<td>58 (grain)</td>
<td>13 (grain)</td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcareous mixed with sand, pH 6.7</td>
<td>RP</td>
<td><em>Azospirillium</em></td>
<td>33</td>
<td>0-33</td>
</tr>
<tr>
<td>Plant: kudzu</td>
<td></td>
<td><em>Penicillium</em></td>
<td>33</td>
<td>0-44</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Unidentified</em></td>
<td>33</td>
<td>22-38</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pseudomonas</em></td>
<td>33</td>
<td>33-50</td>
</tr>
<tr>
<td>Calcareous, pH 7.5</td>
<td>None</td>
<td><em>Aspergillus</em></td>
<td>30</td>
<td>39</td>
</tr>
<tr>
<td>Plant: wheat</td>
<td></td>
<td><em>Penicillium</em></td>
<td>24</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Aspergillus</em></td>
<td>78</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Aspergillus+</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vertic Eplaualf mixed with sand and vermiculite, pH 5.9</td>
<td>RP</td>
<td><em>Enterobacter</em></td>
<td>54 (35 d)*</td>
<td>124</td>
</tr>
<tr>
<td>Plant: Tomato</td>
<td></td>
<td><em>eggiformens</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sandy soil pH 7.6</td>
<td>None</td>
<td><em>Bacillus</em></td>
<td>26</td>
<td>52</td>
</tr>
<tr>
<td>Plant: wheat</td>
<td></td>
<td><em>Cladosporium</em></td>
<td>47</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Bacillus+</em></td>
<td>73</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cladosporium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>RP</td>
<td><em>Bacillus</em></td>
<td>-</td>
<td>248</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Cladosporium</em></td>
<td>-</td>
<td>301</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Bacillus+</em></td>
<td>51</td>
<td>344</td>
</tr>
</tbody>
</table>

* Days after planting.
Table 2.4. Enhancement of the soil P_{i} available by PSM.

<table>
<thead>
<tr>
<th>PSM</th>
<th>Soil</th>
<th>P source</th>
<th>SAP Increase (mg/kg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Aspergillus</em> niger</td>
<td>Fluvaquent, pH 5.4 SAP: 9 mg/kg</td>
<td>Nil P fertilizer</td>
<td>+2</td>
<td>Banik and Dey, 1981b</td>
</tr>
<tr>
<td><em>A. fumigatus</em></td>
<td>Fluvaquent, pH 7.4 SAP: 7 mg/kg</td>
<td>RP and farmyard manure</td>
<td>+3</td>
<td>Banik and Dey, 1982</td>
</tr>
<tr>
<td><em>Penicillium</em> bitai</td>
<td>Molisol, pH 7.7 SAP: 4 mg/kg</td>
<td>RP</td>
<td>+2</td>
<td>Kucey, 1988</td>
</tr>
<tr>
<td><em>Penicillium</em> sp.</td>
<td>Torrifluvent, pH 8.2 SAP: 4 mg/kg</td>
<td>RP</td>
<td>+2</td>
<td>Salih <em>et al.</em>, 1989</td>
</tr>
<tr>
<td><em>A. foetidus</em></td>
<td></td>
<td>TSP</td>
<td>+9</td>
<td></td>
</tr>
<tr>
<td><em>A. awamori</em></td>
<td>Hapludoll, pH 6.2 SAP: 27 mg/kg</td>
<td>Nil P fertilizer</td>
<td>+8</td>
<td>Singh and Singh, 1983</td>
</tr>
<tr>
<td><em>Aspergillus</em> sp.</td>
<td>Ultisol, pH 3.9 SAP: 37 mg/kg</td>
<td>RP</td>
<td>+24</td>
<td>Goenadi <em>et al.</em>, 1995</td>
</tr>
<tr>
<td><em>Aspergillus</em> sp.</td>
<td>Ultisol, SAP: 0.5 mg/kg</td>
<td>Nil P fertilizer</td>
<td>+14</td>
<td>Goenadi, 1995</td>
</tr>
</tbody>
</table>

The effectiveness of PSM to enhance plant P_{i} uptake has been questioned by some authors (Tinker, 1980; Bolan, 1991) because, (i) organic substances required for these microorganisms are scarce in non-rhizospheric sites; (ii) antagonism and competition by other microorganisms in the rhizosphere can reduce the effectiveness of PSM; and (iii) low translocation of solubilized P_{i} through the soil because it can be re-fixed by soil components. This latter point is more important in soils with a high P_{i} fixation capacity as discussed already.
Dual Inoculation of AMF and PSM

Co-inoculation with PSM and AMF of soils with high Pi fixation capacity may overcome the limitations mentioned on the effectiveness of PSM to enhance plant Pi uptake. First, mycorrhizal plants can release higher amounts of carbonaceous substances into the rhizosphere (Rambelli, 1973; Linderman, 1988) than non-mycorrhizal plants. Phosphate solubilizing microorganisms can use these carbon-substrates for their metabolic processes which are responsible for organic acid production in the rhizosphere and/or proton excretion (Azcon and Barea, 1996). Second, the extensive mycorrhizal hyphae network formed around roots can efficiently take up Pi released by PSM, thus minimizing its re-fixation. As long as PSM grow in the rhizosphere (or mycorrhizosphere), there is a great opportunity to satisfy their carbon requirement and deliver Pi into the soil solution.

Synergistic effects have been found in sunflower (Helianthus annuus) with the triple inoculation of two PSM (Azotobacter chroococcum and Penicillium glaucum) and the AMF G. fasciculatum (Gururaj and Mallikarjunah, 1995). Similar effects were found in cotton with the inoculation of Pseudomonas striata and Azospirillum sp. (PSM) and G. fasciculatum (AMF) (Prathiba et al., 1995). In rice, favorable effects were also reported with P. striata (PSM) and Bacillus polymyxa (PSM) and G. fasciculatum (AMF) (Mohod et al., 1991). In chilli (Capsicum annuum) synergistic effects were reported with two AMF, G. fasciculatum or G. macrocarpum, and a PSM P. striata (Sreenivasa and
Krishnaraj, 1992). In tomato beneficial results were found with *E. agglomerans* and *G. etunicatum* (Kim et al., 1998a) (Table 2.5). Moreover, positive results have been obtained in wheat with several combinations that include *P. striata* (PSM) and *G. fasciculatum* (AMF), *P. putida*, *P. aeruginosa* and *P. fluorescens* (PSM) with *G. clarum* (AMF), *P. striata* and *Agrobacterium radiobacter* (PSM) combined with two AMF, *G. fasciculatum* and *Gigaspora margarita* (Gaur et al., 1990).

Kopler et al. (1988) indicated that more legume nodulation was obtained with concurrent inoculation of *Rhizobium* and *Pseudomonas* spp. (PSM). Sturz et al. (1997) found that nodulation by *Rhizobium leguminosarum* b.v. *trifolii* of red clover (*Trifolium pratense*) was promoted when it was co-inoculated with the PSM *Bacillus insolitus*, *B. brevis* or *Agrobacterium rhizogenes*. Similar results were obtained with the inoculation of *G. mosseae* (AMF) and *Azorhizobium caulinodans* (PSM) in *Sesbania rostrata* (Rahman and Parsons, 1997). In soybean, the combination of *Bradyrhizobium japonicum* (N$_2$ fixer) with *P. fluorescens* (PSM) and *G. mosseae* (AMF) has given equally good results (Shabayey et al., 1996). Such results are likely to be due to a higher plant Pi uptake promoted by the combined action of PSM and AMF, which may satisfy the high Pi requirements of the N$_2$ fixing process (Azcon and Barea, 1996; Young et al., 1990).

Peix et al. (2001) found that the N$_2$-fixing bacterium *Mesorhizobium mediterraneum* was able to solubilize Ca$_2$(PO$_4$)$_2$ under *in vitro* and soil conditions. Inoculation with *M. mediterraneum* of seeds of the legume plant
Table 2.5. Effects of *E. agglomerans* (PSM) and *G. etunicatum* (AMF) inoculation on tomato plant growth and Pi uptake 75 days after inoculation.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Shoot dry weight (g/plant)</th>
<th>Root dry weight (g/plant)</th>
<th>Shoot P content (mg/plant)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>42.2 (100)*</td>
<td>4.3 (100)</td>
<td>116.6 (100)</td>
</tr>
<tr>
<td>PSM</td>
<td>48.5 (115)</td>
<td>5.1 (118)</td>
<td>125.3 (107)</td>
</tr>
<tr>
<td>AMF</td>
<td>47.6 (113)</td>
<td>5.6 (130)</td>
<td>120.9 (104)</td>
</tr>
<tr>
<td>PSM+AMF</td>
<td>54.6 (129)</td>
<td>6.8 (158)</td>
<td>134.4 (115)</td>
</tr>
<tr>
<td>LSD (P≤0.05)</td>
<td>1.96</td>
<td>0.5</td>
<td>9.8</td>
</tr>
</tbody>
</table>

*In parenthesis percentage of the control. Source: Kim et al., 1998a.

chickpea (*Cicer arietinum*) and barley (*Hordeum vulgare*) planted in a Calcic Rhodoxeralf significantly increased plant growth and total N and Pi content in both plants. Further increases were observed when *M. mediterraneum* and Ca₃PO₄ were concurrently applied. Benefits of this bacterium were not only due to the symbiotic N₂ fixation when associated with chickpea but also to the enhancement of plant Pi uptake of both plants.

Apparently, there is a certain degree of specificity among PSM, AMF, and P source. Toro et al. (1996) studied the combined effect of AMF (*Glomus* spp.) and eight PSM (bacteria) on plant growth and Pi nutrition of a tropical legume, kudzu (*Pueraria phaseoloides*). The PSM were isolated from an Oxisol and were characterized by their ability to solubilize RP, Al- and Fe-P compounds. In general, PSM inoculation of the kudzu-*Rhizobium*-AMF association increased plant growth, yield, and nutritional status. However, the phenomenon was not observed in all combinations of AMF-PSM. For instance, the three PSM *Azospirillum* sp., *Bacillus* sp., and *Enterobacter* sp. had a higher effect when they
were co-inoculated with *G. Mosseae*. In contrast, *Pseudomonas* sp. and an unidentified isolate had a better performance when they were combined with *G. fasciculatum*. On the other hand, Fe-P solublizers were more effective if they were alone, while Al-P and RP solublizers performed better when they were concurrently inoculated with AMF. Reasons for these differences may be due to interactions between the microorganisms. For instance, some of these PSM could be more effective in stimulating a rapid mycorrhizal colonization, and enhancing the length, distribution, and/or survival of external fungal mycelium. Mycorrhizal fungi might differ in the amount and type of hyphal exudates released into the mycorrhizosphere. In addition, a high capacity to solubilize Pi might stimulate plant growth and favor mycorrhizal activity.

Kucey (1987) inoculated a Mollisol (pH 7.2) of Canada with *P. bilaji*, in which either mycorrhizal or non-mycorrhizal wheat or beans were grown. In the case of wheat, mycorrhizal inoculation alone increased significant Pi uptake (30%), but *P. bilaji* alone did not do so. However, *P. bilaji* increased Pi uptake of mycorrhizal wheat by 10% in the unfertilized soil, but not in the soil fertilized with RP. In the case of bean, mycorrhizal inoculation alone did not increase plant Pi uptake, but *P. bilaji* alone was able to significantly increase it by 31%. Dual inoculation did not increase Pi uptake beyond the level obtained with *P. bilaji*. In other words, there was not synergism between both microorganisms on bean Pi uptake in unfertilized and RP-fertilized soil. Mollisols usually exhibit a low Pi fixation capacity. For this reason, it is not surprising that inoculation with this PSM alone increased bean Pi uptake. Differences between wheat and bean could be due to
different types and amounts of root exudates that would stimulate acid production in the rhizosphere by PSM.

Barea et al. (1975) found that inoculation with PSM (Pseudomonas + Agrobacterium) did not increase plant Pi uptake of mycorrhiza-free and mycorrhizal lavender (Lavandula spica L. cv. Vera) and maize (Zea mays L.) plants grown in an unfertilized red mediterranean soil (pH 7.5, 0.01 M CaCl₂-P: 0.021 mg/L). In contrast, PSM inoculation significantly increased plant Pi uptake of mycorrhizal maize (24%) and mycorrhiza-free lavender (42%), but not of mycorrhizal lavender in a grey-meridional soil (pH 7.6, 0.01 M CaCl₂: 0.008 mg/L). Maize plants achieved 75 and 95% of their maximum yield at 0.008 and 0.025 mg P/L (Fox, 1979), indicating that maize plants (and perhaps lavender too) satisfied most of their P requirements in the red mediterranean soil.

Moreover, estimation of mycorrhizal dependency (MD) of these plants at 0.02 mg/L (0.01 M CaCl₂), as proposed by Habte and Manjunath (1991), indicated that both plants can be classified as marginally dependent on mycorrhizal association (MD for lavender = 5.6%; MD for Maize=15%). Similar results were found by Miyasaka and Habte (2001), who affirmed that maize has a marginal MD. Apparently in the grey meridional soil, lavender satisfied its P requirement via mycorrhizal association and for this reason it did not respond to PSM inoculation. By contrast, mycorrhiza-free lavender required more Pi from the soil solution and it was supplied by microbial Pi solubilization by both bacteria.
Microbial Pi solubilization in temperate vs. tropical soils

Currently, *Penicillium bilaji* is commercially available in North America under the name of Provide™, which has been successfully tested to enhance plant Pi uptake of some crop plants (Whitelaw, 2000). However, the tests have been conducted in Mollisols, which exhibited low Pi fixation capacity. In contrast, little research on PSM has been conducted in highly weathered and volcanic ash soils of the tropics with high Pi fixation capacity. Toro *et al.* (1996) isolated various effective PSM from an Oxisol of Venezuela. However, they were not tested in this soil from which they were originated but in a calcareous soil of Spain in which they were able to improve plant growth and Pi uptake of tropical kudzu (*Pueraria phaseoloides*).

Whitelaw *et al.* (1997) inoculated an acidic Pi-deficient soil of Australia (pH 4.6) with *Penicillium radicum* in combination with several levels of KH₂PO₄ (P: 0-20 kg/ha). The inoculation with this PSF increased wheat Pi uptake by 8% in the unfertilized soil. When the fungus was inoculated in combination with Pi fertilization, plant Pi increased between 2-28% and the increase was highest when the rate of added P was 15 kg of P/ha.

Young *et al.* (1990) found that inoculation with either PSM or AMF significantly increased peanut (*Arachis hypogea*) production in two subtropical-tropical acidic soils of Taiwan. Inoculation with either AMF or PSM in unfertilized soils was as effective as the addition of RP alone (Table 2.6). Inoculation with AMF or PSM of RP-fertilized soils did not increase peanut yield above that
obtained with AMF or PSM inoculation in unfertilized soils. Unfortunately, dual inoculation of AMF and PSM was not evaluated. Note that PSM inoculation alone increased peanut yield by 73% in the less acidic soil (Yuanchang soil), the increase was only 20% in the strongly acidic soil (Hualain soil).

In addition, Young et al. (1990) found that the responses to single or mixed inoculations with PSM and/or AMF had variable effects on plant growth of leucaena grown in three soils of Taiwan. Inoculation with PSM was not as effective as AMF inoculation in enhancing plant growth in the soil with the lowest available Pi level (Hinshe soil) (Table 2.7). In the Wunfun soil (also with a low soil available Pi level), PSM inoculation was ineffective to increase plant growth unlike AMF. In the alkaline soil containing the highest soil available Pi (presumably rich in calcium-phosphates), PSM inoculation alone significantly increased plant growth (40%) above the AMF inoculation effect, which did not increase growth.

Effectiveness of PSM inoculation alone to enhance plant Pi uptake in subtropical and tropical acidic soils is relatively low and variable. The increases recorded were 8% (Whitelaw et al., 1997), 13% (Osorio and Habte, 2001), and 24-25% (Young et al., 1990) compared with those reported in less weathered soils (mostly Mollisols) of temperate zone, where soil Pi fixation capacity is low. By contrast, effectiveness of PSM inoculation to enhance plant Pi uptake of mycorrhizal plants grown in tropical or subtropical soils can be relatively higher compared to data reported in temperate soils (Table 2.7).
Mycorrhizal association in combination with PSM are often needed to obtain improvements in plant P uptake in highly weathered soils, in contrast to results obtained in less weathered soils. In these less weathered soils that normally exhibit low soil P sorption, the inoculation of PSM alone has been enough to increase plant P uptake of non-mycorrhizal plants (Peix et al., 2001; Omar, 1998; Kucey, 1983, 1987, 1988; Asea et al., 1988; Kucey and Legget, 1989; Gleedle, 1993). Most of the soils used by these authors were Mollisols, calcareous soils, or sandy soils, which are characterized by a low P sorption capacity and relatively high soil Ca-Pi content (Cross and Schlesinger, 1995). Therefore, the freshly released Pi by PSM can remain longer in the soil solution until its absorption by the roots.

For instance, Toro et al. (1998) found that the PSM Enterobacter sp. alone was as effective as the mycorrhizal fungus G. mosseae alone in increasing by two-fold the plant P uptake of alfalfa grown in a calcareous soil of Spain. Duponnois et al. (2006) found that the inoculation alone with the fungus Arthrobotrys oligospora increased the P uptake and shoot dry weight of Acacia holoserica grown in a sandy soil of Senegal by 56% and 46%. The increase in plant P uptake and growth were even higher when RP was added with the PSM (74 and 103%, respectively). The addition of RP alone did not increase significantly plant P uptake and growth. Similar results were reported by Wakelin et al. (2004a,b) on wheat with the inoculation of Penicillium radicum in some sandy soils of Australia with neutral to alkaline soil reactivity. In these soils, Wakelin and co-workers observed increases in plant growth between 34 and
76%. Even higher was the effect of *Penicillium thomii* on plant P uptake of mint (*Mentha piperita*) grown in a soilless medium (vermiculite-perlite) fertilized with RP (Cabello *et al.*, 2005). In that experiment the inoculation of the fungus increased plant P content by more than 3-fold compared to the uninoculated plants and unfertilized control. The RP alone was ineffective. The impressive increase in plant P uptake is understandable given the very-low P sorption on this kind of substrates.

Table 2.6. Effect of AMF, PSM, and RP on peanut yield (kg/ha) in two soils of Taiwan.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hualain soil (pH 4.2)</th>
<th>Yuanchang soil (pH 5.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1875 b (100)</td>
<td>3667 c (100)</td>
</tr>
<tr>
<td>RP (660 kg/ha)</td>
<td>2250 a (120)</td>
<td>6167 a (169)</td>
</tr>
<tr>
<td>AMF</td>
<td>2350 a (125)</td>
<td>6208 a (140)</td>
</tr>
<tr>
<td>PSM</td>
<td>2259 a (120)</td>
<td>6333 a (173)</td>
</tr>
<tr>
<td>AMF + RP</td>
<td>2367 a (126)</td>
<td>5125 b (140)</td>
</tr>
<tr>
<td>PSM + RP</td>
<td>2275 a (121)</td>
<td>6083 ab (166)</td>
</tr>
</tbody>
</table>

Mean separation by Duncan's multiple range test (*P* ≤ 0.05). In parenthesis percentage with respect to the control. Source: Young *et al.* (1990).

Table 2.7. Effect of AMF and PSM on growth (g/pot) of leucaena grown in three soils of Taiwan.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hinshe (pH 5.0; P: 2 mg/kg)</th>
<th>Wufun (pH 5.5; P: 3 mg/kg)</th>
<th>Taitung (pH 7.8; P: 95 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.4 c (100)</td>
<td>13.7 b (100)</td>
<td>22.0 b (100)</td>
</tr>
<tr>
<td>PSM</td>
<td>10.4 b (124)</td>
<td>12.9 b (94)</td>
<td>30.8 a (140)</td>
</tr>
<tr>
<td>AMF</td>
<td>13.1 a (156)</td>
<td>27.3 a (199)</td>
<td>26.8 b (122)</td>
</tr>
<tr>
<td>PSM+AMF</td>
<td>12.8 a (152)</td>
<td>26.0 a (180)</td>
<td>23.6 b (107)</td>
</tr>
</tbody>
</table>

Mean separation by Duncan’s multiple range test (*P* ≤ 0.05). In parenthesis percentage of the control. Source: Young *et al.* (1990).
LITERATURE CITED


Toro, M., R. Azcon, and J. M. Barea. 1998. The use of isotopic dilution techniques to evaluate the interactive effects of rhizobium genotypes,


CHAPTER THREE

SYNERGISTIC EFFECT OF A PHOSPHATE SOLUBILIZING FUNGUS AND AN ARBUSCULAR MYCORRHIZAL FUNGUS ON PLANT GROWTH AND PHOSPHATE UPTAKE OF LEUCAENA GROWN IN AN OXISOL

ABSTRACT

An investigation was carried out to assess the role that Pii solubilizing microorganisms (PSM) play in the P nutrition of mycorrhizal and mycorrhiza-free Leucaena leucocephala (Lam.). Soil microorganisms able to solubilize rock phosphate (RP) were isolated from the rhizosphere of L. Leucocephala naturally growing in three different soils of Hawaii. The isolates were screened for their ability to solubilize RP in culture medium. The highest activity was observed with one of the fungal isolates, which was identified as Mortierella sp. It was multiplied and further evaluated with or without the mycorrhizal fungus Glomus aggregatum in a highly weathered soil for its effectiveness to enhance Pi uptake and growth of L. leucocephala. Phosphorus (P) status of L. leucocephala pinnules that were monitored as a function of time revealed that plants colonized by both microorganisms had the highest P content followed by plants inoculated with the mycorrhizal fungus alone. Inoculation of soil with Mortierella sp. alone did not influence P content of plants measured at the time of harvest. However, Mortierella sp. increased the P content of mycorrhizal plants by 13 % in the unfertilized soil and by 73 % in the soil fertilized with RP. Shoot dry weight measurements showed that Mortierella sp. stimulated growth of non-mycorrhizal by 22 %, while it stimulated the

growth of mycorrhizal plants by 29%, regardless of Pi fertilization. The results suggest the existence of synergistic interaction between Pi solubilizing microorganisms and mycorrhizal fungi, although the degree of synergism was more pronounced in terms of Pi uptake than in terms of growth.

INTRODUCTION

Deficiency of Pi and prevalence of soils with high P fixing capacity are serious problems to food production in the tropics (Sanchez and Logan, 1992). The problems can be alleviated through the application of large quantities of Pi fertilizers, but this practice is not cost-effective. One alternative of increasing Pi supply to plants in these soils is to inoculate them with arbuscular mycorrhizal fungi (AMF) (Marschner, 1997). The external hyphae of mycorrhizal fungi are much finer and far more extensive than root hairs, helping the plant explore more of the available soil volume than would be possible with the unaided plant (Barber 1995).

Some soil microorganisms can solubilize insoluble forms of Pi (Di-Simine et al., 1998; Toro et al., 1996; Azcon and Barea, 1996). Soil bacteria particularly the genera Pseudomonas, Enterobacter and Bacillus and some fungi especially Penicillium and Aspergillus are particularly important in this regard (Rao 1992). The mechanism of Pi solubilization appears to be related to the release of organic acids (Kim et al. 1997). However, Bolan (1991) points out that some
factors such as low availability of C in non-rhizospheric sites, antagonism by other microorganisms, and re-immobilization of solubilized Pi by soil are constraints to the use of Pi solubilizing microorganisms (PSM) in practical agriculture.

These obstacles could in part be overcome through a judicious combination of AMF and PSM because of the efficiency of AMF to take up Pi in solution and translocate it to roots (Azcon and Barea, 1996). Although there are a few studies that illustrate the validity of the approach (Kim et al., 1998; Gaur et al., 1990; Sturz et al., 1997; Singh and Kapoor, 1999; Toro et al., 1996), its feasibility in the Pi deficient and high Pi-fixing soils of the tropics is poorly understood.

The objectives of the current investigation were (i) to screen selected soils of the tropics for PSM and (ii) to determine the effectiveness of the best isolates alone or in combination with a highly effective arbuscular mycorrhizal fungus on Pi uptake and growth of *Leucaena leucocephala*.

**MATERIALS AND METHODS**

**Isolation of Pi Solubilizing Microorganisms**

Rhizospheric soil samples (200 g) and portions of fine roots (3 g) were taken from mature *L. leucocephala* plants growing in different soils. Soil samples of the Wahiawa series (very-fine, kaolinitic, isohyperthermic Rhodic Haplustox) (pH 6.1, soil solution P concentration 0.001 mg/L), Tantalus series (medial over
pumiceous or cindery, ferrihydritic, isothermic Typic Hapludand) (pH 6.4, soil
solution P concentration 0.085 mg/L), and Kaena series (very fine, smectitic,
isohyperthermic Typic Natraquert) (pH 7.9, soil solution P concentration 0.066
mg/L) were collected from different locations on Oahu, Hawai‘i. Soil pH was
determined using a soil: water ratio of 1:2 and soil solution P concentration was
determined after equilibrating soil samples in 0.01 M CaCl₂.2H₂O for one hour
(Habte, unpublished data). The soil classification were obtained from
10, 2008).

Phosphate solubilizing microorganisms were isolated on three different media
containing North Carolina rock phosphate (fluoroapatite, 130 g P kg⁻¹; Chien and
Hammond 1978) as the sole source of P. All media were sterilized in an
autoclave for 30 minutes, at 120°C and 0.1 MPa. Medium 1 was a modification of
the medium used by Kim et al. (1997) for isolating PSM and contained 1.0 g
NaCl, 0.2 g CaCl₂.2H₂O, 0.4 g MgSO₄.7H₂O, 1.0 g NH₄NO₃, 10.0 g glucose, 7.0 g
agar, 3.5 g rock phosphate per L of medium. The chemicals were suspended in
deonized water and yeast extract was excluded from the original medium
because it was determined to contain high levels of available P. Medium 2 is a
modification of the medium used by J. C. Perez and N. W. Osorio (unpublished
data) and contained 6.0 g MgSO₄.7H₂O, 20.0 g protease peptone, 15 mL
glycerol, 7.0 g agar, and 3.5 g rock phosphate L⁻¹ of medium. Medium 3 (Rao
1992) contained 0.5 g (NH₄)₂SO₄, 0.2 g KCl, 0.1 g MgSO₄.7H₂O, 0.02 g
MnSO₄·H₂O, 0.02 g FeSO₄·7H₂O, 10.0 g glucose, 7.0 g agar, and 7.7 g rock phosphate per L of medium. Serial dilutions of the test soils were prepared and 0.5 mL aliquots of the suspension from the 10⁻³, 10⁻⁴ and 10⁻⁵ dilutions of each soil prepared in sterile deionized water were transferred to tubes containing 5 mL of a particular medium. Likewise, fine L. leucocephala roots were washed six times in distilled and deionized water and then transferred to similar tubes reserved for root samples. The inoculated media were then separately poured on to Petri plates containing solidified water agar (15 g/L). Plates were incubated at 27 °C for 15 days. At the end of the growth period, large colonies with clear zones around them were selected (Toro et al., 1996). Single colonies were aseptically streaked on petri dishes containing yeast-manitol-agar (YMA) containing 0.5 g of KH₂PO₄, 0.2 g of MgSO₄·7H₂O, 0.1 g NaCl, 10.0 g of mannitol, 1.0 g of yeast extract, 15.0 g of agar per liter. The pH of the medium was adjusted to 6.0 before autoclaving. After 4 days of growth at 27 °C, individual colonies were transferred to YMA slants and stored at 4 °C for further studies.

**In vitro test**

An *in vitro* test was used to evaluate the capacity of selected PSM to solubilize RP. A loopfull of each isolate was aseptically transferred from YMA slant to a 250 mL- Erlenmeyer flasks containing 75 mL of Medium 1 (without agar). Flasks were incubated at 22 °C on an orbital shaker at 100 rpm. After
seven days, the pH of the medium was measured with a pH-meter. Thirty mL of the solution were then centrifuged for ten minutes at 80000x g, and the supernatant was filtered with a Whatman #42 filter paper. Phosphorus in the filtered supernatant was determined by the molybdate-blue method (Murphy and Riley, 1962).

Greenhouse experiment

The soil on which *L. leucocephala* was grown was a subsurface sample (15-30 cm) of the Wahiawa soil series. The soil was passed through a sieve with a 4-mm diameter aperture and it was limed with dolomite. The main soil characteristics were pH, 7.1 (1:1, water), P, 27 mg kg\(^{-1}\) (modified Truog), organic C (Walkley & Black), 10.8 g kg\(^{-1}\), N (Kjeldahl), 2.0 g kg\(^{-1}\), exchangeable Ca, Mg, and K (1 M ammonium acetate): 5.0, 2.8, and 0.8 cmol\(_e\) kg\(^{-1}\), respectively. A P sorption curve was developed for the soil according to the procedure of Fox and Kamprath (1970) (Figure 3.1). For this purpose, aliquots (3 g) of soil were equilibrated with 30 mL of 0.01 \(M\) CaCl\(_2\).2H\(_2\)O containing graded amounts of KH\(_2\)PO\(_4\). Two drops of toluene were added to retard microbial activity. The samples were shaken on an orbital shaker for a 30-minute period twice daily for 6 days. They then were centrifuged (10000 rpm for 10 minutes). The supernatant was filtered with a Whatman #1 filter paper. Phosphorus content of the filtrate was then determined using the molybdate-blue method (Murphy and Riley, 1962).
Plastic pots of 15-cm diameter and 17 cm depth were filled with 2 kg (dry weight) of autoclaved (120°C, 1 h, 0.1 MPa) soil. Blanket nutrients were applied in a solution form at the following rates (mg kg⁻¹): N 50, K 132, Mg 106, S 204, Zn 10, Cu 5, B 0.8, and Mo 0.5. Seeds of *L. Leucocephala* c.v. K-636 were germinated on sterile moist paper towels after the seeds were scarified in concentrated H₂SO₄ for 30 minutes. One germinated seed was planted into each pot. The experiment consisted of 12 treatments in a 3x2x2 factorial combination arranged on a greenhouse bench in a completely randomized design with 4 replicates per treatment. Pots were either not amended with P or received P as North Carolina rock phosphate or KH₂PO₄ at the rate of 100 mg kg⁻¹. The potted soil was uninoculated or inoculated with 75 g of a crude inoculum of *Glomus aggregatum* (Schenk and Smith emend Koske) consisting of sand, spores, hyphal fragments and pieces of mycorrhizal roots per pot. The mycorrhizal inoculum was uniformly mixed with the potted soil. Uninoculated pots received 75 g of sterilized sand along with washings from the crude inoculum after removal of mycorrhizal propagules with Whatman #1 filter paper. A phosphate solubilizing fungus (PSF) identified as *Mortierella* sp. (J. Uchida, University of Hawaii) was multiplied on YMA medium for 5 days and then its mycelium was suspended in sterile deionized water and shaken by hand until the clumps were dispersed. Eleven mL of the fungal suspension containing $3.6 \times 10^6$ colony-forming units (CFU) mL⁻¹ were directly pipetted into the planting holes (2-cm diameter, 3-cm depth) prior to planting.
The initial soil solution P concentration was 0.02 mg/L, which is considered optimum for the activity of AMF in highly mycorrhizal dependent species such as *L. leucocephala* (Habte and Manjunath, 1987). The application of P 100 mg/kg soil as KH₂PO₄ raised the P concentration in the soil solution to 0.04 mg/L (Figure 3.1). Application of RP did not change the soil solution P concentration.

The plants were grown between February 3 to March 20, 2000 under natural light in the greenhouse of the Department of Agronomy and Soil Science, University of Hawaii, Honolulu, HI (21°N, 157°W). The pots were watered to maintain the soil at 60% of the maximum water holding capacity.

Phosphorus status of *L. leucocephala* was monitored as a function of time by determining the P content of the 4th pinnule (counting from the base of the pinna) of the youngest fully expanded leaf starting on the 10th day after planting (Habte *et al.* 1987). Plants were harvested at the end of 45 days of growth. Shoots were dried at 70°C for 48 hours and then dry weights were measured. Phosphorus content of shoots was determined colorimetrically by the molybdate-blue method (Murphy and Riley 1962) after dry-ashing samples at 500°C for 6 hours in a muffle furnace and dissolving the ashes in one mL of 1 M HCl and then diluting with 9 mL of deionized water. Soil solution P concentration was also determined at the time of harvest. For this purpose, samples were suspended in 0.01 M CaCl₂.2H₂O and were shaken for one hour. Supernatant solutions were obtained and analyzed for P as described above. The AMF colonization of roots was determined after clearing roots with 10% KOH and staining them with 0.15 % acid
fuchsin in lactic acid (Habte et al., 1987) and then estimating the proportion of root length colonized by AMF by the grid-line intersection method (Giovannetti and Mosse, 1980). The data were statistically analyzed with SAS (SAS institute, Inc. 1997) employing F-test and Duncan test (P-value of 0.05).

RESULTS

Isolation of PSM

The medium that was most suited for isolating PSM was Medium 1, and many types of PSM were recovered on it. Therefore, only data showing the number of PSM recovered from soil and roots on this medium are reported (Table 3.1). This was also why we used this particular medium for the in vitro evaluation of the activity of selected PSM. Overall, bacteria were the most common PSM we encountered, followed by actinomycetes and fungi (Table 3.1). The frequency of bacteria, actinomycetes and fungi observed varied from soil to soil. The roots we examined contained relatively low numbers of PSM, especially fungi (Table 3.1). Thirty-two isolates were selected and their P solubilizing activity was assessed in vitro. The results showed that the ability of the isolates to solubilize RP varied widely (Table 3.2). There was an inverse relationship between the pH of the growth medium and the concentration of P in the solution determined after 7 days of incubation (Figure 3.2). The greatest reduction in pH and the highest P
solubilization activity was observed with a fungal isolate designated as 27 F, which was identified as Mortierella sp. This isolate was, therefore, the one we selected for the greenhouse study.

Greenhouse experiment

The level of mycorrhizal colonization of roots was significantly affected by inoculation with G. aggregatum (Table 3.3). Inoculated plants were colonized to the extent of 45%, whereas uninoculated ones did not have any mycorrhizal structures in their roots (Table 3.4). Mycorrhizal colonization was not influenced by the application of Pi or inoculation with Mortierella sp.

The concentration of P in the soil solution was significantly increased by initial Pi application, but was not affected by the inoculation treatments (Table 3.3). The unfertilized soils and the soil amended with RP had a solution P concentration of 0.02 mg/L, a value similar to the initial P concentration of the soil (Figure 3.1). The solution P concentration of the soil initially amended with KH₂PO₄ was 0.03 mg/L, a value slightly lower than the initially established target concentration of 0.04 mg/L.

The P content of pinnules was significantly affected by the treatments employed (Table 3.5). In uninoculated plants (-AMF, -PSF), the P content of pinnules decreased drastically and typical symptoms of P deficiency such as chlorosis and defoliation (Habte and Manjunath, 1987; Smith et al., 1992) were
detected 25 days after planting. Plants inoculated only with Mortierella sp. (+PSF) exhibited similar symptoms, although the P content of their pinnules was significantly higher than that of uninoculated plants (Figure 3.3). In contrast, pinnule P content of plants inoculated only with G. aggregatum (+AMF) initially declined rapidly but began to increase on day 20 and continued to increase, reaching a peak value at day 35 (Figure 3.3). Plants inoculated with both microorganisms (+AMF, +PSF) exhibited a pattern similar to the one described above, but had significantly higher pinnule P content at some sampling periods (Table 3.5, Figure 3.3).

At harvest, shoot dry weight was significantly affected by the interaction of both microorganisms regardless of P fertilization (Table 3.3). Uninoculated plants had very low shoot dry weight. When plants were inoculated only with Mortierella sp. (+PSF) there was a significant increase in shoot weight, although, the increase was less than that observed when plants were inoculated with G. aggregatum alone (Figure 3.4). Moreover, plants that were inoculated with both microorganisms (+AMF, +PSF) had shoot dry weight significantly higher than those inoculated only with G. aggregatum. Shoot dry weight of plants grown in soil amended with KH₂PO₄ was significantly higher than shoot dry weight of plants grown in the unfertilized soil. However, shoot dry weights of the former plants were not significantly different from that of plants grown in the soil fertilized with RP.
The content of P in shoots determined at harvest was significantly affected by the interaction of *G. aggregatum* with *Mortierella* sp. and by the three-way interactions among PI-fertilization and inoculation with PSF and AMF (Table 3.3). Plants grown in the unfertilized soil had low shoot P content, but inoculation with *G. aggregatum* increased the P content of shoots significantly. Dual inoculation did not increase shoot P content beyond the level observed by inoculating soil with *G. aggregatum* alone.

In the RP-treated soil, the effect of mycorrhizal inoculation on shoot P status was significantly enhanced if the PSF was present than if it was absent (Table 3.4). The shoot P content of plants grown in soil fertilized with KH₂PO₄ increased significantly in response to inoculation with the PSF or AMF, but the effect of mycorrhizal inoculation was not altered by the presence of the PSF (Table 3.4).

**DISCUSSION**

The methods we used to isolate and test PSM were favorable for obtaining a variety of rhizosphere microorganisms able to solubilize RP. They are straightforward and the materials used are those that are readily available in standard soil and soil biology laboratories. The *in vitro* test we employed for assessing P solubilizing activity was very sensitive and enabled us to separate isolates into several categories based on their effectiveness to solubilize RP. The strong relationship between reduction in the pH of the growth medium and
increase in the concentration of P in the medium after incubation suggest that 
acid production was a major mechanism in the solubilization of RP by the Pi 
solubilizing isolates. In a similar *in vitro* test, Omar (1998) observed a decrease in 
the pH of the medium he used. Likewise, Bar-Yosef *et al.* (1999) noted an inverse 
relationship between changes in pH and Pi solubilizing activity of *Pseudomonas 
cepacia* and proposed the adoption of the approach for RP solubilization in the 
phosphorus fertilizer industry.

Whitelaw (2000) reviewed the literature on the subject matter and noted a 
wide variation in the ability of test microorganisms to depress the pH of test 
media. The most widely held mechanism by which PSM depress the medium pH 
in which they grow is the production of organic acids (Kim *et al.*, 1997; Di Simine 
*et al.*, 1998; Singh and Kapoor 1999) and the release of protons in response to 
ammonium uptake (Kucey, 1983; Asea *et al.*, 1988; Roos and Luckner, 1984).
Some of the organic acids commonly associated with Pi solubilization by PSM *in 
vitro* are gluconic acid (Di Simine *et al.*, 1998), oxalic acid, citric acid, and 2-keto-
D-gluconic acid (Kim *et al.*, 1997). Kim *et al.* (1997) reported similar values of pH 
using a medium comparable to that used by us, except that we did not include 
yeast extract for reasons already explained. Since we were able to quantify the Pi 
solubilized without the need to use an extracting solution, it would appear that the 
Bray and Kurtz (1945) extracting solution they used was also unnecessary.

In the greenhouse experiment, the relative contribution of the PSF and AMF 
tested to the Pi nutrition of the indicator plant was clearly delineated in terms of
pinnule P content which was monitored as a function of time (Habte et al., 1987). Pinnule P data showed that the synergistic interaction between the AMF and the PSF was first detected on the 20th day after planting and lasted through the 35th day (Figure 3.3). Beyond this period the synergistic effect became no significant as P was translocated from leaves to other plant parts (Habte et al., 1987). Since we were able to detect synergistic effect as early as 20 days, the pinnule technique can save a great deal of time in the screening of different PSM/AMF combinations for synergistic efficacy. Kim et al. (1998) grew tomato in the presence of G. etunicatum and then introduced the PSM Enterobacter agglomerans 15 days later. They observed maximum synergistic effect 50 days after planting, the effect diminishing there after.

It appears that in the current study the network of mycorrhizal hyphae and roots formed was effective in removing the P solubilized by Mortierella sp. as early as 20 days after planting. It is also likely that the mycorrhizal roots were releasing exudates (Raminelli 1973; Linderman 1988) required by the PSF to produce organic acids (Bolan 1991) which are at least in part responsible for the solubilization of Pi. Organic acids mobilize Pi by sequestering P fixing metals like Al and Fe and also by replacing phosphate adsorbed on soil colloids (He and Zhu, 1997).

Pinnule P content was significantly (P≤ 0.05) increased by inoculation of soil with Mortierella sp. alone (Figure 3.3). However, this increase was not sufficient to satisfy the Pi requirement of leucaena and the plants developed Pi deficiency
symptoms and their growth was stunted. This may be due to the re-immobilization of a large portion of the Pi solubilized by the fungus on adsorption sites of the soil (Bolan, 1991).

Most of the studies on PSM have been carried out in lowly weathered soils, particularly Mollisols, which exhibit a low P fixation capacity (Asea et al., 1988; Kucey, 1987, 1988; Kucey and Legget, 1989; Singh and Singh, 1993). In these soils, inoculation with PSM alone has increased plant P uptake by a 25 to 73% in the absence of P fertilization. By contrast, inoculation with Mortierella sp. alone did not increase P uptake in our test plant. This suggests that the effect of PSM on plant P uptake may be diminished in highly weathered soils with high Pi fixation capacity as result of re-immobilization of the Pi released by the PSM. These limitations may in part be overcome with the presence of the mycorrhizal association due to the higher efficiency of AM hyphae to take up Pi from the soil solution.

Rock phosphate enhanced the effectiveness of the PSF as well as the synergistic interaction between AMF and PSF with respect to shoot P content in our study. Using a lowly weathered soil, Omar (1988) reported that RP increased the positive effect of two PSF on wheat, but AMF inoculation diminished it. On the other hand, Singh and Kapoor (1999) noted that RP had not influence on the effectiveness of two Pi solubilizing bacteria unless the soil was inoculated with Glomus sp. The positive effect of North Carolina rock phosphate may have to do with its high reactivity (Chien and Hammond, 1978), which can be explained by
its high surface area (Hammond and Leon, 1992). These properties allow it to interact with the soil solution and eventually with the protons released by PSM.

Soluble Pi amendment enhanced the effect of the PSF, but this effect disappeared when both AMF and PSF were present. Soluble Pi sources could be rapidly converted to immobile Pi forms. Such freshly precipitated Pi are likely to be more readily solubilized by PSM, because of their high surface area exposed to the soil solution (Brady and Weil, 1999), we believe that these Pi forms were subsequently remobilized by the PSF. However, this effect may be masked by a rapid mycorrhizal removal of Pi from soil solution, which in turn leads to the release of more Pi ions from the recently immobilized Pi (Murdoch et al., 1967; Sanders and Tinker, 1985; Bolan, 1991).

The fact that there was significant effect of the PSF in the unfertilized soil suggests that in addition to the solubilization of RP there were indigenous forms of insoluble soil Pi and/or mechanisms of Pi desorption may have been involved. He and Zhu (1997) suggested that soil microorganism were able to mobilize sorbed P on the surface of soil minerals such as kaolinite, goethite, and Al oxides, which are commonly present in Oxisols such as the one we used in the current investigation (USDA, 1972).

The level of mycorrhizal colonization observed in the current study was relatively low compared to values previously observed under comparable conditions (Habte et al., 1987; Habte and Fox, 1989). The fact that the variable was not influenced by any other factor except by inoculation allowed the
assessment of the effect of the PSF at constant AMF colonization. It is conceivable that the effect of the PSF could be enhanced at higher levels of AMF colonization. The relatively low level of colonization noted in the current study is most likely caused by the low level of infective propagules in the inoculum used (M. Habte, unpublished data).
Table 3.1. Number of colony forming units of PSM per gram of rhizosphere soil (S) or roots (R) isolated using medium 1.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Bacteria</th>
<th>Actinomycetes</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wahiawa</td>
<td>S 1300000</td>
<td>200000</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>R 5000</td>
<td>0</td>
<td>800</td>
</tr>
<tr>
<td>Tantalus</td>
<td>S 195000</td>
<td>10000</td>
<td>2000</td>
</tr>
<tr>
<td></td>
<td>R 300</td>
<td>0</td>
<td>900</td>
</tr>
<tr>
<td>Kaena</td>
<td>S 297000</td>
<td>10000</td>
<td>3000</td>
</tr>
<tr>
<td></td>
<td>R 700</td>
<td>100</td>
<td>200</td>
</tr>
</tbody>
</table>

Table 3.2. Changes in pH and P concentration in medium 1 inoculated with selected PSM (means with the same lower case letter are not significantly different at $P \leq 0.05$, Duncan Test).

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Solution pH</th>
<th>Solution P (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.02 a</td>
<td>0.06 d</td>
</tr>
<tr>
<td>1 B</td>
<td>4.57 b</td>
<td>10.53 c</td>
</tr>
<tr>
<td>4 B</td>
<td>4.49 b</td>
<td>15.47 b</td>
</tr>
<tr>
<td>6 B</td>
<td>3.93 d</td>
<td>25.97 a</td>
</tr>
<tr>
<td>10 B</td>
<td>4.45 bc</td>
<td>17.85 b</td>
</tr>
<tr>
<td>16 B</td>
<td>4.44 bc</td>
<td>16.83 b</td>
</tr>
<tr>
<td>18 A</td>
<td>6.91 a</td>
<td>0.15 d</td>
</tr>
<tr>
<td>25 F</td>
<td>3.87 d</td>
<td>16.73 b</td>
</tr>
<tr>
<td>27 F</td>
<td>3.45 e</td>
<td>29.68 a</td>
</tr>
<tr>
<td>28 F</td>
<td>3.84 d</td>
<td>15.44 b</td>
</tr>
<tr>
<td>29 F</td>
<td>6.86 a</td>
<td>0.098 d</td>
</tr>
<tr>
<td>32 F</td>
<td>4.27 c</td>
<td>15.28 b</td>
</tr>
</tbody>
</table>

A = actinomycete, B = bacterium, F = fungus
### Table 3.3. Level of significance (P) associated with shoot dry weight, P content of shoots, P in the soil solution and mycorrhizal colonization at the time of harvest (NS: not significant).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Shoot dry weight (g/plant)</th>
<th>P in shoots (mg/plant)</th>
<th>P in soil solution (mg/L)</th>
<th>Mycorrhizal colonization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>0.0139</td>
<td>NS</td>
<td>0.0050</td>
<td>NS</td>
</tr>
<tr>
<td>AMF</td>
<td>0.0001</td>
<td>0.0001</td>
<td>NS</td>
<td>0.0001</td>
</tr>
<tr>
<td>PSF</td>
<td>0.0001</td>
<td>0.0125</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PxAMF</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PxPSF</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AMFxPSF</td>
<td>0.0186</td>
<td>0.0360</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>PxAMFxPSF</td>
<td>NS</td>
<td>0.0463</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

C.V. (%) 18.3 28.3 21.3 19.8

### Table 3.4. Effects of RP, KH$_2$PO$_4$ (KP), and inoculation with G. aggregatum and a PSF on shoot P content and mycorrhizal colonization of L. leucocephala roots (means with the same letter are not significantly different at P<0.05, a Duncan test for each group of P source).

<table>
<thead>
<tr>
<th>P source</th>
<th>Inoculation $^a$</th>
<th>Shoot P content (mg/plant)</th>
<th>Mycorrhizal colonization (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMF</td>
<td>PSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>-</td>
<td>0.11a</td>
<td>0 b</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>0.12a</td>
<td>0 b</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>1.68b</td>
<td>48 a</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>1.91c</td>
<td>48 a</td>
</tr>
<tr>
<td>RP</td>
<td>-</td>
<td>0.14a</td>
<td>0 b</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>0.18a</td>
<td>0 b</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>1.27b</td>
<td>48 a</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>2.20c</td>
<td>39 a</td>
</tr>
<tr>
<td>KP</td>
<td>-</td>
<td>0.21a</td>
<td>0 b</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>0.29a</td>
<td>0 b</td>
</tr>
<tr>
<td>+</td>
<td>-</td>
<td>1.83b</td>
<td>44 a</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>1.82b</td>
<td>49 a</td>
</tr>
</tbody>
</table>

$^a$ - uninoculated, + inoculated

74
Table 3.5. Levels of significance (P) of sources of variation with respect to P content of pinnules of *L. leucocephala* at indicated days after planting (DAP) (NS: not significant)

<table>
<thead>
<tr>
<th>Factor</th>
<th>Time (DAP)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>P</td>
<td>0.0039</td>
</tr>
<tr>
<td>AMF</td>
<td>NS</td>
</tr>
<tr>
<td>PSF</td>
<td>NS</td>
</tr>
<tr>
<td>PxAMF</td>
<td>NS</td>
</tr>
<tr>
<td>PxPSF</td>
<td>0.0027</td>
</tr>
<tr>
<td>AMFxPSF</td>
<td>NS</td>
</tr>
<tr>
<td>PxAMFxPSF</td>
<td>NS</td>
</tr>
<tr>
<td>C.V. (%)</td>
<td>12.4</td>
</tr>
</tbody>
</table>
Figure 3.1. Phosphorus sorption curve for the Wahiawa soil.
Figure 3.2. Relationship between pH and P in liquid medium inoculated or not inoculated with phosphate solubilizing microorganisms.
Figure 3.3. P content of pinnules of *L. leucocephala* monitored as a function of time in the presence (+) or absence (-) of *G. aggregatum* (AMF) and *Mortierella* sp. (PSF).
Figure 3.4. Effects of *G. aggregatum* (AMF) and *Mortierella* sp. (PSF) on shoot dry weight (SDW) of *L. leucocaphala*. 
The bar chart shows the dry weight (SDW) per plant under different conditions.

- **-AMF** shows lower SDW compared to **+AMF**.
- The bars labeled **a**, **b**, **c**, and **d** represent different groups within the -AMF and +AMF conditions, indicating differences in SDW.

Legend:
- **- PSF**
- **+ PSF**

**-AMF** conditions:
- **d** is the lowest SDW.

**+AMF** conditions:
- **a** is the highest SDW.
- **b** is the second-highest SDW.
- **c** is the lowest SDW among the +AMF conditions.
LITERATURE CITED


CHAPTER FOUR

EFFECTIVENESS OF A PHOSPHATE SOLUBILIZING MICROORGANISM TO ENHANCE SOIL AVAILABLE PHOSPHATE VIA ROCK PHOSPHATE DISSOLUTION IN THE PRESENCE OF SOIL MINERALS AND SOILS DIFFERING IN THEIR PHOSPHATE SORBING CAPACITY

ABSTRACT

Two in vitro experiments were carried to assess the effectiveness of Mortierella sp., a phosphate (Pi) solubilizing fungus (PSF), to enhance solution Pi concentration of a liquid medium containing rock phosphate (RP) as the sole source of P in the presence of soil minerals and soil samples differing in their Pi sorption capacity. The soil minerals used were Allophane, Bauxite, Goethite, Kaolinite, Halloysite, Illite, and Montmorillonite. Soil samples were collected from Colombia and consisted of four Andisols, an Oxisol, an Ultisol, and a Mollisol. Mortierella sp. was capable of decreasing the pH of the liquid medium from 7.7 to 3.0, even in the presence of soils with high pH buffering capacity. The results showed that the different soil minerals present in the medium controlled the effectiveness of Mortierella sp. to increase Pi in solution to different degrees. Montmorillonite and Kaolinite did not affect solution Pi concentration, while Halloysite, Illite, and Bauxite decreased it. The effect was particularly severe in the presence of Goethite and Allophane. Likewise, in the presence of very-high Pi fixing soils, soil solution Pi concentration was depressed despite the dissolution of RP by the PSF. By contrast, the PSF increased the concentration of Pi in solution due to the dissolution of RP (by oxalic acid) in the presence of...
soils having lower Pi sorbing capacity. The results also suggest that Mortierella sp. was able to grow on the surface of some soils and soil minerals. Higher fungal P uptake in the presence of high Pi sorbing soils indicates that Mortierella sp. was capable of desorbing sorbed Pi. However, under this condition Mortierella sp. tended to immobilize more of the Pi into its cells than was released into the growth medium.

INTRODUCTION

Phosphate (Pi) deficiency is a major constraint to plant productivity in agricultural soils, particularly in the tropics (Bolan et al., 1999; Sanchez and Logan, 1992). Many soils of the tropics exhibit a high Pi fixing capacity, particularly the highly weathered soils and those formed from volcanic ash (Trolove et al., 2003). Consequently, large amounts of soluble Pi fertilizers are required to achieve adequate concentration of solution Pi in these soils (Havlin et al., 1999). Juo and Fox (1977) proposed several categories of soil Pi sorption capacity in tropical soils based on the $P_{0.2}$ value. This parameter represents the amount of P (mg/kg) required to be added to soil in order to achieve a soil solution P concentration of 0.2 mg/L, which is the critical value of available P for many crops. The categories are: Very Low (VL, $P_{0.2}$<10 mg/kg), Low (L, $P_{0.2}$: 10-100 mg/kg), Medium (M, $P_{0.2}$: 100-500 mg/kg), High (H, $P_{0.2}$: 500-1000 mg/kg), and Very High (VH, $P_{0.2}$>1000 mg/kg).

Although, rock phosphates (RP) can be used effectively in high P fixing soils (Gyaneshwar et al., 2002; Brady and Weil, 1999), the low reactivity of most RP is
a draw back to their widespread use (Shrivastava et al., 2007; Ojo et al., 2007; Chien and Hammond, 1978). The use of phosphate solubilizing microorganisms (PSM) to enhance RP dissolution has been considered a viable approach to improve the efficacy of RP as a source of Pi (Vyas et al., 2007; Delvasto et al., 2006; Welch et al., 2002).

Many authors have obtained positive effects on Pi uptake and yield, of several plant species due to inoculation of soil with PSM (Barea et al., 2002; Whitelaw, 2000; Kucey and Legget, 1989). Most of these positive results have been obtained in low Pi sorbing soils (Mollisols, sandy soils, calcareous soils) (Peix et al., 2001; Omar, 1998; Kucey, 1983, 1987, 1988; Asea et al., 1988; Kucey and Legget, 1989; Gleddie, 1993). However, in high P sorbing soils of the tropics and volcanic ash soils, where cost-effective approaches are needed to overcome the effects of high Pi fixation by soils, PSM have been little studied. The effectiveness of these microorganisms to enhance plant Pi uptake has been questioned by some authors (Tinker, 1980; Bolan, 1991) because of the concern that the solubilized Pi will be rapidly re-fixed by soil constituents. However, there are no published data to refute or confirm this argument. My hypothesis is that the effectiveness of PSM in increasing soluble P, via RP dissolution, may be controlled by soil Pi sorption capacity. The objective of the current investigation was to determine the effectiveness of the Mortierella sp., a PSF, to increase Pi availability by dissolving RP in the presence of soil minerals and soils differing in their Pi sorbing capacity.
MATERIAL AND METHODS

The effectiveness of *Mortierella* sp. to solubilize RP was evaluated in the absence or presence of soil minerals or soils widely differing in their Pi sorbing capacity. To two hundred fifty-mL Erlenmeyer flasks, I added 75 mL of a liquid medium that contained per liter: 1.0 g NH₄NO₃, 1.0 g NaCl, 0.2 g CaCl₂.2H₂O, 0.4 g MgSO₄·7H₂O, 10.0 g glucose, and 3.5 g of Hulla RP. The Hulla RP which was passed through a 0.5-mm aperture sieve had a P content of 130 g/kg, and its empirical formula is Ca₀.₆₉Na₀.₂₂Mg₀.₀₉(PO₄)₁₄(CO₃)₀.₈₈F₂.₃₄ as indicated by Chien and Hammond (1978). The liquid medium was also amended with soil minerals (<0.5-mm diameter) or different soils (0.5 to 2.0-mm diameter) at the rate of 0.6 g/flask. The medium was then autoclaved (120°C, 0.1 MPa, 30 min). The medium was either not inoculated or inoculated with two mL of a three-day-old suspension containing 3x10⁵ colony forming units (CFU) of *Mortierella* sp. per mL. Uninoculated flasks received two-mL of sterile deionized water.

*Mortierella* sp. was originally isolated from an Andisol of Hawaii (Osorio and Habte, 2001) and has been multiplied and stored on yeast mannitol agar (YMA) slants at 4°C. For this study, the fungus was multiplied in Petri dishes on YMA medium for three days at 28°C. Then its mycelium was removed from the surface of the agar with a sterile loop and suspended in sterile deionized water and shaken by hand until the clumps were dispersed. The minerals tested were Montmorillonite, Illite, Halloysite, Kaolinite, Bauxite, Goethite, and Allophane, and the soils tested were Guane, La Selva, Naranjal, Letras, Caucasia, Carimagua,
and Neira. Soil minerals were supplied by the soil mineralogy lab of the University of Hawai‘i. The origin and properties of soils are given in Appendices A, B, D, and Table 4.1.

Flasks were continuously shaken on an orbital shaker (model Innova 4400, New Brunswick Scientific Co., Inc., Edison, NJ) at 100 rpm at 25°C for seven days.

After the incubation period, the medium was filtered through a Whatman No. 42 filter paper fitted to a Buchner funnel. Vacuum was exerted at 350 mm-Hg. Fresh fungal matter of Mortierella sp. was collected on the filter paper and then dried in an oven at 60°C for 30 h for dry mass determination after correcting for the weight of remaining RP and minerals or soils. The filtrate was centrifuged at 5000×g for 15 minutes and passed through a Millipore membrane filter (0.45 μm) for solution pH determination by means of a pH-meter. Solution P concentration was measured by the molybdate blue method (Murphy and Riley, 1962). For each soil mineral or soil sample, the difference in solution P between samples not inoculated and inoculated with PSF was designated as Net solubilized P. In order to determine Fungus P content and concentration, fungus samples (3-5 mg) were oven-dried and then ashed in a muffle furnace at 500°C for 3 hours. The ash was dissolved in one-mL of 0.1 M HCl and then brought up to 10 mL with deionized water. Fungal P content was determined with the molybdate blue method (Murphy and Riley, 1962). Regression analyses were carried to establish the significance of the relationship between the P_{0.2} value (Appendix D) and other variables under study.
Treatments were arranged factorially in a completely randomized design with three replicates per treatment. Data were subjected to analysis of variance and mean separation was achieved by employing Duncan’s multiple range test or the LSD test at a $P$-value of 0.05, provided the $F$ statistics is significant. Analysis of data was achieved by employing the statistical package Statgraphics version 4.0 (Statpoint, Inc., Herdon, Virginia).

RESULTS

The extent to which pH decreased due to the growth of Mortierella sp. in the growth medium was not impaired by the presence of soil minerals (Figure 4.1). In fact, pH declined to a greater extent if Montmorillonite and Illite were also present in the liquid medium (Table 4.2). Solution pH of the uninoculated flasks remained close to the original pH value (7.7), and it was not significantly affected by the presence of soil minerals.

The concentration of Pi detected in the medium due to Mortierella-induced dissolution of RP varied depending on the type of soil mineral present in the medium (Figure 4.2; Table 4.2). Although Mortierella sp. did solubilize RP in the presence of Allophane and Goethite, the solution Pi concentration did not increase significantly compared to the uninoculated control. The concentration of Pi detected in the growth medium was also reduced significantly in the presence of Bauxite, Illite, and Halloysite with respect to the inoculated flask without soil mineral (Figure 4.2). Montmorillonite and Kaolinite did not influence the
concentration of Pi in solution and in that way the effectiveness of Mortierella sp. to increase solution Pi was higher. The net amount of Pi detected in solution followed a trend that was similar to the above results (Figure 4.3).

Dry matter of Mortierella sp. was significantly affected by the type of soil present in the liquid medium (Table 4.2). The fungus grew significantly more ($P \leq 0.05$) in the presence of soil minerals than in their absence, except in the presence of Allophane. In the presence of Allophane fungal dry mass was similar to that found in the flask without minerals (Figure 4.4). The dry matter of Mortierella sp. was significantly higher in the presence of soil minerals than in their absence. The highest value was observed in the presence of Illite and Kaolinite, followed by Halloysite, Goethite, Montmorillonite, and Bauxite.

Fungal P content was not measured in this experiment due to the strong adhesion of soil minerals to Mortierella sp. The separation of soil particles from the fungal mycelium was tedious and not always successful.

Significant ($P \leq 0.05$) inverse relationship was noted between the net amount of P solubilized and the $P_{0.2}$ value (Figure 4.5). Fungal dry matter yield also was inversely related to this parameter (Figure 4.6).

Mortierella sp. exhibited a strong capacity to reduce pH even in the presence of soils with high pH buffer capacity as indicated by the high lime requirement of these soils (Appendix C) (Table 4.2). The decline in pH produced due to the activity of Mortierella sp. was higher in the presence of some soils (Letras, Naranjal, Carimagua, Caucasia, and Guarme ($pH \leq 3.0$)) than in their absence (Figure 4.7).
The effectiveness of *Mortierella* sp. to increase solution Pi concentration was significantly affected by the presence of soil samples in the growth medium (Table 4.3). Its effectiveness to enhance solution Pi was significantly impaired by the presence of soils with very-high Pi sorbing capacities (Guarne, La Selva, and Naranjal). This effect was more severe with two of the soils having the highest Pi sorbing capacity (Figure 4.8 and 4.9). In contrast, with the presence of the other soils in the liquid medium the concentration of soluble Pi released by *Mortierella* sp. was significantly higher than in their absence, even though they had exhibited Pi-sorbing capacity. The extent of this favorable effect on *Mortierella* activity was in the order Letras (M) > Carimagua (M) = Caucasia (H) > Neira (L) (see Table 4.1) (letters in parenthesis represent the Pi sorbing capacity of the soils measured by the P_0.2 value). Net solubilized P (difference between uninoculated and inoculated) followed a similar pattern (Figure 4.9).

*Mortierella* sp. dry matter was significantly higher (*P* < 0.05) in the presence of soils than in their absence, except in the presence of the Letras soil (Figure 4.10; Table 4.3). The highest fungus dry matter yield was observed in the presence of the Neira soil (the soil with the lowest Pi sorbing capacity).

Fungus P uptake was significantly stimulated by the presence of some soils, and not by others [Letras soil (M) and Caucasia (H)] (Figure 4.11). Surprisingly, *Mortierella* sp. took up significantly more Pi in the presence of high Pi-fixing soils (Guarne and Naranjal) than in the presence of low Pi-fixing soils (Figure 4.11). The lowest fungus P content was observed in flasks not amended with soil and in
those amended with the Letras soil. Similar results were obtained for tissue P concentration of Mortierella sp. (Figure 4.12).

The relative proportions of microbial-P and soluble-P in the growth medium varied depending on the type of soil present in the medium (Table 4.4). In the absence of soil samples in the medium, Mortierella sp. mobilized only 3.9 mg of P, leaving 96% of the P in solution (Figure 4.13). In the two soils with the highest P-sorbing capacity (Guame and La Selva) the proportion of soluble-P was only 27-31%, the balance being immobilized in the fungus mycelium as microbial-P. In the Naranjal soil, also a soil with very-high Pi sorption capacity, but lower than the other two Andisols, the distribution of P was significantly different, soluble-P being 62% while microbial-P being 38%. The values observed in the presence of soils that stimulated the effectiveness of Mortierella to increase Pi in solution were comparable to those observed in the soil-free medium. In the absence of soil, soluble-P ranged 84-99% while microbial-P was around 1-16% (Table 4.14).

Results of regression analysis revealed that the net amount of P solubilized was inversely related to the P_{0.2} value \( r^2 = 0.672 \) (Figure 4.14). On the other hand, the microbial-P fraction (immobilized) was directly related to the P_{0.2} value \( r^2 = 0.8226 \), whereas the soluble-P-fraction was inversely related to the P_{0.2} value \( r^2 = 0.8471 \) (Figure 4.15).

**DISCUSSION**
The capacity of Mortierella sp. in increasing solution Pi concentration was significantly affected by the presence of soil minerals and soils in the liquid medium. The results indicate that Mortierella sp. did dissolve RP by oxalic acid production (appendix H) and Pi was released into solution. However, the presence of soil minerals and soils controlled to different degrees the amount of Pi that remained in solution. In the presence of a mineral or a soil with very-high Pi sorption capacity ($P_{0.2} > 1000$ mg/kg) less Pi was left in solution; conversely, more Pi remained soluble in the presence of minerals and soils that exhibited less Pi sorption capacity.

It is very well documented that a major mechanism for the microbial dissolution of RP is acid production (Vyas et al., 2007; Chen et al., 2006; Radersma and Grierson, 2004; Welch et al., 2002; Whitelaw et al., 1999; Illmer and Schinner, 1995). In the current investigation, the presence of Mortierella sp. was associated with decreases in the pH of the growth medium despite the presence of soil minerals and soils which were expected to buffer the pH (Bolan et al., 1999; Prabhakaran, 1996). The lowest pH observed in the presence of these materials was 2.75 (Figures 4.1 and 4.7), which is lower than what is commonly reported in the literature (pH 3.5-4.5) (Pandey et al., 2006; Cerezine et al., 1988). This reduction in the pH of the growth medium indicates that dissolution of RP took place under the current experimental conditions. An inverse relationship between pH and solution Pi concentration was obtained by Osorio and Habte (2001) (Chapter 3) under similar experimental conditions but without soils.
Although RP dissolution occurred in the presence of all soil minerals and soils, the effectiveness of *Mortierella* sp. in increasing solution P varied widely (Figures 4.3 and 4.9). The active surfaces of some of the soil minerals and the soil samples controlled the extent to which Pi released by *Mortierella* can stay in solution. The presence of Allophane and Goethite was particularly effective in lowering the concentration of Pi measured in the liquid medium. These results are understandable in light of the tendency of Allophanes to strongly sorb large quantities of Pi in volcanic ash soils (Bolan *et al.*, 1994; Shoji *et al.*, 1993; Bolan *et al.*, 1999). Goethite is the major factor responsible for Pi sorption in highly weathered soils of the tropics (Jones 1981; Sposito, 1984; Parfitt, 1989). This fact explains why the concentration of Pi in the liquid medium was low if either Allophane or Goethite were present in the liquid medium along with *Mortierella* sp. Consistently, the three Andisols with very high Pi sorbing capacity (Guame, La Selva, and Naranjal) depressed the quantity of Pi released by *Mortierella* by adsorbing it on their surfaces. These types of soils are very well known for their very high Pi sorbing capacity (Jackman *et al.*, 1997; Shoji *et al.*, 1993) due to the large active surface area they have coupled by the high binding strength they exert on Pi. These properties appear to be responsible for the low level of Pi detected in the solution when *Mortierella* sp. was incubated in the presence of soils compared to that level of Pi observed in their absence. Among the very high Pi sorbing soils studied, Guame and La Selva were most effective in adsorbing Pi released from RP due to the solubilization activity of *Mortierella* sp. To obtain a soil solution concentration of 0.2 mg/L in their presence, it was necessary to add
to the growth medium 4126 and 2222 mg of P/kg (i.e. P_{0.2} of 4126 and 2222, respectively). Naranjal was the Andisol with lowest Pi sorbing capacity (P_{0.2} = 1429 mg/kg); apparently this lower capacity was reflected in a lower reduction in the concentration of Pi detected in the medium in the presence of *Mortierella* sp. (Figures 4.8 and 4.9). The results obtained with these three Andisols support the hypothesis of Tinker (1980) and Bolan (1991) regarding low or nil effectiveness of PSM in presence of high Pi sorbing soils. However, it is interesting to note that some soils, even those with relatively high (P_{0.2}: 500-1000 mg/kg) and medium (P_{0.2}: 100-500 mg/kg) Pi sorbing capacity, enhanced the concentration of Pi in solution, suggesting that in their presence *Mortierella* sp. was more effective in solubilizing RP than in their absence. For instance, in the presence of the Caucasia soil, an Ultisol with a high Pi sorbing capacity (P_{0.2}=714 mg/kg) the concentration of Pi in the liquid medium was in excess of twice that noted in the absence of the soil (Figure 4.8). The effect of the Carimagua soil (an Oxisol with medium P fixing capacity (P_{0.2}= 417 mg/kg) was similar to that of the Caucasia soil. Kaolinite is the dominant soil mineral in these two soils, a soil mineral that did not reduce the concentration of Pi measured in solution after the growth of *Mortierella* in the medium in the presence of RP.

The positive effects on the dissolution of RP by *Mortierella* sp. observed in the presence of Neira (Montmorillonite as the dominant soil mineral) and the Letras soils were not unexpected because of their low Pi-sorbing capacities (Letras P_{0.2} = 123, Neira P_{0.2} = 45). However, the magnitude of the positive effect they had on the dissolution of RP by *Mortierella* is surprising, particularly
regarding the Letras soil (Vitrand). This soil has a sandy texture, 94% sand and only 2% clay, which explains, at least in part, why it did not impair the effectiveness of Mortierella sp. in solubilizing RP.

The positive effect of the presence of soil minerals and soils (except Letras) had on dry mass, P content, and P concentration of Mortierella sp. is hard to explain. However, some explanations may lie in the growth characteristics exhibited by the fungus. First, Mortierella sp. has a tendency to grow on solid surfaces, its mycelium tending to adhere onto internal flask walls, RP particles, minerals, soil particles, and also on root surfaces, forming a biofilm-like pattern (Wood, 1980; Atlas and Bartha, 1997; Moore, 1998). This ability to grow on soil mineral surfaces may be advantageous for the fungus because these surfaces contain large quantities of sorbed Pi. The capacity of this fungus to produce oxalic acid (Appendix H) could help this fungus to desorb Pi for its own uptake. That PSM can utilize sorbed Pi was recently demonstrated by Hoberg et al. (2005). For Mortierella sp. to grow on these Pi-adsorbent surfaces is an advantage since it can remove Pi from the immediate vicinity of these adsorbent surfaces. It is probable that essential nutrients were also concentrated on these surfaces (Burns, 1980; Stotzky, 1980). Mortierella sp. could displace and utilize these anions with oxalic acid that it excretes. Mortierella sp. could make them available for its own demands without the need to leave behind much soluble Pi. Although Pi desorption has been suggested as a possible mechanism of PSM by a few authors (Hoberg et al. 2005, He and Zhu, 1997) it has not been adequately
demonstrated to my knowledge. Evidence showing that this hypothesis is indeed possible will be presented in chapter 5 of this dissertation.

The self-immobilization of Mortierella sp. cells on surfaces might explain the better performance of the fungus in the presence of some minerals and soils. Vassilev et al. (2001) observed that immobilized cells of PSF were more effective in dissolving RP under in vitro conditions than if freely suspended.

The higher ability of Mortierella sp. to absorb Pi in the most Pi fixing soils suggests that more Pi ions were present on the soil surfaces (Figure 4.15). This alternative pool of Pi for uptake by the fungus explain how, despite the very low concentration of Pi in solution, Mortierella overcame Pi starvation by growing on the Pi-rich surfaces of the soils and desorbing Pi from the surfaces.

It is important to keep in mind that Mortierella sp. was isolated from the Tantalus soil series (an Andisol of Hawai‘i), where it was growing probably by desorbing Pi from soil adsorption sites since the soil’s Pi sorption capacity is very high. The fungus is probably the first PSM isolated from a very high Pi sorbing soil.

Data on the relative distribution of Pi between microbial tissue and the growth medium (Figures 4.13 and 4.15; Table 4.4) clearly showed that under limited Pi availability (as when the very high P fixing soils were present) the tendency of Mortierella sp. was to accumulate Pi in its cells instead of mobilizing it into the growth medium. Notice that the relative amount of microbial-P is higher than that of soluble-P in the presence of the Guarne and La Selva soils (the most P fixing soils). In the presence of the Naranjal soil that rated third in Pi sorbing capacity,
the proportion of microbial P observed was significantly lower than that observed in the presence of the very high Pi fixing soils. Likewise, other soils with much lower Pi sorbing capacity had still a lower proportions of microbial P (0.8-15.6%) and obviously lower fungal dry mass. The soil in which Mortierella released the highest quantity of Pi in solution was the Letras soil, but was the worst regarding Pi uptake by Mortierella sp. As mentioned before, the Letras soil is very sandy (94% sand and only 2% clay), and hence has a very low capacity to sorb Pi on its limited active surfaces, making it unfavorable for the growth of Mortierella sp. but highly favorable to the accumulation of Pi in the growth medium. The results of my investigation concur with earlier observations that PSM can be effective in increasing soil Pi availability in soils having low Pi fixing capacity ($P_{0.2} < 100$ mg/kg) (e.g., Mollisols and sandy soils) as reported by many authors (Peix et al., 2001; Omar, 1998; Kucey, 1983, 1987, 1988; Asea et al., 1988; Kucey and Legget, 1989; Gleddie, 1993). In the presence of soils with very-high Pi sorbing capacity ($P_{0.2} > 1000$ mg/kg) most of the Pi mobilized from RP is prevented from remaining in solution by the extremely high P adsorption capacity of the soils. Ramirez et al. (2002) reported that Pseudomonas aeruginosa, a known PSM, did not increase plant Pi uptake of leucaena grown in an Andisol, presumably due to re-fixation of released Pi. That is the only known report of a study of PSM in Andisols.

According to my results, in soils with medium ($100 < P_{0.2} < 500$) and high ($500 < P_{0.2} < 1000$) Pi sorbing capacity, it is expected that Mortierella sp. can
increase soil available Pi and increase plant Pi uptake (Osorio and Habte 2001). Additional evidence is provided in Chapter 8 of this dissertation.

The *in vitro* approach proposed in the current work represents a simple, rapid, and relatively inexpensive approach for predicting whether or not a PSM can release Pi into soil solution in sufficient quantities for plant growth. This approach has made it abundantly clear that a PSM may not release adequate Pi to the soil solution in soils characterized by high to very-high Pi fixing capacities. It is probable that the presence of arbuscular mycorrhizal fungi might considerably change the picture if the fungi succeed in capturing the Pi solubilized by a PSM before it is re-immobilized by the Pi adsorbing sites.
Table 4.1. Soil classification, P₀.2 value, and Pi sorption category.

<table>
<thead>
<tr>
<th>Soil</th>
<th>USDA Soil taxonomy</th>
<th>P₀.2 value (mg/kg)</th>
<th>Soil Pi sorption category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanme</td>
<td>Melanudand</td>
<td>4000</td>
<td>Very High (VH)</td>
</tr>
<tr>
<td>La Selva</td>
<td>Endoaquand</td>
<td>2222</td>
<td>Very High</td>
</tr>
<tr>
<td>Naranjal</td>
<td>Melanudand</td>
<td>1429</td>
<td>Very High</td>
</tr>
<tr>
<td>Caucasia</td>
<td>Paleoudult</td>
<td>714</td>
<td>High (H)</td>
</tr>
<tr>
<td>Carimagua</td>
<td>Haplustox</td>
<td>417</td>
<td>Medium (M)</td>
</tr>
<tr>
<td>Letras</td>
<td>Vitrand</td>
<td>123</td>
<td>Medium</td>
</tr>
<tr>
<td>Neira</td>
<td>Haplustoll</td>
<td>45</td>
<td>Low (L)</td>
</tr>
</tbody>
</table>

Table 4.2. Significant P-values of ANOVA for solution pH, solution P, Net solubilized P in the experiment involving soil mineral suspensions.

<table>
<thead>
<tr>
<th>Source</th>
<th>Solution pH</th>
<th>Solution P</th>
<th>Net solubilized P</th>
<th>Fungus dry matter*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil mineral (A)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PSM inoculation (B)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AxB</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.5</td>
<td>24.9</td>
<td>16.6</td>
<td>11.9</td>
</tr>
</tbody>
</table>

* Only inoculated experimental units were considered

Table 4.3. Significant P-values of ANOVA for the variables under study in the experiment involving soils suspensions.

<table>
<thead>
<tr>
<th>Source</th>
<th>Solution pH</th>
<th>Solution P</th>
<th>Fungus dry mass</th>
<th>Fungus P content</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil (A)</td>
<td>0.0182</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PSF inoculation (B)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AxB</td>
<td>0.0180</td>
<td>&lt;0.0001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV (%)</td>
<td>1.5</td>
<td>24.9</td>
<td>14.8</td>
<td>30.8</td>
</tr>
</tbody>
</table>

* Only inoculated experimental units were considered
Table 4.3. (Continued) Significant $P$-values of ANOVA for the variables under study in the experiment involving soil suspensions*.

<table>
<thead>
<tr>
<th>Source</th>
<th>Fungus $P$ concentration</th>
<th>Net solubilized P</th>
<th>Microbial + Soluble P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PSF inoculation (B)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AxB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CV (%)</td>
<td>29.6</td>
<td>7.6</td>
<td>15.1</td>
</tr>
</tbody>
</table>

* Only inoculated experimental units were considered

Table 4.4. Distribution of microbial-P and soluble P (mg) in a liquid medium containing rock phosphate and soil samples seven days after Mortierella sp. inoculation. Values followed by different lower case letters are significantly different (Duncan test $P \leq 0.05$). Each value is the mean of three replicates.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Soluble (%)</th>
<th>Microbial (%)</th>
<th>Soluble (%)</th>
<th>Microbial (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.7</td>
<td>0.2</td>
<td>96.0 a</td>
<td>4.0 a</td>
</tr>
<tr>
<td>Guama</td>
<td>0.9</td>
<td>1.9</td>
<td>31.0 d</td>
<td>69.0 a</td>
</tr>
<tr>
<td>La Selva</td>
<td>0.3</td>
<td>0.7</td>
<td>27.3 d</td>
<td>72.7 a</td>
</tr>
<tr>
<td>Naranjal</td>
<td>2.4</td>
<td>1.5</td>
<td>62.0 c</td>
<td>38.0 b</td>
</tr>
<tr>
<td>Caucasia</td>
<td>8.6</td>
<td>0.2</td>
<td>96.8 a</td>
<td>3.2 a</td>
</tr>
<tr>
<td>Carimagua</td>
<td>8.3</td>
<td>0.6</td>
<td>93.5 a</td>
<td>6.5 a</td>
</tr>
<tr>
<td>Letras</td>
<td>11.0</td>
<td>0.1</td>
<td>99.2 a</td>
<td>0.8 a</td>
</tr>
<tr>
<td>Neira</td>
<td>4.9</td>
<td>0.9</td>
<td>84.4 b</td>
<td>15.6 c</td>
</tr>
</tbody>
</table>
Figure 4.1. Solution pH of the liquid medium containing rock phosphate and different soil minerals seven days after inoculation with Mortierella sp.
Figure 4.2. Solution P concentration of the liquid medium containing rock phosphate and different soil minerals seven days after inoculation with Mortierella sp.
None  Montmorillonite  Illite  Halloysite  Kaolinite  Bauxite  Goethite  Allophane

Soil mineral x PSM inoculation

LSD_{0.05} = 3.5

CV = 23.4%
Figure 4.3. Net solubilized P by Mortierella sp. activity in a liquid medium containing rock phosphate and different soil minerals seven days after inoculation. Columns with different low case letters are significantly different (Duncan test $P \leq 0.05$).
Figure 4.4. Dry mass of Mortierella sp. grown in a liquid medium containing rock phosphate and different soil minerals seven days after inoculation. Columns with different low case letters are significantly different (Duncan test $P \leq 0.05$).
Figure 4.5. Relationship between net solubilization of P by Mortierella sp. and the $P_{0.2}$ value in a liquid medium containing rock phosphate and soil minerals.
$y = 65.2564 - 7.5765 \ln(x)$

$P < 0.0001$

$r^2 = 0.8492$
Figure 4.6. Relationship between Mortierella sp. dry mass and the $P_{0.2}$ value in a liquid medium containing soil minerals and rock phosphate.
$y = 34463x^{-0.7662}$

$r^2 = 0.8033$
Figure 4.7. Solution pH of a liquid medium containing rock phosphate and soils seven days after inoculation with Mortierella sp.
Figure 4.8. Solution P concentration of the liquid medium containing rock phosphate and soil seven days after inoculation with *Martierella* sp.
Figure 4.9. Net solubilized P (mg/L) by Mortierella sp. in a liquid medium containing rock phosphate and soils seven days after inoculation.
Figure 4.10. *Mortierella* sp. dry mass in a liquid medium containing rock phosphate and soils seven days after its inoculation. Columns with different lower case letters are significantly different (Duncan test $P \leq 0.05$).
Figure 4.11. *Mortierella* sp. P content (µg) in a liquid medium containing rock phosphate and soil seven days after inoculation. Columns with different lower case letters are significantly different (Duncan test $P \leq 0.05$).
Figure 4.12. *Mortierella* sp. P concentration (%) in a liquid medium containing rock phosphate and different soils seven days after inoculation. Columns with different low case letters are significantly different (Duncan test $P \leq 0.05$).
Figure 4.13. Microbial and Soluble P (mg/flask) in a liquid medium containing rock phosphate and soils seven days after Mortierella sp. inoculation. Columns with different low case letters are significantly different (Duncan test $P \leq 0.05$).
Figure 4.14. Relationship between net solubilized P (mg/L) and the $P_{0.2}$ value in a liquid medium containing rock phosphate, soil, and Mortierella sp. inoculum.
$y = 125.91 - 0.0713x + 0.00001x^2$

$P < 0.001$

$r^2 = 0.672$
Figure 4.15. Relationship between the soluble-P fraction and microbial-P fraction (%) and the $P_{0.2}$ value in a liquid medium containing rock phosphate, soil and Mortierella sp. inoculum.
\[
y = 0.000003x^2 - 0.0305x + 100.65 \\
P < 0.001 \\
r^2 = 0.8471
\]

\[
y = -0.000005x^2 + 0.0372x - 2.6022 \\
P < 0.001 \\
r^2 = 0.8226
\]
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Phosphate solubilizing bacteria from subtropical soil and their tricalcium 

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CHAPTER FIVE

PHOSPHATE DESORPTION FROM THE SURFACE OF SOIL MINERALS AND SOILS INDUCED BY A PHOSPHATE SOLUBILIZING FUNGUS

ABSTRACT

A series of in-vitro experiments were carried out to evaluate the effectiveness of Mortierella sp. to desorb Pi from soil minerals and six soil samples differing in their Pi sorption capacity. The results indicate that the fungus was effective in desorbing Pi from all the minerals tested by producing oxalic acid, except from an Allophane. The extent of its Pi desorption effectiveness was in the order Montmorillonite > Kaolinite > Goethite > Allophane (nil). Consistently, Mortierella sp. also desorbed Pi from the surfaces of different soils tested and its effectiveness to do so was in the order Mollisol > Oxisol > Ultisol > Andisols. With both soil minerals and soil samples, the quantity of soluble Pi released by microbial desorption was higher when the amount of sorbed Pi was high. Mortierella sp. effectiveness was dependent on the soil Pi sorption capacity expressed as $P_{0.2}$ value (amount of P required to achieve a soil solution P of 0.2 mg/L). This parameter could be used as a predictor of Mortierella sp. effectiveness to increase soluble P via desorption it from soils. These findings represent the first conclusive report on Pi desorption form soils by a PSM.
INTRODUCTION

Phosphate (Pi) fixation is a serious problem in agricultural soils, particularly in highly weathered soils and those formed from volcanic ash (Trolove et al., 2003; Shoji et al., 1993; Sanchez and Uehara, 1980). It is estimated that 1018 million ha in the tropics have a high Pi fixation capacity Sanchez and Logan (1992). The term Pi-fixation is used to describe reactions that remove bioavailable Pi from the soil solution and incorporate it into the soil solid phase (Barber, 1995).

Phosphate sorption is particularly strong on iron and aluminum hydrous-oxides (crystalline or non-crystalline) that predominate in the highly weathered soils of humid regions and acid savannas (Mattlingly, 1975). Jones (1981) found that the surface area of goethite was a primary factor responsible for Pi sorption by 11 Puerto Rican soils, whereas gibbsite contributed little to Pi sorption, and hematite had essentially no contribution. Jackman et al. (1997) investigated the phenomenon using Hawaiian soils and obtained similar results. They also found that Pi sorption was significantly correlated with the sum of surface area of soil minerals and their potential Pi sorption sites. Hence, Pi sorption was satisfactorily predicted by soil mineralogical composition.

In soils derived from volcanic parent materials, humus-Al/Fe complexes, Allophane, Ferrihydrite, and Goethite are responsible for the strong Pi sorption exhibited by the soils (Parfitt, 1989; Schwertmann and Herbillon, 1992; Jackman et al., 1997; Shoji et al., 1993). Fox and Kamprath (1970) showed that the degree of Pi fixation varies among soils, with those classified as Andisols (US soil
taxonomy) having a very high Pi fixation capacity (Boul et al., 1997), followed by Ultisols and Oxisols. Juo and Fox (1977) proposed several categories for soil Pi sorption capacity in tropical soils based on the $P_{0.2}$ value. This value represents the amount of P (mg/kg) required to achieve a soil solution P concentration of 0.2 mg/L. The categories are: Very Low ($P_{0.2}<10$ mg/kg), Low ($P_{0.2}:10-100$ mg/kg), Medium ($P_{0.2}:100-500$ mg/kg), High ($P_{0.2}:500-1000$ mg/kg), and Very High ($P_{0.2}>1000$ mg/kg).

Many organic acids are effective in reducing Pi sorption (Hue, 1991). These acids are produced by roots (Kirk et al., 1999) and soil microorganisms (Gyaneshwar et al., 2002; Marschner, 1997). Bolan et al. (1994) studied the influence of monocarboxylic (acetic, formic, and lactic), dicarboxylic (malic, tartaric, and oxalic), and tricarboxylic (citric) acids on the solubilization and sorption of Pi on an Andisol (Hydric Dystrandept) and an Alfisol (Typic Fragiaquaf) of New Zealand and observed that the organic acids significantly decreased Pi sorption on allophane surface. The effectiveness of the acids in this regard followed the order tricarboxylic > dicarboxylic > monocarboxylic. Bolan and collaborators explained this phenomenon based on the formation of complexes between aluminum (Al) with the conjugate anion of each organic acid and their respective stability constant (Log $K_{AI}$) proposed by Hue et al. (1986).

Hue (1991) found similar results on the availability of soil P when he added organic acids on two Andisols, an Oxisol, an Ultisol, and a Vertisol of Hawai‘i. The effectiveness of the acids to reduce Pi sorption from a soluble source ($KH_{2}PO_{4}$) was higher with malic acid (monohydroxy dicarboxylic), followed by
protocatechuic acid (dihydroxy monocarboxylic), and acetic acid (monocarboxylic).

Many soil microorganisms have the ability to produce and release organic acids (citric, oxalic, gluconic, malic, among others) or their conjugate bases capable of dissolving P compounds (Pandey et al., 2006; Welch et al., 2002; Reddy et al., 2002). Therefore, these microorganisms can desorb Pi from adsorptive surfaces of soils as suggested by He and Zhu (1998). They indicated that Pi sorbed on surfaces of soil minerals was displaced when a culture medium was inoculated with soil samples containing microbes (unidentified). Since other soil components (e.g., organic matter, humic acids, organic acids) can desorb Pi (Guppy et al., 2005; lyamuremye and Dick, 1996), the results of He and Zhu cannot be completely attributed to microorganisms present in the soil used as inoculum. Recently, Hoberg et al. (2005) found that two microorganisms were able to take up Pi adsorbed on Goethite. However, in that experiment soluble Pi decreased over time due apparently to immediate absorption of all Pi desorbed by the microorganisms. A major characteristic required of effective PSM is that they must increase soluble Pi, which can be used by other organisms (e.g., plants and/or mycorrhizal fungi).

The hypothesis tested was that Mortierella sp. can increase soil solution Pi by desorbing Pi adsorbed on the surface of soil minerals and that its effectiveness will depend on the Pi sorption capacity and the extent of saturation of Pi sorption sites in the soil. The effectiveness of the fungus in desorbing Pi will be controlled by the soil mineralogy and soil type. The objective of this
investigation was to determine the effectiveness of *Mortierella* sp. to increase soluble Pi by desorbing Pi from the surfaces of soil minerals and from soils differing in their Pi sorption capacity.

**MATERIAL AND METHODS**

**Preliminary Pi sorption isotherms**

Samples of four soil minerals (Allophane, Goethite, Kaolinite, and Montmorillonite) (Appendix B) were passed through a sieve of 0.5 mm aperture sieve. Then, Pi sorption isotherms were constructed in triplicates following the procedure developed by Fox and Kamprath (1970) (Figure 5.1). To avoid microbial activity the 50-mL plastic centrifuge tubes containing two g of a soil mineral, 20-mL of 0.01 M CaCl₂ solution, and graded amounts of KH₂PO₄ were autoclaved (120°C, 0.1 MPa, 30 minutes) instead of the traditional use of two drops of toluene. The centrifuge tubes and their contents were shaken in a reciprocal shaker for seven days, twice a day, 30 minutes each time.

The P₀.₂ value (amount of P (mg/kg) required to achieve a solution P of 0.2 mg/L) was estimated. This allowed to readily quantify the Pi sorption capacity of each soil mineral (Appendix D).

A similar procedure was employed with triplicate samples of six Colombian soils (Neira, Carimagua, Caucasia, Naranjal, La Selva, and Guame) differing in their Pi sorption capacity (increasing in the order they are written above) (Figure 5.2). Soil samples were passed through a 2-mm aperture sieve and retained in a
0.5-mm aperture sieve. Soil collection, locations, and their chemical and physical properties of the soils are presented in Appendix A. Additional relevant information for this experiment is presented in table 5.1.

**Inoculum of Mortierella sp.**

*Mortierella* sp. was originally isolated from an Andisol of Hawaii (Osorio and Habte, 2001) and has been multiplied and stored on YMA slants at 4°C. For this study, the fungus was multiplied in Petri dishes on YMA medium for three days at 28°C. Then its mycelia were removed with a sterile loop and suspended in sterile deionized water and shaken by hand until the clumps were dispersed. The fungal suspension contained $4 \times 10^5$ colony forming units of *Mortierella* sp.

**Microbial Pi desorption experiments**

Based on the Pi sorption isotherms, graded amounts of KH$_2$PO$_4$ in 0.01 M CaCl$_2$ solution were mixed with two-g portions of each soil mineral or soil sample in 50-mL centrifuge tubes in order to establish target solution P concentrations of 0.05, 0.1, and 0.2 mg/L (Table 5.2). The initial solution P concentration of a soil mineral or soil sample was determined by adding to it 0.01 M CaCl$_2$ solution alone instead of 0.01 M CaCl$_2$ + KH$_2$PO$_4$. Suspension pH was adjusted to 6.0 with drops of 0.1 M NaOH. The tubes were horizontally shaken on a reciprocal shaker at 100 rpm seven days at 25°C for. The, the solution was removed and the soil minerals or soil samples were washed three times with de-ionized water. The Pi remaining on the soil minerals or soils was considered as sorbed. The soil
mineral/soil samples were oven-dried at 60°C for two days in the centrifuge tubes.

At the end of the drying treatment, each tube received 19 mL of a nutrient solution that contained 1.0 g NaCl, 0.2 g CaCl₂.2H₂O, 0.4 g MgSO₄.7H₂O, 1.0 g NH₄NO₃, and 10.0 g glucose per L. The sole source of P was Pi adsorbed on the surfaces of the soil minerals or soil-samples. Then, the tubes and their contents were autoclaved (120°C, 30 min, 0.1 MPa). One-mL of a fungal suspension of Mortierella sp. was aseptically transferred into each tube. Tubes not inoculated with the fungus received 19 mL of the nutrient solution and one-mL of deionized sterile water. The tubes were continuously shaken on a reciprocal shaker at 100 rpm for six days at 25°C.

At the end of the incubation period, the suspensions were centrifuged at 5000xg for 15 minutes and the supernatant was filtered through a Whatman No. 42 filter paper followed by filtration through a Millipore membrane filter (0.45 μm). Solution P concentration in the filtrates was determined using the molybdate-blue method (Murphy and Riley, 1962). The presence of Pi in solution was considered a evidence of Pi desorption. The effectiveness of Mortierella sp. to desorb Pi was calculated by subtracting solution P concentration measured measured in the absence of the fungus from that measured in its presence, and was designated as microbial desorption Pi. This value was related to the P₀.₂ value.

The treatments consisted of a factorial combination (4x2) of four levels of sorbed-P (at four target solution P at equilibrium) and two levels of PSM inoculation (with or without Mortierella sp.) with three replicates per treatment.
arranged in a completely randomized design. Each mineral or soil was evaluated in separated experiments. Data were subjected to analysis of variance (ANOVA) and LSD test (P-value of 0.05) was used to evaluate the significance of treatments when the F-statistics was significant. For this purpose the software Statgraphics Plus version 4.0 (Statpoint, Inc.; Herdon, Virginia) was used.

RESULTS

Soil minerals

Phosphate sorption isotherms of soil minerals (Figure 5.1) clearly showed that the minerals widely differed in their Pi sorbing capacity, making them suitable for evaluation of Mortierella sp. effectiveness under a wide range of Pi sorption conditions. Surprisingly, Montmorillonite exhibited high Pi sorbing capacity, even higher than Goethite, a known high Pi fixing mineral (Appendix D).

In all soil minerals, except in the Allophane, Mortierella sp. was capable of increasing solution P concentration significantly (Table 5.2). However, the effect of the fungus was significantly modified by the concentration of Pi initially sorbed on the surface of the minerals.

In the case of Allophane, Mortierella sp. did not increase solution P at any level of sorbed P (Figure 5.3). The very high Pi sorbing capacity of the mineral was apparently responsible for the ineffectiveness of the fungus to desorb Pi. In contrast, Mortierella sp. inoculation increased soluble P concentration significantly in all other soil minerals evaluated except when P was not added to them prior to incubation with the fungus. The magnitude of Pi desorption induced
by *Mortierella* sp. increased with increase of sorbed Pi (higher saturation of Pi sorbing sites). This effect was highest with Montmorillonite, followed by Kaolinite, and Goethite.

With the exception of the Allophane, the $P_{0.2}$ values estimated from the Pi desorption isotherms of inoculated and unincubated minerals were significantly different (Table 5.4). Inoculation with *Mortierella* sp. led to a significant decrease in the $P_{0.2}$ values of Kaolinite (31%), Goethite (37%), and Montmorillonite (61%) (Table 5.4).

*Mortierella* sp. was not effective in desorbing Pi sorbed on Allophane (Figure 5.4). In contrast, the fungus was effective in desorbing sorbed-Pi from the surface of Goethite, Kaolinite, and Montmorillonite. More Pi was desorbed if the level of sorbed Pi was high than if it was low. The extent of the effectiveness of *Mortierella* sp. in desorbing P from the soil minerals followed the order Montmorillonite > Kaolinite > Goethite > Allophane (nil).

**Soil minerals**

The Pi sorption capacity of the soils ranged from low to very high (Figure 5.2) (Juo and Fox, 1977). The Guarne soil had the highest Pi sorption, followed by La Selva and Naranjal. The Pi sorption capacity of the remaining soils ranged from high for the Caucasia soil (a clayey and Al-rich Ultisol), medium for the Carimagua soil (Oxisol), and low for the Neira soil (Mollisol).
Inoculation with *Mortierella* sp. significantly increased soil solution P in the presence of all soils (Figure 5.5); but the effect of *Mortierella* sp. was significantly affected by the Pi concentration of Pi adsorbed on soils with the exception of the Neira soil (Table 5.5). In this soil, *Mortierella* sp. increased soil solution Pi regardless of the concentration of Pi initially sorbed on the soil. The magnitude of Pi desorbed by *Mortierella* sp. effectiveness was in excess of 0.25 mg/L.

The effectiveness of *Mortierella* sp. in desorbing sorbed Pi from the Carimagua soil is striking (Figure 5.5). However, solution Pi did not increase in the unfertilized soil. The capacity of *Mortierella* sp. to increase soluble P in this soil was enhanced with an increase in the quantity of sorbed Pi. Soluble Pi in the inoculated soil reached values in excess of 0.2 mg of P/L. Similar results were obtained when the Caucasia soil was inoculated with *Mortierella* sp. (Figure 5.5). Thus, *Mortierella* sp. desorbed Pi from all of the three Andisols studied, except when they were unfertilized with KH$_2$PO$_4$ (Figure 5.5). However, the magnitude of its effectiveness was lower compared to its effectiveness with other soils due certainly to the very-high Pi-sorbing capacity. Phosphate desorption was particularly restricted in the most Pi fixing soil (Guarne) (Figure 5.5 and 5.6). Despite this fact, the effect of the fungus was enhanced by increasing the level of sorbed Pi. Concentration of soil solution P in the inoculated and fertilized Andisols ranged from 0.057 to 0.131 mg/L.

In the presence of *Mortierella* sp., $P_{0.2}$ values were significantly lower for all soils than in the absence of the fungus (Table 5.6). For the Andisols, the
percentage of reduction ranged from 16 to 70%, while for other soils it ranged from 22.5 to 100%.

DISCUSSION

The capacity of Mortierella sp. to desorb sorbed-Pi was demonstrated in five of six soil minerals and in all six soils representing a wide range of Pi adsorbing capacities (Figures 5.3 and 5.5). The differences among these materials to sorb Pi can be associated with the degree of crystallization and type of counter ions retained on soil mineral surfaces. Montmorillonite was saturated with calcium, which can precipitate Pi to form insoluble calcium-phosphate compounds.

The effectiveness with which the fungus desorbed Pi from soils and minerals is in general governed by the Pi adsorbing capacity of these materials (Figure 5.6) and by variables related to it (Appendix D and Table 5.1). These variables include $P_{0.2}$ which represents the amount of P required to be added to a mineral or soil in order to obtain 0.2 mg P/L in the solution. While the capacity of Mortierella sp. to augment soil solution Pi concentration is directly correlated with the quantity of Pi absorbed on the surface of minerals/soils, it is inversely correlated with the Pi sorption capacity of a mineral/soil and with the above mentioned variable derived from it. Hence, the fungus is expected to be less effective or ineffective in the presence of Allophane or soils whose mineralogy is dominated by it (Guarne, Naranjal, and La Selva) (Figure 5.4 and Figure 5.6). The limited effectiveness of Mortierella sp. in the presence of these materials is
largely due to the strength with which Pi is bound to them. On the other hand, the fungus is very effective in desorbing Pi from Montmorillonite and Kaolinite and soils dominated by these minerals. Based on soil classification (Buol et al., 1997) and Pi sorption categories proposed by Juo and Fox (1977), the Neira soil is considered as Montmorillonite-dominated and hence is one of the soils in which Mortierella sp. exhibited very high capacity to desorb Pi (Figure 5.6). The other soil in which Mortierella sp. has a high capacity to desorb Pi is the Kaolinite-dominated Carimagua soil (IGAC, 1991). The effectiveness of Mortierella's in desorbing Pi from the remaining soil (Caucasia) was intermediate between the two extremes (the Andisols on the one hand and Carimagua and Neira on the other hand) considered above. The most probable mineral composition of the Caucasia soil is a mixture of Kaolinite and Goethite, whereas the Allophane is dominant in the three Andisols (Guarne, La Selva, Naranjal).

The effectiveness of Mortierella sp. to increase solution Pi ensures the availability of sufficient Pi for itself but also makes Pi available to other organisms, including plants and mycorrhizal fungi. This phenomenon may in part explain the synergistic interaction of this fungus and the mycorrhizal fungus Glomus aggregatum to stimulate Pi uptake and growth of Leucaena leucocephala in a highly weathered Oxisol of Hawai‘i with moderate Pi sorbing capacity (Osorio and Habte, 2001).

The tendency of Mortierella sp. to desorb a higher concentration of Pi when more Pi is sorbed on the soil surface is not surprising (Figure 5.5). It is well known that the strength with which Pi is sorbed tends to decrease with an
increase in the degree to which the adsorptive surfaces are saturated with Pi (Do Carmo Harta and Torrent, 2007; Bohn et al., 1985). This is true for five of the six soils tested, the Neira soil being the sole exception in which microbial desorption of native Pi occurred. This of course is consistent with the low Pi sorption capacity of the soil. It is possible that the phenomenon was operating in several studies conducted using soils similar to the Neira soil (Peix et al., 2001; Omar, 1998; Kucey, 1983, 1987, 1988; Asea et al., 1988; Kucey and Legget, 1989; Gleddie, 1993), although the authors apparently were not aware of it. On the other hand, the magnitude of the microbial Pi desorption and the further increase in soil solution Pi with increase in the level of adsorbed Pi in Carimagua and Caucasia soils suggest that these soils are conducive for the Pi-desorbing activity of PSM. The effectiveness of Mortierella in the Caucasia soil is very interesting, because this Ultisol contained very high amounts of 1M KCl extractable Al (13.7 cmol/kg) and an Al saturation of 96.5%.

Although the Pi desorption induced by Mortierella sp. in the three Andisols was very limited, its effect in raising soil solution Pi concentration when sorbed Pi increased is important. It is evident from the data that Pi can be raised to a level sufficient for mycorrhiza-dependent growth of many plant species (Habte and Manjunath, 1991, 1987). However, relatively large amounts of Pi fertilizers will be required to enhance the Pi desorption activity of Mortierella sp., and the cost-benefit ratio of the practice ought to be considered carefully. In Andisols with a long history of Pi fertilization, it is probable that PSM will increase the supply of Pi in the soil solution and hence increase plant Pi uptake.
He and Zhu (1998) presumed soil microorganisms were able to use sorbed-Pi from soil minerals on the basis of a study in which soil was used as an inoculum. Unfortunately, their study did not take into account the possible involvement of other soil components (soil organic matter, humic and fulvic acids, low molecular weight organic acids/anions, inorganic anions), which can also desorb Pi from the surfaces of soil minerals (Jara et al., 2006; Guppy et al., 2005; Iyamuremye and Dick, 1996).

The mechanism of Pi desorption is the release of oxalic acid/oxalate by the PSF during its normal metabolic activity. It is very well known that organic acids/anions can desorb Pi from sorbing sites (Jara et al., 2006; Sato and Comerford, 2006; Jones et al., 2003; Trolove et al., 2003; Welch et al., 2002; Ramirez and Osorio, 2005). Their effects were studied by Sato and Comerford (2006), Bolan et al. (1994), and Hue (1991) in highly weathered soils and in volcanic ash soils similar to those used in the current study. The ability of PSM to produce organic acids/anions is most prominent in the rhizosphere whereby carbonaceous compounds are released by roots (Corrales et al., 2007; Amos and Walters, 2006; Le Bayon et al., 2006; Marschner et al., 2006; Marschner, 1997). Once the root exudates are released they are metabolized by rhizosphere microorganisms leading to the production of organic acids/anions (Gyaneswar et al., 2002). Although this excretion appears to be genetically determined (Rodriguez et al., 2006, 2000), environmental conditions such as Pi deficiency or high ammonium concentration could trigger or enhance the production of organic acid/anions (Vyas et al., 2007; Chen et al., 2006; Whitelaw, 2000).
Results of the current study suggest that the value of $P_{0.2}$ obtained from the initial soil Pi sorption isotherm can be used for predicting the degree to which a PSM is effective in desorbing Pi from a given soil. This could save a lot of time in research and in making decisions on whether or not to inoculate with a PSM. The effectiveness of Mortierella sp. in desorbing Pi might have some practical implications such as lowering soil Pi fertilizer requirement and enhancing the residual effect of soluble Pi fertilizers (Table 5.6).

To my knowledge, the results presented here on the effectiveness of Mortierella sp. in increasing soil solution Pi by desorbing Pi sorbed on soils is the first of its kind. In another study, I have established the effectiveness of Mortierella sp. in dissolving Pi from rock phosphate (N.W. Osorio, unpublished data). The effectiveness of this fungus in increasing soil solution Pi by dissolving rock phosphate and desorbing sorbed Pi can play an important role in the alleviation of Pi deficiency in soils, particularly in the tropics where the high Pi sorption capacity of soils constrains plant productivity. Moreover, the role that PSM play in soil Pi availability ought to be an important consideration in the development of biotechnological approaches to the managements of soils.
Table 5.1. Soil classification, location, and $P_{0.2}$ value.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Soil classification</th>
<th>Latitude, longitude</th>
<th>$P_{0.2}$ value (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guame</td>
<td>Melanudand</td>
<td>6°15'N, 75°30'W</td>
<td>4000</td>
</tr>
<tr>
<td>La Selva</td>
<td>Endoaquand</td>
<td>6°08'N, 75°25'W</td>
<td>2222</td>
</tr>
<tr>
<td>Naranjal</td>
<td>Melanudand</td>
<td>4°58'N, 75°39'W</td>
<td>1429</td>
</tr>
<tr>
<td>Caucasia</td>
<td>Paleoudult</td>
<td>8°03'N, 75°07'W</td>
<td>714</td>
</tr>
<tr>
<td>Carimagua</td>
<td>Haplustox</td>
<td>4°34'N, 71°20'W</td>
<td>417</td>
</tr>
<tr>
<td>Neira</td>
<td>Haplustoll</td>
<td>5°08'N, 75°35'W</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 5.2. Amount of P added (mg/kg) to soil minerals and soil to achieve four target solution P concentrations at equilibrium.

<table>
<thead>
<tr>
<th>Target solution P (mg/L)</th>
<th>Soil minerals</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Allophane</td>
</tr>
<tr>
<td>Initial</td>
<td>0 (0.002)*</td>
</tr>
<tr>
<td>0.05</td>
<td>3009</td>
</tr>
<tr>
<td>0.1</td>
<td>3578</td>
</tr>
<tr>
<td>0.2</td>
<td>4146</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Target solution P (mg/L)</th>
<th>Soils</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guame</td>
<td>0 (0.005)*</td>
</tr>
<tr>
<td>La Selva</td>
<td>2254</td>
</tr>
<tr>
<td>Naranjal</td>
<td>2944</td>
</tr>
<tr>
<td>Caucasia</td>
<td>3635</td>
</tr>
</tbody>
</table>

* In parenthesis initial solution P at equilibrium (mg/L) found in soil minerals and soils without $KH_2PO_4$ addition.
Table 5.3. Significant P-values of ANOVA tests for solution Pi concentration as a function of initial target P level at equilibrium in the presence of PSF and soil minerals.

<table>
<thead>
<tr>
<th>Source</th>
<th>Allophane</th>
<th>Goethite</th>
<th>Kaolinite</th>
<th>Montmorillonite</th>
</tr>
</thead>
<tbody>
<tr>
<td>P level (A)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PSF(B)</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AxB</td>
<td>0.0318</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CV (%)</td>
<td>10.7</td>
<td>15.9</td>
<td>11.5</td>
<td>10.7</td>
</tr>
</tbody>
</table>

Table 5.4. The initial and final $P_{0.2}$ value for soil minerals seven days after inoculation with Mortierella sp. or not inoculated.

<table>
<thead>
<tr>
<th>Soil mineral</th>
<th>Initial $P_{0.2}$ (mg/kg)</th>
<th>Final $P_{0.2}$ value (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uninoculated</td>
<td>Inoculated</td>
</tr>
<tr>
<td>Allophane</td>
<td>4146</td>
<td>5103 a*</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5330 a</td>
</tr>
<tr>
<td>Goethite</td>
<td>224</td>
<td>319.4 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>202.2 b</td>
</tr>
<tr>
<td>Kaolinite</td>
<td>94</td>
<td>229.2 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>157.9 b</td>
</tr>
<tr>
<td>Montmorillonite</td>
<td>340</td>
<td>444.1 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>172.4 b</td>
</tr>
</tbody>
</table>

*Values of $P_{0.2}$ followed by different lower case letters are significantly different. Horizontal comparisons.

Table 5.5. Significant P-values of ANOVA tests for solution Pi concentration as a function of initial target P level at equilibrium in the presence of a PSF and six soils.

<table>
<thead>
<tr>
<th>Source</th>
<th>Guane</th>
<th>La Selva</th>
<th>Naranjal</th>
<th>Caucasia</th>
<th>Carimagua</th>
<th>Neira</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sorbed P (A)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PSF (B)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AxB</td>
<td>0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0008</td>
<td>0.1484</td>
</tr>
<tr>
<td>CV (%)</td>
<td>8.2</td>
<td>13.2</td>
<td>16.8</td>
<td>12.7</td>
<td>13.1</td>
<td>16.5</td>
</tr>
</tbody>
</table>
Table 5.6. Value of $P_{0.2}$ (mg/kg) and its relative decrease in six soils after an incubation/desorption period in the presence or absence of Mortierella sp. Each value is the mean of three replicates. In each soil, means followed by different letters are significantly different from each other ($P \leq 0.05$).

<table>
<thead>
<tr>
<th>PSM</th>
<th>Guame</th>
<th>La Selva</th>
<th>Naranjal</th>
<th>Caucasia</th>
<th>Carimagua</th>
<th>Neira</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninoculated (U)</td>
<td>5172 a</td>
<td>5525 a</td>
<td>4704 a</td>
<td>626 b</td>
<td>600 a</td>
<td>42 a</td>
</tr>
<tr>
<td>Inoculated (I)</td>
<td>4332 b</td>
<td>2544 b</td>
<td>1428 b</td>
<td>485 b</td>
<td>292 b</td>
<td>0 b</td>
</tr>
</tbody>
</table>

Relative decrease of $P_{0.2} = 100 \times \frac{(U-I)}{U}$

<table>
<thead>
<tr>
<th></th>
<th>Guame</th>
<th>La Selva</th>
<th>Naranjal</th>
<th>Caucasia</th>
<th>Carimagua</th>
<th>Neira</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.2</td>
<td>54.0</td>
<td>69.6</td>
<td>22.5</td>
<td>51.4</td>
<td>100.0</td>
<td></td>
</tr>
</tbody>
</table>

* Values followed by different lower case letters are significantly different. Vertical comparisons.
Figure 5.1. Soil mineral P sorption isotherms. The Y-axis on the right is for Allophane.
Figure 5.2. Soil P sorption isotherms of seven Colombian soils.
Figure 5.3. Concentration of soluble P (mg/L) desorbed from four soil minerals as a function of four initial target solution P concentrations at equilibrium and inoculation with Mortierella sp. The LSD ($P \leq 0.05$) value for each soil mineral is used to compare uninoculated and inoculated treatments.
Figure 5.4. Concentration of P in solution desorbed by Mortierella sp. from sorbed-P on soil minerals. Before inoculation, the four soil minerals had four target concentrations of P at equilibrium (X-axis).
Figure 5.5. Concentration of soluble P (mg/L) desorbed by Mortierella sp. from six soils as a function of four initial target solution P concentrations at equilibrium and inoculation with Mortierella sp. The LSD ($P \leq 0.05$) value for each soil is used to compare uninoculated and inoculated treatments.
Figure 5.6. Concentration of P in solution desorbed by *Mortierella* sp. from P sorbed on six different soils. Before inoculation, the six soils had four target concentrations of P in solution at equilibrium (X-axis). For each soil the P₀.2 value from the soil P isotherm is shown in parenthesis.
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CHAPTER SIX

EFFECT OF NITROGEN FORM ON THE EFFECTIVENESS OF A PHOSPHATE SOLUBILIZING FUNGUS TO DISSOLVE ROCK PHOSPHATE

ABSTRACT

An in vitro experiment was carried out to evaluate the effect of nitrogen (N) form (NH₄⁺ or NO₃⁻) on the dissolution of rock phosphate (RP) by a phosphate solubilizing fungus (PSF) identified as Mortierella sp. In the presence of NH₄Cl or NH₄NO₃ the solution pH significantly decreased from an initial value of 7.6 to 3.37 and 3.67, respectively. In the presence of KNO₃ the pH went down only to 6.69. Consequently, the relative amount of RP dissolved by this fungus was significantly higher when NH₄Cl (37%) was the sole source of N than when the source of N was KNO₃ (8%) or a mixture of NO₃⁻ and NH₄⁺ (NH₄NO₃) (32%). As a result of that, significantly more P in solution was detected in the presence of NH₄Cl (129.65 mg/L) than with NH₄NO₃ (109.25 mg/L); in the presence of KNO₃ the concentration of P in solution was significantly lower (0.08 mg/L). The excess NH₄⁺ apparently imposed a stress on Mortierella sp. that negatively affected its growth. However, this may have promoted a more active H⁺-pumping that decreased solution pH. In the presence of NO₃⁻ as the only source of N, Mortierella sp. not only dissolved a small amount of Pi from the RP but also immobilized most of it in its mycelia (99.8%). In contrast, in the presence of NH₄Cl Mortierella sp. was effective to dissolve RP and most of Pi released remained in solution (78%) and little was immobilized (22%).
INTRODUCTION

There is an increasing interest in developing strategies to improve the effectiveness of RP as a direct source of Pi for plant growth in parts of the tropics endowed with local deposits of the material (Shrivastava et al., 2007; Ojo et al., 2007; Yusdar et al., 2007; Randhawa et al., 2006; Msolla et al., 2005). One of these strategies involves the use of phosphate-solubilizing microorganisms (PSM) (Wakelin et al., 2004; Gyaneshwar et al., 2002; Osorio and Habte, 2001; Whitelaw, 2000; Gadd, 1999). Microbial dissolution of RP is brought about by a number of mechanisms including (i) release of organic acids (Pandey et al., 2006; Fransson et al., 2004; Bar-Yosef et al., 1999), (ii) formation of complexes between organic anions and cations such as Al $^{3+}$ and Ca$^{2+}$ (Welch et al., 2002; Jones et al., 2003), and (iii) excretion of protons due to NH$_4^+$ uptake (Whitelaw, 2000; Abd-Alla, 1994). The participation of the last mechanism in the microbial solubilization of RP has not been fully investigated for fungal P solubilizers (Fransson et al., 2004). Given the limited N supply of most soils (Brady and Weil, 1999; Havlin et al., 1999), the N applied either as NO$_3^-$ or NH$_4^+$ fertilizers can control the extent of acid production by PSM, which is crucial in the efforts to evaluate the suitability of Mortierella sp. as an effective RP solubilizer in the rhizosphere.

The hypothesis of this experiment is that the effectiveness of PSM in dissolving RP may be influenced by the N form present in the growth medium. This effectiveness may be enhanced if the PSM is supplied with NH$_4^+$ as the sole
source of N. The objective of the current investigation was to assess the effects of N form (NH$_4^+$ or NO$_3^-$) on RP solubilization activity of Mortierella sp. under in vitro conditions.

**MATERIALS AND METHODS**

*Mortierella* sp. was originally isolated from an Andisol of Hawaii (Osorio and Habte, 2001) and maintained on YMA slants at 4°C. For this study, the fungus was multiplied in Petri dishes on YMA medium for three days at 28°C. Mycelia were removed from the surface of the agar with a sterile loop and suspended in sterile deionized water and shaken by hand until the clumps were dispersed.

One-mL of a *Mortierella* sp. suspension containing 5.9x10$^5$ CFU was aseptically transferred into 250-mL Erlenmeyer flasks that containing 75-mL of an autoclaved (30 min., at 120°C and 0.1 MPa) liquid medium. The medium consisted of 1.0 g NaCl, 0.2 g CaCl$_2$.2H$_2$O, 0.4 g MgSO$_4$.7H$_2$O, 28 mg Fe-EDTA, 28 mg Cu-EDTA, 28 mg Mn-EDTA, 14 mg Zn-EDTA, 10.0 g glucose, and 3.5 g of Huila RP per liter. The RP was passed through a 0.5-mm aperture sieve. The P content of the Huila RP was 130 g kg$^{-1}$, and its empirical formula is Ca$_{9.69}$Na$_{0.22}$Mg$_{0.08}$(PO$_4$)$_{6.14}$(CO$_3$)$_{0.88}$F$_{2.34}$ (Chien and Hammond, 1978). The medium contained 0.35 g N/L, the source of N was either NH$_4$NO$_3$ (1.0 g L$^{-1}$), NH$_4$Cl (1.34 g L$^{-1}$), or KNO$_3$ (2.53 g L$^{-1}$). The flasks containing NH$_4$NO$_3$ and NH$_4$Cl also received KCl (1.87 g L$^{-1}$) in order to supply similar amount of potassium in all treatments. The initial solution pH was adjusted with 0.1 M
NaOH to pH 7.6. Flasks were continuously shaken at 150 rpm on an orbital shaker (model Innova 4400, New Brunswick Scientific Co., Inc., Edison, NJ) at 25°C for seven days.

After the incubation period, 50 mL of the suspension was pipetted into plastic tubes for centrifugation at 5000xg for 15 minutes. The supernatant was filtered through a Whatman No. 42 filter paper followed by filtration through a membrane filter (0.45 µm). Solution pH was measured with a pH-meter. Solution P concentration was determined using the molybdate-blue method (Murphy and Riley, 1962). The fungal mats were transferred onto a filter paper, oven-dried (60°C for 48 h), and weighted for fungal dry weight (FDW) determination after removal of remaining RP particles. Fungal P content (FPC) was determined by the molybdate-blue method after dry-ashing samples in a muffle furnace at 500 °C for 3 hours and dissolving the ash in one mL of 1 M HCl and then bringing up the solution to 10 mL with deionized water. Total P solubilized (TPS) by Mortierella sp. consisted of the sum of soluble-P and fungal-P.

Treatments were arranged in a completely randomized design and consisted of three different N sources (KNO₃, NH₄NO₃, or NH₄Cl), and there were four replicates per treatment. Analyses of variance and Duncan multiple range test were used to evaluate the significance of treatment effects ($P$-value ≤ 0.05). Data were analyzed by means of the software package Statgraphics, version 4.0 (Statpoint, Inc., Herdon, Virginia).
RESULTS

The effect of N source was significant for all measured variables (Table 6.1). Solution pH was significantly lower when Mortierella sp. was supplied with N was supplied as ammonium than as nitrate (Figure 6.1). When the fungus grew in the presence of NH$_4$Cl or NH$_4$NO$_3$ the pH went down to 3.37 and 3.67, respectively, while in the presence of KNO$_3$ the pH went down only to 6.69. There was an inverse relationship between pH and P concentration in the culture medium inoculated with the fungus. In the presence of Mortierella sp. the concentrations of P in solution were 0.08, 109.25, and 129.65 mg/L if the N source was KNO$_3$, NH$_4$NO$_3$, and NH$_4$Cl, respectively (Figure 6.2). Despite the very high RP solubilizing activity noted in the presence of NH$_4^+$ compared to that observed in the presence of NO$_3^-$, fungal dry mass was significantly higher when KNO$_3$ was the sole source of N (Figure 6.3). Fungal P content (mg/flask) did not show significant differences as a function of the N sources. However, the fungal P concentration (%) was significantly higher in the presence of NH$_4$Cl or NH$_4$NO$_3$ than in the presence of KNO$_3$ (1.93, 2.00, and 1.71%, respectively) (Figure 6.4). That is, Mortierella sp. absorbed more P when the sole source of N was NH$_4$Cl or NH$_4$NO$_3$. The total amount of P solubilized by Mortierella sp. (P in fungal mycelium and P remaining in solution) clearly showed that significantly more P was solubilized if N was supplied as NH$_4^+$ than if it was supplied as a mixture of the two ions (Figure 6.5).

The relative proportion of soluble-P and fungal-P significantly varied with N source. When Mortierella sp. was supplied with NH$_4$Cl, the P remaining in
solution represented 78% of the total solubilized P, compared to only 0.2% if the N source was KNO₃. If N was supplied as NH₄NO₃ 75% of the total P solubilized remained in solution (Figure 6.5). The fraction of Pi immobilized by Mortierella sp. in the presence of the different N sources was in the order KNO₃ > NH₄NO₃ > NH₄Cl.

DISCUSSION

The results of this study clearly showed that in the presence of NH₄⁺ Mortierella sp. acidified the growth medium to a greater extent than in the presence of NO₃⁻; most likely by increasing proton-pumping in the fungal cell membrane (Cooke and Whipps, 1993; Slayman et al., 1990). The fraction of RP dissolved by Mortierella sp. varied with the N form in the liquid medium, this was 37, 32, and 8% when NH₄⁺, NO₃⁻ and both ions were supplied, respectively (Figure 6.5). The positive effect of NH₄⁺ as N source on RP solubilization observed has been reported previously (Whitelaw, 2000; Cerezine et al., 1988).

It is very well known that microbial cells must keep an internal balance of electrical charges in order to maintain a functional cell membrane (Hall et al., 1995). This is achieved by maintaining a near neutral pH and a more negatively charged cytoplasm than the external solution (Hall et al., 1995; Slayman et al., 1990). Since N is a major nutrient for fungi (Griffin, 1993), the form in which it is taken up by fungal cells can shift the electrical charge in the cytoplasm one way or the other. Since the net charge in the cytoplasm must remain constant,
imbalance caused by the uptake of excess cations must be countered by a very active proton-pumping, which expels H\(^+\) into the external medium through a K\(^+\)/H\(^+\) antiport mechanism (Griffin, 1993).

Furthermore, the assimilation of NH\(_4^+\) in the fungal cell for amino acid synthesis could reduce the cytosolic pH because NH\(_4^+\) is converted to NH\(_3\) and the excess H\(^+\) is introduced into the cytoplasm (Garraway and Evans, 1984). This H\(^+\) is released into the external solution to maintain cytoplasmic pH, acidifying the medium surrounding the fungal cells and thereby favoring RP dissolution. Although NH\(_4^+\) is a nutrient for fungi, it can become toxic at high concentrations (Garraway and Evans, 1984). One problem due to an excessive level of NH\(_4^+\) is its interference with the uptake of other fungal nutrients (Mg\(^{2+}\), Na\(^+\), Ca\(^{2+}\), etc.) as clearly seen in plants (Marschner, 1997). However, the most negative effect of NH\(_4^+\) is probably its interference with the electrochemical gradient that must be maintained between the cytoplasm and the external medium (Slayman et al., 1990; Garraway and Evans, 1984).

It is possible that excess positive charge in the cytoplasm may also trigger the synthesis of organic anions (Davies, 1986) that later can be released in the cytoplasm in order to balance charges. I detected the capacity of Mortierella sp. to produce oxalic acid/oxalate (Appendix H). Organic acids/anions are commonly synthesized in the Kreb’s cycle or from compounds derived from it to be used for normal metabolic functions and to maintain fungal growth (Gadd, 1999; Griffin, 1993). However, during periods of stress imposed by an excess of positive charges, these compounds could be used for the purpose of charge-balancing.
This hypothesis may explain the lower dry mass of Mortierella sp. observed when the fungus was supplied with NH$_4^+$ compared to when it was supplied with NO$_3^-$ as a sole source of N (Figure 6.3).

The final outcome of the decrease in pH and the release of oxalic acid or oxalate associated with NH$_4^+$ assimilation by Mortierella sp. is an increase in RP dissolution. The phenomenon is explained by the Apatite dissolution reaction (Lindsay, 2001) which is given below:

$$\text{Ca}_5(\text{PO}_4)_3\text{OH} + 7\text{H}^+ \leftrightarrow 3\text{H}_2\text{PO}_4^{2-} + 5\text{Ca}^{2+} + \text{H}_2\text{O} \quad (K=10^{14.5})$$

Increasing H$^+$ drives the reaction to the right, so that Apatite (RP) dissolves and releases H$_2$PO$_4^{2-}$. The release of oxalate into the growth medium by Mortierella allows Pi to remain available by tying up Ca$^{2+}$, a Pi-fixing cation, as an organic complex (log $K$= 3.44). Welch et al. (2002) found that the dissolution of apatite was favored by the formation of Ca$^{2+}$-oxalate complex due to production of oxalate by microorganisms. This reaction occurred not only in solution but also at the mineral surface.

The potential practical implications of these results could be appreciable if farmers manage N fertilizer application with the view of enhancement of RP solubilization by PSM and prevention of re-adsorption of Pi. Increasing the efficacy of nitrification inhibitors will be part of the management strategy. This effort, of course, must take into account the fact that excess of NH$_4^+$ can affect plant growth negatively (Osorio et al., 2003). This negative effect on plant growth
should reduce the amount of root exudates on which the RP solubilization activity is dependent (Lynch and Ho, 2005).

On the other hand, an excess of H⁺ in the external medium could reduce the fungal Pi uptake since fungal Pi uptake is accomplished by an H⁺-symport mechanism (Griffin, 1993). As a result of this more Pi will remain in solution. Sugar uptake might be affected in a similar manner (Garraway and Evans, 1984), which is also a likely explanation for lower growth of Mortierella sp. when grown with NH₄Cl as N source.

The benefits that a plant could derive from a PSM lies in the ability of the microorganism to increase the soil solution P in the rhizosphere. This ability will hardly occur if Mortierella sp. is supplied with NO₃⁻ as the sole N source because not only the fungus is ineffective in dissolving RP, but also immobilizes the small amount of Pi released in solution under this condition. From the standpoint of plant P nutrition this relationship is as important as P solubilization per se.
Table 6.1. Analysis of variance for the variables studied as a function of N source.

<table>
<thead>
<tr>
<th>Source</th>
<th>Solution pH (mg/L)</th>
<th>Solution P (mg/flask)</th>
<th>FDW concentration (%)</th>
<th>Fungal P Content (mg/flask)</th>
<th>Fungal P Solubilized (mg/flask)</th>
<th>Total P Solubilized (mg/flask)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>0.0056</td>
<td>0.01</td>
<td>NS</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.1</td>
<td>4.7</td>
<td>7.3</td>
<td>5.7</td>
<td>8.4</td>
<td>5.1</td>
</tr>
</tbody>
</table>


Figure 6.1. Solution pH of a liquid medium inoculated with *Mortierella* sp. as a function of N source.
Solution pH

<table>
<thead>
<tr>
<th>N source</th>
<th>KNO₃</th>
<th>NH₄NO₃</th>
<th>NH₄Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>CV</td>
<td>4.1%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The bar graph shows the pH levels of different N sources: KNO₃, NH₄NO₃, and NH₄Cl. The pH levels are indicated by the bars, with KNO₃ having the highest pH level.
Figure 6.2. Solution P concentration (mg/L) of a liquid medium inoculated with *Mortierella* sp. as a function of N source.
Solution P (mg/L)

KNO$_3$  | NH$_4$NO$_3$  | NH$_4$Cl

N source

CV = 4.7%
Figure 6.3. Fungal dry weight (FDW) of Mortierella sp. as a function of N source.
FDW (mg/flask)

<table>
<thead>
<tr>
<th>N source</th>
<th>FDW (mg/flask)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNO₃</td>
<td>160</td>
</tr>
<tr>
<td>NH₄NO₃</td>
<td>b</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>b</td>
</tr>
</tbody>
</table>

CV = 7.3%
Figure 6.4. Fungal P content (FPC, mg/flask) and fungal P concentration (%) of Mortierelia sp. as a function of N source.
Figure 6.5. Total P solubilized by Mortierella sp. as a function of N source. Solution-P in white columns and fungal-P in black columns.
Soluble P

Fungal P

N source

CV = 5.1%
LITERATURE CITED


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CHAPTER SEVEN

THE REACTION OF Mortierella sp., A PHOSPHATE SOLUBILIZING FUNGUS, TO ACIDITY AND ALUMINUM UNDER IN VITRO CONDITIONS

ABSTRACT

Although Mortierella sp. is an effective phosphate solubilizing fungus (PSF) its use in phosphate (Pi) deficient soils may be limited by soil acidity and high levels of Al$^{3+}$, two major soil constraints in the tropics. For that reason, an in vitro investigation was conducted in order to determine the tolerance of the fungus to acidity and high levels of free-Al$^{3+}$. With this purpose a liquid medium was adjusted with 0.1 M HCl or NaOH to obtain nine initial pH values ranging from 3.0 to 7.0. In a separate experiment, a liquid medium received graded amounts of AlCl$_3$ in order to achieve seven initial levels of free-Al$^{3+}$ activity ranging from 0.0 to 3.0 mg/L at pH 4.0. Dry mass of Mortierella sp. decreased significantly as pH decreased, particularly below pH 5.0. However, its P uptake was unaffected by the initial pH value. On the other hand, increases in the concentration of free-Al$^{3+}$ in solution did not affect Mortierella sp. dry mass and this was associated with increases in P uptake by the fungus. The results indicate that this fungus was sensitive to H$^+$, particularly below pH 5.0, and was highly tolerant to Al$^{3+}$ toxicity. The tolerance of Mortierella sp. to Al$^{3+}$ can be associated to its capacity to produce oxalic acid which can form stable complex Al-oxalate. Therefore, its eventual use should be limited to agricultural soils that normally exhibit pH above 5.0.
INTRODUCTION

Phosphate solubilizing microorganisms (PSM) have a potential use in the management of soil Pi deficiency (Whitelaw, 2000; Peix et al., 2001; Omar, 1998; Kucey and Legget, 1989). These microorganisms can accelerate the dissolution of rock phosphates (RP) and, thereby, increase the supply of plant-available Pi (Vyas et al., 2007; Delvasto et al., 2006; Wakelin et al., 2004 a,b; Reddy et al., 2002).

Most of the research on PSM has been conducted in neutral or alkaline soils and little has been done in strongly acidic soils. One of the concerns is whether or not PSM can carry out their functions in the presence of high concentrations of exchangeable and soluble Al\(^{3+}\) (Vyas et al., 2007), a condition commonly found in P deficient soils of the tropics (Sanchez and Logan, 1992; Wolt, 1994).

We hypothesize that PSM may exhibit tolerance to acidity and Al toxicity. The production of oxalic acid /oxalate with which this fungus solubilize RP can protect it against Al toxicity. These organic acids/anions are not only responsible for P solubilization (Chen et al., 2006; Takeda and Knight, 2006; Radersma and Grierson, 2004; Welch et al., 2002) but also responsible for Al tolerance in plants (Hue, 1991; Bolan et al., 1994; Miyasaka et al., 1991; Trolove et al., 2003). The objective of the current work was to evaluate the tolerance of Mortierella sp. to acidity and Al toxicity in a liquid medium.
MATERIALS AND METHODS

Acidity tolerance experiment

Seventy-five-mL of a liquid medium containing 1.0 g NH₄NO₃, 1.0 g NaCl, 0.2 g CaCl₂·2H₂O, 0.4 g MgSO₄·7H₂O, 1.87 g KCl, 28 mg Fe-EDTA, 28 mg Cu-EDTA, 28 mg Mn-EDTA, 14 mg Zn-EDTA, 10.0 g glucose, and 0.5 g KH₂PO₄ (as in the YMA medium) per liter were transferred into 250-mL Erlenmeyer flasks. The medium pH was adjusted with drops of 0.1 M NaOH or 0.1 M HCl to obtain an initial pH range from 3.0 to 7.0. The flasks and their contents were autoclaved at 120°C, 0.1 MPa for 30 minutes and then inoculated with one-mL a fungal suspension containing 6.2x10⁶ CFU of Mortierella sp.

The fungus was originally isolated from an Andisol of Hawaii (Osorio and Habte, 2001), multiplicated and stored in YMA slants at 4°C. For this study, the fungus was multiplied in Petri dishes on YMA medium for three days at 28°C. Then its mycelia were removed with a sterile loop and suspended in sterile deionized water and shaken by hand until the clumps were dispersed. Then this fungal suspension was aseptically transferred into the liquid medium as explained above. Flasks were continuously shaken on an orbital shaker (model 4400, New Brunswick Scientific Co., Inc., Edison, NJ) at 100 rpm at 25°C for 7 days. Treatments consisted of nine pH levels with three replicates per pH level, arranged in a completely randomized design.
After the incubation period, the liquid medium was filtered through a Whatman No. 42 filter paper placed in a Buchner funnel. Vacuum was applied at 350 mm-Hg. Fresh fungal mass of Mortierella sp. was collected on the filter paper and then dried in an oven at 60°C during 48 hours for the fungal dry mass determination. Final solution pH of the filtrates was determined by means of a pH-meter. Solution P concentration was measured by the molybdate-blue method (Murphy and Riley, 1962). Fungal P content was measured after ashing mycelium sample in a muffle furnace at 500°C for 3 hours. The ash was dissolved in one-mL of 0.1 M HCl and then diluted with nine-mL of deionized water. The P content was determined by employing the molybdate blue-method (Murphy and Riley, 1962).

Testing for aluminum tolerance

Seventy five-mL of a liquid medium containing 1.0 g NH₄NO₃, 1.0 g NaCl, 0.2 g CaCl₂.2H₂O, 0.4 g MgSO₄.7H₂O, 1.87 g KCl, 28 mg Fe-EDTA, 28 mg Cu-EDTA, 28 mg Mn-EDTA, 14 mg Zn-EDTA, 10.0 g glucose, and 0.1 g KH₂PO₄ per liter were transferred into 250-mL Erlenmeyer flasks. Based on the software Species (Barak, 1990), the amount of AlCl₃ were estimated to achieve seven initial activity values of free-Al³⁺ ranging from 0.0 to 3.0 mg/L. The medium pH was adjusted with drops of 0.1 M NaOH or 0.1 M HCl to obtain an initial pH of 4.0. The flasks and their contents were autoclaved at 120°C, 0.1 MPa for 30 minutes. One-mL of a fungal suspension containing 6.2x10⁶ CFU of Mortierella
sp. was aseptically transferred into the liquid medium. *Mortierella* sp. was multiplied as mentioned above. Flasks were continuously shaken on an orbital shaker (model 4400, New Brunswick Scientific Co., Inc., Edison, NJ) at 100 rpm at 25°C for 7 days. Treatments consisted of seven levels of free-Al³⁺ activity arranged in a completely randomized design with three replicates/treatment.

After the incubation period, the liquid medium was filtered with a Whatman No. 42 filter paper placed in a Buchner funnel. Vacuum was applied at 350 mm-Hg. Fresh fungal material of *Mortierella* sp. was concentrated on the filter paper and then oven dried at 60°C for 48 h for fungal dry mass determination. Final pH of the growth solution was determined by means of a pH-meter. Solution P concentration was measured by the molybdenum blue method (Murphy and Riley, 1962). Fungal P content was determined after drying mycelium samples (3-5 mg), and ashing them in a muffle furnace at 500°C for 3 hours. The ash was dissolved in one-mL of 0.1 M HCl and then diluted with nine-mL of deionized water. Phosphorus content of samples was determined by employing the molybdate blue method (Murphy and Riley, 1962).

**Statistical analysis**

Data were subjected to analyses of variance (ANOVA) and regression analyses (*P*-value ≤ 0.05), with the aid of the software Statgraphics Plus, version 4.0 (Statpoint, Inc.; Herdon, Virginia).
RESULTS

_Acidity tolerance_

_Mortierella_ sp. significantly reduced the pH of the growth medium and the final pH values observed ranged from 3.0 to 4.0 regardless of the initial pH (Figure 7.1). The fungus was sensitive to low initial pHs; its dry mass was significantly reduced at low-initial pH levels, particularly below 5.0 (Figure 7.2). The best fungal growth was detected at pH 7.0 (157.4 mg/flask) while the lowest one was observed at pH 3.0 (93.1 mg), which represented 56% of the relative fungal dry weight observed at pH 7.0.

However, fungal P content of _Mortierella_ sp. was not significantly affected by the initial pH (Figure 7.3 A). Fungal P concentration was significantly higher at low initial pH values (Figure 7.3 B) and decreased as initial pH increased. The P concentration in the fungus was very high ranging from 3.2 to 6.2%.

_Aluminum tolerance_

Dry mass of _Mortierella_ sp. was not affected by any of the _Al^{3+}_ activity levels tested (0.0-0.3 mg/L) (Figure 7.4). The final pH ranged from 3.5 to 4.0 and it was not significantly affected by the treatments. Furthermore, both fungal P content and concentration of uptake by _Mortierella_ sp. increased with the increase in _Al^{3+}_ activity (Figure 7.5 A, B).
Fungal cell materials tended to form larger aggregates as the concentration of free-Al$^{3+}$ increased (Figure 7.6). In the absence of Al, the size of fungal aggregates was lower than 1 mm in diameter while at 3.0 mg/L of free-Al$^{3+}$, the diameter of the fungal aggregates increased to 4.5 mm.

DISCUSSION

The results indicate that *Mortierella* sp. is highly Al tolerant and moderately sensitive to low pH. The differences found regarding the degree of Al and acidity tolerance may be due to different mechanisms exhibited by the fungus against these constraints. The fungal tolerance to high levels of H$^+$ seems to be associated with the capacity of the fungus to expel H$^+$ from the cytoplasm to the external medium (Booth, 1985; Neidhardt et al., 1990). In this way, the fungus can maintain a negative charge and nearly neutral pH in the cytoplasm. This mechanism allows the fungus to maintain an electrochemical gradient across the cell membrane required for nutrient uptake (Garraway and Evans, 1984; Slonczewski et al., 1981). Proton pumping is facilitated if the external medium is neutral or slightly acidic (Griffin, 1993; Cooke et al., 1993); however, in highly acidic environments, as imposed in the current study (pH<4.0), the maintenance of neutral electrical charge in the cytosol is made difficult by the unfavorable H$^+$ gradient (Slayman et al., 1990) most likely requiring the expenditure of additional energy (Griffin, 1993). This hypothesis might well explain the decrease in *Mortierella* sp. growth observed under very acidic conditions (Figure 7.2).
On the other hand, the most probable mechanism of Al-tolerance of this fungus is the exudation of oxalic acid/oxalate (appendix H) that forms stable complexes with \( \text{Al}^{3+} \) (Gadd, 1999; Beveridge et al., 1997). It is highly probable, therefore, that free \( \text{Al}^{3+} \) did not reach the fungal cytoplasm. That some soil microorganisms can produce organic anions has been amply demonstrated (Fransson et al., 2004; Gyaneshwar et al., 2002). These organic acids/anions are also responsible for plant tolerance to Al (Jemo et al., 2007; Mariano and Keltjens, 2003; Marschner, 1997; Miyasaka et al., 1991). Fungi are particularly very efficient in the production of low molecular weight organic acids (Gadd, 1999). However, not all PSM that are capable of RP dissolution are Al tolerant. For instance, Vyas et al. (2007) found that the growth and RP dissolution capacity of the PSF *Eupenicillium parvum* were reduced at high levels of \( \text{Al}^{3+} \) in solution.

The positive relationship observed between \( \text{Al}^{3+} \) concentration in solution and the size of *Mortierella* mycelial aggregates in the growth medium (Figure 7.6) is interesting, although its significance is difficult to ascertain. Aluminum is known to flocculate organic materials and mineral particles in water (Brady and Well, 1999). It appears that \( \text{Al}^{3+} \) is flocculating the mycelium of *Mortierella* sp. into large aggregates, although there is no evidence in the published literature to this effect. Perhaps, the tolerance of *Mortierella* to \( \text{Al}^{3+} \) may also be related to the binding of the cation to fungal cell wall. This mechanism is known to be responsible in the protection of fungi against toxicity of heavy metals such as Mn, Cu, and Zn (Ross, 1993). There are some indications that arbuscular mycorrhizal
fungi protect associated host plants against aluminum toxicity by sequestering the cation in their hyphae. Binding Al$^{3+}$ to root cell walls is also the mechanism by which plants are protected against aluminum toxicity (Marschner, 1997). This mechanism of protection may be secondary to oxalic acid production detected (Appendix H).

On the other hand, the fact that the fungal P uptake was unaffected by low levels of initial pH (Figure 7.3) and favored in the presence of high levels of Al$^{3+}$ activity (Figure 7.5) is intriguing. This is unexpected because part of the Pi applied to the growth medium is bound to be complexed by Al species, resulting in very low concentration of Pi in solution. The mechanisms that lie behind these phenomena are unknown and were not considered in this investigation. Hall et al. (1995) indicated that fungi expel H$^+$ by a K$^+$/H$^+$ antiport mechanism, whereas Griffin (1993) and Garraway and Evans (1984) point out that the absorption of K$^+$ can facilitate the fungal Pi uptake. Whether or not these mechanisms were connected to favor fungal Pi uptake under low pH has to be investigated. In any case, the fungal Pi uptake as well as mycelial P concentration data suggest that Mortierella sp. has an extraordinary capacity to take up Pi and accumulate it at concentrations far in excess (10-20 times) of those fungi are known to normally accumulate (Madigan et al., 1997).

The survival of Mortierella sp. at low pH levels in the presence of high levels of free-Al$^{3+}$ makes it an ideal PSM for acid soils containing toxic levels of Al$^{3+}$. 

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Figure 7.1. Final pH of the liquid medium as a function of initial pH seven days after inoculation with Mortierella sp.
Figure 7.2. Fungal dry weight (mg/flask) as a function of the initial pH, seven days after inoculation with *Mortierella* sp.
Fungal dry mass (mg/flask)

\[ y = 40.429 + 16.273x \]

\[ P < 0.001 \]

\[ r^2 = 0.937 \]

CV = 4.8%
Figure 7.3. Fungal P content (mg/flask) (A) and Fungal P concentration (%) (B) as a function of the initial pH of the liquid medium, seven days after inoculation with Mortierella sp.
A. 

Fungal P content (mg/flask) vs. Initial pH

B. 

Fungal P concentration (%) vs. Initial pH

\[ y = 10.958 - 1.694x + 0.1105x^2 \]

\[ P < 0.001 \]

\[ r^2 = 0.704 \]
Figure 7.4. Fungal dry mass (mg/flask) as a function of the initial free-$\text{Al}^{3+}$ activity seven days after inoculation with Mortierella sp.
Fungal dry mass (mg/flask) vs. Initial free-Al^{3+} (mg/L) with CV = 11.4%.
Figure 7.5. Fungus P content (μg/flask) and concentration (%) as a function of the initial free-AI activity seven days after inoculation with Mortierella sp.
$y = 322.49 - 23.58x + 120.95x^2 - 31.81x^3$

$P < 0.001$

$r^2 = 0.735$

$y = 0.499 + 0.122x$

$P < 0.001$

$r^2 = 0.779$
Figure 7.6. Size of fungal aggregates (mm) as a function of the initial free-Al$^{3+}$ activity (mg/L) seven days after inoculation with Mortierella sp.
y = 0.694 + 2.118x - 0.247x²  
\[ P<0.001 \]  
\[ r^2 = 0.981 \]  

Initial free-AI³⁺ (mg/L)  
CV= 7.4%


ABSTRACT

A greenhouse experiment was carried out to determine the synergistic effects of a phosphate solubilizing fungus (*Mortierella* sp., PSF) and an arbuscular mycorrhizal fungus (*Glomus fistulosum*, AMF) in enhancing plant Pi uptake and growth of *Leucaena leucocephala* (Lam.) grown in an Oxisol fertilized with graded amounts of rock phosphate. For this purpose, a surface soil sample (0-20 cm) of a clay loam, kaolinitic, isohyperthermic Typic Haplustox was fertilized with six levels of the Huila rock phosphate (RP) (P: 0-2400 mg/kg) and inoculated with none, one or both fungi. Both microorganisms established in the leucaena rhizosphere. In the unfertilized soil leucaena plants grew poorly and there were not plant responses to individual or dual inoculation. When RP was added *G. fistulosum* significantly increased plant Pi uptake and growth, the effect of inoculation was significantly higher if the soil was amended with RP at the rate of 300-600 mg of P/kg. In contrast, *Mortierella* sp. inoculation was ineffective in increasing P uptake and growth of non-mycorrhizal leucaena across the RP gradient tested. However, *Mortierella* sp. was highly effective in increasing plant P uptake and growth in the presence of mycorrhizal association, particularly when RP was added at the rate 150, 300, and 600 mg of P/kg. The synergistic
effects of dual inoculation was more evident on plant Pi uptake than on growth. The results indicate that the synergistic effect of PSF and AMF was limited by low soil Ca-P content and the sorption of P by the soil. The first of these limitations was overcome through the use of moderate amounts of RP (P≤600 mg/kg), which increased the level of Ca-P in the soil, which is considered the P fraction most susceptible to solubilization by PSM through acid production. The second limitation was overcome by the mycorrhizal association, which allowed a more efficient capture of the Pi released due to RP solubilization. Phosphorus fractionation performed on soil samples from the rhizosphere at the end of the growth period indicated that only the Ca-P fraction was significantly diminished by inoculation with Mortierella sp., while other P fractions (Al-P and Fe-P) were unaffected. In some cases the Al-P fraction increased.

INTRODUCTION

Phosphate (Pi) deficiency is a major constraint to plant productivity in many soils of the world, particularly in highly weathered soils of the humid tropics and acidic savannas (Dabin, 1980; Mattlingly, 1975). In these environments, Pi can be strongly sorbed by soil minerals and/or precipitated with free ions in the soil solution (Al³⁺, Fe²⁺, Ca²⁺) (Jackman et al., 1997; Barber, 1995; Smith, 2002). Sanchez and Logan (1992) estimated that 1018 million ha in the tropics have a high Pi fixation capacity. Because of this problem, high rates of Pi fertilizers are required to achieve normal plant growth (Brady and Weil, 1999). Unfortunately,
this is very expensive and not always affordable in many under-developed countries (Randhawa et al., 2006; Yusdar et al., 2007; Havlin et al., 1999).

A viable alternative is the use of locally available rock phosphates (RP). However, the effectiveness of most of these materials is limited by their very low solubility (Yusdar et al., 2007; Chien and Hammond, 1978; Hammond and Leon, 1992). There is an increasing interest in developing strategies to improve RP effectiveness in tropical soils (Shrivastava et al., 2007; Ojo et al., 2007; Randhawa et al., 2006; Msolla et al., 2005). One of these strategies is increasing Pi supply to plants in these soils by inoculating them with arbuscular mycorrhizal fungi (AMF) (Marschner, 1997). The much finer and far more extensive external hyphae of mycorrhizal fungi are more efficient than root hairs in taking up Pi because they can explore more of the available soil volume than would be possible with root hairs alone (Barber 1995). However, mycorrhizal hyphae can only take up Pi that is already in solution (Cardoso et al., 2006; Bolan, 1991); hence, a high amount of RP is required to obtain significant benefits from a mycorrhizal-dependent plant Pi uptake (Satter et al., 2006; Ba and Guissou, 1996; Manjunath et al., 1989).

The quantity of RP required can be substantially reduced by increasing its solubility. One of the biological approaches of achieving this objective is through the use of RP solubilizing microorganisms (Vyas et al., 2007; Delvasto et al., 2006; Barea et al., 2002; Bar-Yosef et al., 1999). However, most of the research on PSM has been conducted in less weathered soils that exhibit low P sorption capacity (Mollisols, sandy soils) (Peix et al., 2001; Omar, 1998; Kucaey, 1983,
1987, 1988; Asea et al., 1988; Kucey and Legget, 1989; Gleedle, 1993) and little has been studied in highly weathered soils (Osorio and Habte, 2001) where the need to alleviate Pi deficiency is greatest. Moreover, most researchers working in the area have focused their attention on plant responses to inoculation with PSM and very few studies have been undertaken on the interaction of these microorganisms and soil factors that control P availability. Consequently, the effectiveness of PSM in increasing plant P uptake in highly weathered soils and the variables governing it are not clearly understood.

The hypotheses tested in the current study were: (i) in highly weathered soils, the effectiveness of PSM to increase plant Pi uptake and growth may be enhanced by the presence of mycorrhizal association, the extensive hyphal network of AMF would avoid the Pi re-sorption by soil minerals. (ii) The effectiveness of PSM can be limited by low soil calcium-P (Ca-P) content of these soils, and this deficiency can be ameliorated through the addition of RP. The objective of the investigation was to determine the synergistic interaction of a phosphate solubilizing fungus (PSF, Mortierella sp.) and a mycorrhizal fungus (Glomus fistulosum) in enhancing plant growth and Pi uptake by Leucaena leucocephala (Lam.) in an Oxisol fertilized with graded amounts of rock phosphate.

MATERIALS AND METHODS

Soil preparation
A surface soil sample (A horizon, 0-15 cm) was collected from the Agricultural Experimental Station of CIAT-Carimagua (4°34'N, 71°20'W) in the Eastern Plains of Colombia (kindly supplied by Carmen-Rosa Salamanca from the Colombian institute of Agriculture, Villavicencio). The soil was classified as a clay loam, kaolinitic, isohypertermic Typic Haplustox by the soil survey staff of the Colombian Geographic Institute “Agustin Codazzi” -IGAC- (1991). Chemical and physical properties of the soil were analyzed in the Soil Fertility Laboratory of the National University of Colombia at Medellin (Appendix A). I also determined the soil P isotherm of the soil (Fox and Kamprath, 1970) (Appendix D) and performed P fractionation on it following the procedure of Kuo (1996) (Table 8.1), which confirmed that the soil had low Ca-P content as previously reported by Benavides (1963). The soil was passed through a 4-mm diameter aperture sieve. Soil pH was adjusted to 6.0 with Ca(OH)₂ (Appendix C) following the procedure of Uchida and Hue (2000). Then the soil was mixed with quartzitic sand (3.3:1, W:W), autoclaved twice (120°C, 0.1 MPa for 1 hour) and transferred into polystyrene foam pots measuring 11.5-cm in diameter and 15.5 cm in depth, which were filled with 900 g (dry weight) of the mixture of soil and quartz.

**Inoculum preparation**

*Glomus fistulosum* Skou and Jakobsen was obtained from the Soil Microbiology Lab at the National University of Colombia. It was multiplied using a mixture of sorghum (*Sorghum bicolor*) and kudzu (*Pueraria phaseoloides*) grown
as nurse plants which were grown in a substrate composed of soil and quartzitic sand (2:1, by weight) for 4 months as described by Osorio and Habte (2001). The inoculum consisted of spores, hyphal fragments, and pieces of mycorrhizal roots in the soil-quartz matrix. The crude inoculum contained 40 infective propagules per g, which was determined by employing the most probable number (MPN) technique (Porter, 1979).

*Mortierella* sp. was originally isolated from an Andisol of Hawaii (Osorio and Habte, 2001) and has been maintained on YMA slants at 4°C. For this study, the fungus was multiplied in Petri dishes on YMA medium for three days at 28°C. Then its mycelium was removed with a sterile loop and suspended in sterile deionized water and shaken by hand until the clumps were dispersed. Plate counting on YMA medium indicated that there were $5.8 \times 10^6$ colony-forming-units/cm$^3$.

**Host plant**

Seeds of *L. leucocephala* cv. K-11 were scarified in concentrated H$_2$SO$_4$ for 30 minutes and then rinsed several times with deionized water. They were placed onto sterile moist paper towels to germinate for two days. Two germinated seeds were planted into each pot.
Treatments

At planting (March 4th 2006), the potted soil-sand mixture was amended with six P levels (0, 150, 300, 600, 1200, and 2400 mg/kg) using Huilla rock phosphate (HRP) as P source, which was passed through a 500-μm diameter aperture sieve. The P content of HRP was 130 g/kg, and its empirical formula, Ca₉.₆₉Na₀.₂₂Mg₀.₀₈(PO₄)₅.₁₄(CO₃)₀.₈₈F₂.₃₄, was obtained from Chien and Hammond (1978). Concurrently, the soil-sand mixture was either not inoculated or inoculated with 34 g per pot of the crude inoculum of G. fistulosum. The mycorrhizal inoculum was uniformly mixed with the upper-half of the potted soil. Uninoculated pots received 34 g of sterilized soil-sand mixture (1:1) along with 10 cm³ of washings from the crude inoculum after removal of mycorrhizal propagules with Whatman No. 1 filter paper.

Soil was also either uninoculated or inoculated with 10 cm³ of a suspension (in 5% glucose) containing 5.8x10⁶ colony-forming-units/cm³ of Mortierella sp., which was directly pipetted into the planting holes (2-cm diameter, 3-cm depth) prior to transplanting.

At planting, based on soil test I selected the following nutrient sources and rates to be applied (mg/kg of soil): 475 of (NH₄)₂SO₄, 240 of K₂SO₄, 414 MgO, 111 of Zn-EDTA, 55 of Cu-EDTA, 4 of Na₂B₉O₁₃·₄H₂O, and 0.9 of (NH₄)₆Mo₇O₂₄·₄H₂O. The nutrient sources were mixed thoroughly with substrate.

The plants were grown under natural light in the greenhouse of the Soil Microbiology Lab, National University of Colombia, Medellin, Colombia (6° 15’N,
75° 35' W, and 1495 m altitude). The soil-quartz mixture was watered to maintain it at 50-60% of maximum water holding capacity (moisture content of 39%). Twenty-five cm$^3$ per pot of P-free Hoagland's solution was applied to each pot once a week. Plants were harvested on April 20$^{th}$, 2006.

**Measured variables**

Phosphorus status of *L. leucocephala* leaves was monitored as a function of time by determining P content of the 4$^{th}$ pinnule (counting from the base of the pinna) of the youngest fully expanded leaf (Habte *et al.*, 1987) at 14, 24, 32, 37, and 47 days after transplanting. Plants were harvested at the end of 47 days of growth, shoots were dried at 70 °C for 48 hours before shoot dry weight was measured. **Shoot P content** was determined colorimetrically by the molybdate-blue method (Murphy and Riley, 1962) after dry-ashing samples at 500°C for 3 hours in a muffle furnace and dissolving the ash in 2.5-mL of solution containing 0.3 mM antimony potassium tartrate, 3.89 mM ammonium molybdate, 24.3 mM ascorbic acid, and 0.83 M H$_2$SO$_4$ and then diluting with 10-cm$^3$ of deionized water (Habte and Osorio, 2001). **Soil PI fractions** were determined on samples of rhizosphere soil. The P fractionation method used was that proposed by Kuo (1996). Briefly, triplicate samples of one-g (dry weight basis) were passed through a 2-mm aperture sieve and then subjected to sequential extraction in plastic 50-mL centrifuge tubes with 1 M NH$_4$Cl (0.5 h), 0.5 M NH$_4$F (1 h), 0.1 M NaOH (17 h), and 0.25 M H$_2$SO$_4$ (1 h). Centrifuge tubes were shaken for
durations indicated in parenthesis on an end-to-end shaker (Indulab, Medellin, Colombia). The reductant P fraction was not determined. According to Kuo, the NH₄Cl-P fraction corresponds to soluble and loosely bound Pi (Labile-Pi), the NH₄F-P fraction represents aluminum phosphate (Al-P), the NaOH-Pi fraction corresponds to iron phosphate (Fe-P), and the H₂SO₄-P corresponds to calcium phosphate (Ca-P). In addition, separate soil samples were suspended in triplicate in 0.01 M CaCl₂.2H₂O and shaken for one hour in order to estimate the fraction designated as soil solution Pi (Olsen and Sommers, 1982). After shaking, the respective suspensions were centrifuged for 15 min at 5000xg and then filtered through Millipore membrane filters (0.45 µm). Phosphorus concentration in the filtrates was determined by the molybdate-blue method (Murphy and Riley, 1982).

Mycorrhizal colonization of roots was determined after clearing roots with 10% KOH and staining them with 0.15 % acid fuchsin in lactic acid (Habte et al., 1987) and then estimating the proportion of root length colonized by AMF by the grid-line intersection method (Giovannetti and Mosse, 1980). Colony forming units of Mortierella sp. were counted in a selective yeast mannitol agar (YMA, 0.5 g of KH₂PO₄, 0.2 g of MgSO₄.7H₂O, 0.1 g NaCl, 10.0 g of mannitol, 1.0 g of yeast extract, 15.0 g of agar per liter) containing streptomycin sulfate (500 µg/g), benomyl (75 µg/g), and cycloheximide (100 µg/g) (N.W. Osorio, unpublished; Appendix F). In order to determine the extent of the rhizoplane colonized by Mortierella sp. Twenty root fragments of 1-cm length were randomly taken from the root system and transferred into petri-dishes that contained 15 cm³ of the
sterilized selective medium. The absence or presence of fungal growth around the root segments was tallied after which time the growth was examined under a Scanning Electron Microscope (JEOL, JSM-5910V model, Japan).

Shoot Zn and Cu contents were measured by triplicate, to this purpose shoots were ashed in a muffle furnace for 12 hours at 500°C. Then, the ashes were dissolved in 15 mL of 1.5 M HCl. The concentrations of Zn and Cu were determined using Atomic Absorption (Perkin Elmer™, AAAnalyst 300 model) at wave-lengths of 214 and 325 nm, respectively. Soil soluble fluoride (F⁻) was measured in 0.01 M CaCl₂ soil extracts according to the method proposed by Adriano and Doner (1982) and using a selective fluoride electrode (Orion™ 96-09). These analyses were conducted in the soil fertility lab of the National University of Colombia at Medellín.

Experimental design

The treatments consisted of a factorial combination of six levels of RP, two levels of mycorrhizal inoculation, and two levels of PSF inoculation (6×2×2) in a completely randomized design with four replicates per treatment. Data were subjected to analysis of variance (ANOVA) and the LSD test was employed for mean separation (P-value ≤0.05). The statistical package used was Statgraphics Plus version 4.0 (Statpoint, Inc.; Herdon, Virginia).
**Incubation study**

An incubation study was conducted concurrently with the above study in order to monitor the changes in the levels of different mineral P forms following the addition of HRP to the soil-quartz mixture. Triplicate samples of the autoclaved mixture of the Carimagua soil and quartz (500 g dry basis) were amended with HRP at the rate of 0-2400 mg/kg and incubated in 500-mL plastic pots. However, the soil was neither planted nor inoculated. The pots were incubated on a bench next to the planted pots in the greenhouse for 90 days. The soil was subjected to weekly cycles of wetting and drying. Deionized water was used to raise the soil moisture content up to 60% of the maximum water holding capacity during the wetting cycle. At the end of the incubation period, soil P fractionation was conducted (Kuo, 1996). For this study, a completely randomized design was employed. Data for each mineral P form were subjected to regression analysis with the software Statgraphics Plus, version 4.0.

**RESULTS**

**Incubation study**

The incubation study indicated that the addition of RP significantly increased the pool of Ca-P (0.25 M H₂SO₄-Pi) in the soil tested. The values ranged from 1.2 in the unfertilized soil to 58 mg/kg in the soil fertilized with the highest rate of RP
(Figure 8.1). The native Ca-P fraction represented only 5% of the total inorganic P, but with the addition of the highest rate of RP it was increased to 63%. The relationship between the Ca-P fraction and the quantity of P added as rock phosphate is expressed by the following regression model:

\[ \text{Ca-P (mg/kg)} = 4.681 + 0.0234X \text{(mg/kg)} \] \( (P<0.0001; r^2 = 96.3\%) \)

The model shows that 96% of the variability was explained by RP amendment.

However, the addition of RP also increased other P fractions. For instance, there was a significant increase in the AI-P fraction (extracted by 0.5 \( M \) NH\(_4\)F), which is described by the following regression model (Figure 8.1).

\[ \text{AI-P (mg/kg)} = 2.092 + 0.0014X \] \( (P<0.0001; r^2 = 91.3\%) \)

The overall increase in this fraction due to fertilization of soil with the highest level of RP was 2.7-fold.

The Fe-P fraction (extracted by 0.1 \( M \) NaOH) also significantly increased (1.3-fold) with the addition of RP. This fraction was dominant in the unfertilized soil (86%), but its relative proportion diminished progressively with the increase in RP fertilization, reaching a value of 31% with the highest level of RP added. The regression model for this fraction was:

\[ \text{Fe-P (mg/kg)} = 22.96 + 0.0069X - 0.000002X^2 \] \( (P=0.0018; r^2 = 51.3\%) \)

In contrast, the addition of RP did not significantly change the soil solution P concentration obtained after equilibration with 0.01 \( M \) CaCl\(_2\) (Figure 8.1). The values observed ranged between 0.010 and 0.012 mg P per L. The soil P fraction
extracted with 1 M \( \text{NH}_4\text{Cl} \) (labile-Pi) was also not significantly affected by RP addition (Figure 8.1).

**Plant responses**

Mycorrhizal colonization was only detected in the roots of plants inoculated with \( G. \) fistulosum (+AMF). However, the extent of mycorrhizal colonization was significantly increased by the addition of RP (Figure 8.2). In the unfertilized soil, the level of mycorrhizal colonization observed was 38%, but the value increased significantly to 58, 62, and 64% if RP was added at 150, 300, and 600 mg P/ kg, respectively. Mycorrhizal colonization was decreased significantly if the soil was amended with 1200 and 2400 mg of P/kg, and the values observed were 25 and 13%, respectively. Inoculation with Mortierella sp. significantly reduced the extent of mycorrhizal colonization at all levels of P added (Figure 8.2).

*Mortierella* sp. was detected only in root samples of plants grown in soil inoculated with the fungus (+PSF) (Figures 8.3, 8.4). Regardless of the level of added P, inoculation of soil with \( G. \) fistulosum significantly decreased the presence of Mortierella sp. on roots. In mycorhiza-free roots the relative frequency of Mortierella sp. on root surface was 58%, while the value observed in mycorrhizal roots was only 24% (Figure 8.3).

The P content of pinnules of leucaena was significantly influenced by treatments (Table 8.2). In plants grown in uninoculated soil, pinnule P content decreased drastically and typical symptoms of P deficiency such as chlorosis and
defoliation of pinnules of lower leaves occurred (Habte and Manjunath, 1987; Smith et al., 1992) were detected 25 days after planting. Plants inoculated only with Mortierella sp. exhibited similar symptoms, the P content of their pinnules being similar to that of plants grown in uninoculated soil (Figure 8.5).

In the unfertilized soil, pinnule P content of plants grown in soil inoculated with G. fistulosum (+AMF) declined progressively over time and P deficiency symptoms were also observed. However, when the soil was fertilized with RP (150-1200 mg P/kg) pinnule P content initially decreased but increased beginning on Day 25 and continued to increase until harvest (Figure 8.5). At the highest rate of P (2400 mg/kg), P pinnule P content of mycorrhizal plants continued to decline until Day 25, remaining constant thereafter.

Plants inoculated with both microorganisms exhibited a pattern similar to the one described above for mycorrhizal plants (+AMF), but had significantly higher pinnule P content at some sampling periods (37 and 47 days after transplanting) if soil was amended with RP at the rate of 150-600 mg P/kg (Figure 8.5). At harvest, dual inoculation increased pinnule P content by 31, 43, and 38 % over those of plants grown in soil inoculated with G. fistulosum alone (Figure 8.6).

Uninoculated plants had very low shoot dry weight, although they responded significantly to RP applications up to 1200 mg of P/kg (Figure 8.7). When soil was inoculated only with Mortierella sp. they exhibited a similar pattern and shoot dry mass was not significantly different from that of plants grown in soil not inoculated with the fungus.
Inoculation with *G. fistulosum* significantly increased shoot dry weight if soil was fertilized with RP, reaching a peak value at 600 mg of P/kg (Figure 8.7). Shoot dry weight of plants grown in soil inoculated with AMF and PSF followed the same pattern as that of plants grown in soil inoculated with AMF alone. However, when P was added at the rate of 300 mg/kg, shoot dry weight of plants grown in dually inoculated soil was significantly higher (by 16.2%) than those grown in soil inoculated with AMF alone. At 1200 and 2400 mg of P/kg, dual inoculation of soil significantly decreased shoot dry weight compared to inoculation with AMF alone.

Uninoculated plants exhibited a very low P content (<0.6 mg/shoot) regardless the rate of RP applied (Figure 8.8). Shoot P content of plants grown in soil inoculated with *Mortierella* sp. alone did not increase at any of the rates of RP addition. On the other hand, inoculation of soil with *G. fistulosum* alone significantly increased shoot P content of plants grown in soil fertilized with RP, but not in unfertilized soil. At 600 and 1200 mg/kg of added P, the shoot P content of mycorrhizal plants reached 2.28 and 2.34 (mg/plant), respectively, which were significantly higher than values observed at other levels of RP (Figure 8.8). In the unfertilized soil, dual inoculation was as ineffective as not inoculating at all. However, dual inoculation significantly increased shoot P content (40 and 66%) beyond that obtained with mycorrhizal inoculation alone if RP was added at 150 and 300 mg of P/kg, respectively. At higher RP levels, the synergistic effect began to decline. Moreover, at 2400 mg/kg of P, inoculation of
soil with both fungi significantly decreased shoot P content compared to that observed in plants grown in soil inoculated with *G. fistulosum* alone.

At the end of the growth period, all soil P fractions except the CaCl₂-P (soil solution P) fraction increased significantly with the addition of RP (Table 8.2) (Figure 8.9). As expected, the increase was more notorious in the Ca-P fraction. In the absence of AMF, inoculation with *Mortierella* sp. significantly reduced the level of labile Pi fraction only if RP was applied at 150 and 1200 mg/kg. By contrast, the presence of AMF and PSF led to a significant increase in the level of this fraction of P if soil was amended with 1200 mg of P/kg (Figure 8.9). The fraction of P associated with Al-P in the rhizosphere of mycorrhizal leucaena was significantly increased if soil was inoculated with *Mortierella* sp. but not in mycorrhizal-free rhizosphere (Figure 8.9). The content of the P fraction associated with iron phosphate in the rhizosphere soil was unaffected by inoculation with *Mortierella* sp. (Figure 8.9). The fraction of Pi associated with calcium phosphate in the rhizosphere of leucaena was significantly reduced by the inoculation of soil with *Mortierella* sp. regardless of the presence or absence of mycorrhizal association and P level added (Figures 8.9 and 8.10).

Except in the unfertilized soil (P: 0 mg/kg), shoot Zn and Cu were significantly higher in mycorrhizal plants than in non-mycorrhizal plants. The effect was significantly higher at the P level of 600 mg/kg. However, the content of Cu was significantly lower when P was added at the rates of 1200 and 2400 mg/kg respect to the level found at 600 mg/kg (Table 8.4). The concentration of soluble
F⁻ in all soil extracts (0.01 M CaCl₂), regardless the RP level added and
AMF/PSF inoculation, was very low (<10⁻⁵ M, 0.1 mg/L).

DISCUSSION

Both G. fistulosum and Mortierella sp. were successfully established in the
rhizosphere of L. leucocephala after inoculation into soil (Figures 8.1, 8.2, and
8.3). The outcomes of their interaction at times had synergistic effect on the host
while its effect was nil or negative at other times, depending on the level of RP.
The poor growth of leucaena seedlings and the lack of response to microbial
inoculation in the unfertilized soil suggest that low soil Ca-P content was a
limiting factor for beneficial soil-plant-microbe interactions (Figure 8.5). This
inference is supported by the significantly positive growth responses observed if
the soil Ca-Pi content was increased by adding RP (150-600 mg/kg) (Figure 8.1).
Once the Ca-P inadequacy was removed Mortierella sp. was able to stimulate
plant P uptake. However, it is evident that Mortierella sp. was effective in
increasing plant P uptake and growth only in the presence of mycorrhizal
association (Figures 8.5, 8.6, 8.8).

Although inoculation with Mortierella sp. alone did not increase plant P
uptake of non-mycorrhizal plants (Figure 8.8), increases in Pi associated with the
labile and soluble and aluminum phosphate fractions in the rhizosphere suggest
that the fungus dissolved part of the RP added (Figure 8.9). This observation
illustrates the fact that dissolution of RP by Mortierella sp. does not necessarily
lead to enhanced P uptake by the host, the latter phenomenon being dependent on other variables such as the presence of effective mycorrhizal association and the Pi sorption capacity of the soil of interest.

The magnitude of the increase in Pi uptake by mycorrhizal plants due to inoculation with the PSF ranged between 40 and 60%. The presence of mycorrhizal hyphae insure that at least part of the Pi freshly released due to Mortierella activity is intercepted by AMF hyphae before it is re-sorbed by soil constituents. This phenomenon is particularly true for plant species such as L. leucocephala that are inefficient in taking up P from soil because of the few and short root hairs they have on the surface of their roots (Manjunath and Habte, 1991).

Synergistic effects between AMF and PSM have been observed in different plant species including sunflower (Gururaj and Mallikarjunaiah, 1995), cotton (Prathiba et al., 1995), rice (Mohod et al., 1991), chili (Sreenivasa and Krishnaraj, 1992), wheat (Gaur et al., 1990), alfalfa (Toro et al., 1998), tomato (Kim et al., 1998), and leucaena (Osorio and Habte, 2001; Young et al., 1990).

Data on the effectiveness of PSM to enhance non-mycorrhized plant Pi uptake in subtropical-tropical acidic soils is particularly rare and varied, with reported increases ranging from 8 to 25% (Young et al., 1990; Whitelaw et al., 1997; Osorio and Habte, 2001). Differences in the Pi sorption capacities of the soils used probably explain the different values reported and the differences between what was observed in the current investigation and what is reported in the literature. In less weathered soils characterized by low P sorbing capacities
PSM has been shown to increase plant P uptake of non-mycorrhizal plants (Peix et al., 2001; Omar, 1996; Kucey, 1983, 1987, 1988; Asea et al., 1988; Kucey and Legget, 1989; Gleddie, 1993). Most of the soils used by these authors were Mollisols, calcareous soils, or sandy soils, which are characterized by a low P sorption capacity and relatively high inherent Ca-Pi content (Crews et al., 1995; Cross and Schlesinger, 1995). In these types of soils, Pi released by PSM can remain in the soil solution long enough to be absorbed by roots (Table 8.3). Although the benefit of mycorrhizal fungi to plants in these soils can be very substantial (Muthkumar et al., 2001; Toro et al., 1996; Barea et al., 2002), the presence of the fungi in highly weathered soils of the tropics appears to be mandatory if Pi released from the dissolution of RP by PSM is to be prevented from re-adsorption by highly active Pi adsorbing surfaces in these soils.

Although the amendment of soil with RP was necessary for maximizing the effectiveness of dual inoculation on host P uptake and growth, the addition of very large quantities of RP (P: 1200 and 2400 mg/kg) curtailed the benefits that leucaena can obtain from dual inoculation with G. fistulosum and Mortierella sp (Figures 8.5, 8.6, 8.7, and 8.8). This negative effect appears to be related to a reduction in the Cu uptake by leucaena (Table 8.4). The low concentration of Cu (2 to 6 µg/g) is evident when the data are compared to those reported in leucaena by Habte and Manjunath (1991). The high release of Pi to the soil solution at very high rates RP added apparently was accompanied by a reduction in the Cu availability (Lindsay, 2001). Duponnois et al. (2006) also observed that the growth of the fungus Arthrobotrys oligospora (a PSF) was significantly
reduced as the concentration of the three rock phosphate increased in a *in-vitro* culture medium. However, this problem is not likely to occur under more realistic rates of rock phosphate addition (≤ 600 mg of P/kg). On the other hand, the very low concentration of soil soluble F\(^{-}\) (<10\(^{-5}\) M) found under all treatments suggest that this ion was not involved in the reduction of plant growth, mycorrhizal activity and fungal colonization on the roots at the two high levels of RP addition.

The positive interactions between RP application and mycorrhizal activity in tropical soils has been amply discussed elsewhere (Manjunath et al., 1989; Ba and Guissou, 1996; Lange and Vleck, 2000; Takacs et al., 2006). However, it must be understood that AMF do not participate in RP solubilization and that their primary function is to remove Pi that is released into the soil solution. This activity, of course, can further enhance the dissolution process, as the removal of Pi from solution will tend to shift the RP dissolution equation to the right. The dissolution of RP by PSM is carried out by two mechanisms that follow the solubility product principle. These mechanisms are the production of H\(^{+}\) and the formation of Ca\(^{2+}\)-complex by oxalic acid/oxalate released by *Mortierella* sp. as reported with other microorganisms (Whitelaw, 2000; Welch, 2002). The ineffectiveness of *Mortierella* sp. in the unfertilized soil thus can be explained by the inadequacy of Ca-P for the dissolution reaction. The lack of Ca-P fraction is a common feature that characterizes highly weathered soils (Sanchez, 1976; Hedley *et al*., 1994; Crews *et al*., 1995; Maroko *et al*., 1999; Trolove *et al*., 1996, 2003).
The lack of effectiveness in the unfertilized soil also represents an indirect evidence that the fungus cannot solubilize Fe-P even though it was present at relatively high concentration (22.4 mg/kg) (Table 8.1). With the addition of RP, and its further dissolution in the rhizosphere, the Fe-Pi content increased significantly (Figure 8.9). Despite this effect, inoculation of soil with Mortierella sp. did not alter the concentration of Fe-P (Table 8.2). The Al-P fraction was influenced in a similar manner. Moreover, in the presence of AMF, inoculation with Mortierella sp. led to significant increase in the concentration of the Al-P fraction, presumably due to a more active release of Pi from RP which was precipitated with Al, thus ruling out the availability of Al-P as a source of P for the microbial dissolution, at least in highly weathered soils. On the other hand, the significant decrease of the Ca-P fraction in the rhizosphere of plant inoculated with Mortierella sp. (Figure 8.10) suggest that it is the primary source of Pi that is acted upon by PSM.

Nevertheless, some authors have observed that PSM can solubilize Al-P and Fe-P compounds under in-vitro conditions (Illmer and Schinner, 1995; Toro et al., 1996). This is conceivable in the absence of soil minerals, but certainly not in highly weathered soils. The results of the earlier study of Bar-Yosef et al. (1999) lends support to this view. They noted that dissolution of RP in the presence of kaolinite by Pseudomonas cepacia decreased the pH of the growth medium to 3.0, which in turn favored the dissolution of Kaolinite and and the concomitant release of Al$^{3+}$. The presence of Al$^{3+}$ promoted the formation of Al-P at the end of the incubation. This, in fact, is expected to occur in soil (Lindsay, 2001;
Mahmoud et al., 1999). Thus the low solubility of Al-P and Fe-P under acidic conditions makes them poor candidates for solubilization by PSM in highly weathered soils.

Although both test microorganisms were established in the rhizosphere, they appeared to interact competitively for colonization sites on the rhizoplane (Figures 8.2 and 8.3). To my knowledge, this is the first report of a competitive interaction between AMF and PSM. Despite this antagonistic interaction between them, the effect they had on Pi uptake and growth was significantly better if they were present together than if only one or the other was present, at least in soil amended with moderate amounts of RP. On the other hand, the reduction in mycorrhizal colonization with the increase of RP level could be caused by the adverse effect of available Pi (Marschner, 1997; Habte and Osorio, 2001), particularly in the presence of Mortierella sp. It is also probable, that at the two highest levels of RP added the low availability of Cu could have some effect.

In summary, the effectiveness of Mortierella sp. to increase plant Pi supply in highly weathered soils appears to be limited by the low soil Ca-P content and the high soil P sorption capacity. These limitations can be overcome through (i) the application of moderate amounts of RP (P ≤ 500 mg/kg) in order to elevate the pool of P susceptible to dissolution by PSM, and (ii) inoculation with arbuscular mycorrhizal fungi in order to insure the efficient capture of the Pi (and Ca²⁺) released. Further research should focus on the screening of tropical soils, particularly Oxisols and Ultisols for conduciveness to the effectiveness of PSM. Work is also needed to predict the efficacy of PSM under field conditions.
Table 8.1. Soil P fractionation of the Carimagua soil according to the method of Kuo (1996).

<table>
<thead>
<tr>
<th></th>
<th>Labile-P $^1$</th>
<th>Al-P $^2$</th>
<th>Fe-P $^3$</th>
<th>Ca-P $^4$</th>
<th>Total mineral-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/kg</td>
<td>0.25</td>
<td>2.02</td>
<td>22.40</td>
<td>1.29</td>
<td>25.97</td>
</tr>
<tr>
<td>%</td>
<td>1.0</td>
<td>7.8</td>
<td>86.3</td>
<td>5.0</td>
<td>100.0</td>
</tr>
</tbody>
</table>

$^1$1 M NH$_4$Cl (0.5 h); $^2$0.5 M NH$_4$F (1 h); $^3$0.1 M NaOH (17 h); and $^4$0.25 M H$_2$SO$_4$ (1 h).

Table 8.2. Significant P-values of ANOVA test.

<table>
<thead>
<tr>
<th>Source</th>
<th>Pinnule P at (days after transplanting)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>14</td>
</tr>
<tr>
<td>Treatments</td>
<td></td>
</tr>
<tr>
<td>RP (A)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AMF (B)</td>
<td>NS</td>
</tr>
<tr>
<td>PSF (C)</td>
<td>NS</td>
</tr>
<tr>
<td>AxB</td>
<td>NS</td>
</tr>
<tr>
<td>AxC</td>
<td>NS</td>
</tr>
<tr>
<td>BxC</td>
<td>NS</td>
</tr>
<tr>
<td>AxBxC</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 8.2. (Continued) Significant P-values of ANOVA test.

<table>
<thead>
<tr>
<th>Source</th>
<th>SDW</th>
<th>SPC</th>
<th>MC</th>
<th>PSFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RP (A)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>NS</td>
</tr>
<tr>
<td>AMF (B)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PSF (C)</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AxB</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>NS</td>
</tr>
<tr>
<td>AxC</td>
<td>0.0367</td>
<td>0.0264</td>
<td>0.0002</td>
<td>NS</td>
</tr>
<tr>
<td>BxC</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AxBxC</td>
<td>0.0396</td>
<td>0.0085</td>
<td>0.0002</td>
<td>NS</td>
</tr>
</tbody>
</table>

SDW= shoot dry weight; SPC= shoot P content; MC= mycorrhizal colonization; PSFR= presence of Mortierella sp. on the rhizoplane.

253
Table 8.2. (Continued) Significant P-values of ANOVA test.

<table>
<thead>
<tr>
<th>Source</th>
<th>Soil solution P (CaCl₂-P)</th>
<th>Labile-P (NH₄Cl-P)</th>
<th>Al-P (NH₄F-P)</th>
<th>Fe-P (NaOH)</th>
<th>Ca-P (H₂SO₄)</th>
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<td></td>
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<tr>
<td>Treatments</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>RP (A)</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AMF (B)</td>
<td>NS</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>NS</td>
</tr>
<tr>
<td>PSF (C)</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>0.0371</td>
</tr>
<tr>
<td>AxB</td>
<td>NS</td>
<td>0.0214</td>
<td>0.0051</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AxC</td>
<td>NS</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>BxC</td>
<td>NS</td>
<td>0.0002</td>
<td>0.0001</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AxBxC</td>
<td>NS</td>
<td>0.0089</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>CV (%)</td>
<td>24.9</td>
<td>13.3</td>
<td>7.8</td>
<td>28.9</td>
<td></td>
</tr>
</tbody>
</table>

Table 8.3. Increases in plant P uptake due to the sole inoculation of soils/substrate with PSM.

<table>
<thead>
<tr>
<th>PSM</th>
<th>Soil</th>
<th>Plant</th>
<th>Increase in P uptake due to PSM (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low P sorbing soils</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arthrobotrys oligospora</td>
<td>Sandy soil of Senegal</td>
<td>Acacia holoserica</td>
<td>56 / 74</td>
<td>Duponnois et al., 2006</td>
</tr>
<tr>
<td>Pencillium radicum</td>
<td>Unfertilized/ RP fertilization</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesorhizobium mediterraneum</td>
<td>Sandy soils of Australia with neutral to alkaline soil reactivity</td>
<td>Wheat</td>
<td>34 to 76</td>
<td>Wake In et al., 2004</td>
</tr>
<tr>
<td>Enterobacter sp.</td>
<td>Calcereous Alfisol of Spain</td>
<td>Chickpea</td>
<td>100</td>
<td>Peix et al., 2001</td>
</tr>
<tr>
<td>Penicillium thomii</td>
<td>Vermiculite-perlite substrate</td>
<td>Alfalfa</td>
<td>103</td>
<td>Toro et al., 1998</td>
</tr>
<tr>
<td></td>
<td>fertilized with RP</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Table 8.4. Shoot Zn and Cu content of leucaena as a function of the P level added and mycorrhizal inoculation.

<table>
<thead>
<tr>
<th>P level (mg/kg)</th>
<th>Shoot Zn (mg/shoot)</th>
<th>Shoot Cu (mg/shoot)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-AMF</td>
<td>+AMF</td>
</tr>
<tr>
<td>0</td>
<td>3.4</td>
<td>3.1</td>
</tr>
<tr>
<td>150</td>
<td>3.0</td>
<td>6.0</td>
</tr>
<tr>
<td>300</td>
<td>3.6</td>
<td>7.2</td>
</tr>
<tr>
<td>600</td>
<td>3.3</td>
<td>8.8</td>
</tr>
<tr>
<td>1200</td>
<td>4.1</td>
<td>8.0</td>
</tr>
<tr>
<td>2400</td>
<td>4.8</td>
<td>8.3</td>
</tr>
<tr>
<td>LSD 0.05</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td>CV (%)</td>
<td>17.1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 8.1. Content of inorganic P forms in the Carimagua soil as a function of the level of P added (HRP used as P source) after an incubation period of 60 days.
Figure 8.2. Mycorrhizal colonization of roots of leucaena in soil amended with rock phosphate and not inoculated (-) or inoculated (+) with Gliomus fistulosum (AMF) and Mortierella sp. (PSF) at 47 days after transplanting.
LSD_{0.05} = 6.0

Mycorrhizal colonization (%)

Added P (mg kg\(^{-1}\))

CV = 22.9%
Figure 8.3. Presence of Mortierella sp. in the rhizoplane of leucaena in soil amended with rock phosphate and not inoculated (-) or inoculated (+) with Glomus fistulosum (AMF) and Mortierella sp. (PSF) at 47 days after transplanting.
LSD_{0.05} = 6.2

Inoculants (AMF x PSF)

M. robertsii, sp. in rhizoplane (%)
Figure 8.4. SEM-images of *Leucaena leucocephala* roots showing the presence of *Mortierella* sp. in the rhizoplane 47 days after transplanting. A = Uninoculated root, B = Inoculated root, C = Magnified view of the rhizoplane heavily colonized by the mycelium of *Mortierella* sp., D = Sporangia of *Mortierella* sp.
Figure 8.5. Pinnule P content of *Leucaena leucocephala* in soil amended with rock phosphate and not inoculated (-) or inoculated (+) with *Glomus fistulosum* (AMF) and *Mortierella* sp. (PSF) at different sampling dates after transplanting.
Figure 8.6. Pinnule P content of *Leucaena leucocephala* in a soil amended with rock phosphate and not inoculated (-) or inoculated (+) with *Glomus fistulosum* (AMF) and *Mortierella* sp. (PSF) at 47 days after transplanting. The presence of an asterisk indicates significant difference between the pair (+PSF vs. -PSF) compared.
LSD$_{0.05}$ = 1.17

**Ponule P (mg/pinnule)**

Added P (mg kg$^{-1}$)

CV = 24.3%
Figure 8.7. Shoot dry weight (SDW) of *Leucaena leucocephala* in a soil amended with rock phosphate and not inoculated (-) or inoculated (+) with *Glomus fistulosum* (AMF) and *Mortierella* sp. (PSF) at 47 days after transplanting. The presence of an asterisk indicates significant difference between the pair (+PSF vs. -PSF) compared.
Added P (mg kg⁻¹)

SDW (g/pot)

LSD_{0.05} = 0.12

CV = 15.6%
Figure 8.8. Shoot P content (SPC) of *Leucaena leucocephala* in a soil amended with rock phosphate and not inoculated (-) or inoculated (+) with *Glomus fistulosum* (AMF) and *Mortierella* sp. (PSF) at 47 days after transplanting. The presence of an asterisk indicates significant difference between the pair (+PSF vs. -PSF) compared.
LSD$_{0.05}=0.56$

CV=28.4%
Figure 8.9. Soil P fractions in the rhizosphere of *Leucaena leucocephala* as a function of the level of P added, mycorrhizal inoculation (AMF), and *Mortierella* sp. (PSF) inoculation 47 days after transplanting. The asterisks in the NH₄Cl-Pi fraction indicates significant differences between the pairs compared. NS: No significant differences.
Figure 8.10. Soil P fractions in the rhizosphere of *Leucaena leucocephala* as a function of *Mortierella* sp. (PSF) inoculation 47 days after transplanting. Different lower case letter above the column indicate significant differences ($P \leq 0.05$, LSD) between the pair compared (-PSF and +PSF). NS: not significant. The initial value for each soil P fraction is presented for comparison.
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CHAPTER NINE

ASSESSMENT OF THE EFFECT OF SOIL P SORPTION CAPACITY ON THE SYNERGISM BETWEEN A PHOSPHATE SOLUBILIZING FUNGUS AND AN ARBUSCULAR MYCORRHIZAL FUNGUS TO ENHANCE PLANT P UPTAKE AND GROWTH

ABSTRACT

A greenhouse experiment was carried out to evaluate the synergistic effect of a PSF (Mortierella sp.) and a mycorrhizal fungus (Glomus fistulosum) to enhance plant growth and Pi uptake of Leucaena leucocephala (Lam.) in soils differing in their P fixing capacity. The low P sorbing soil was represented by a Mollisol (Neira), while the high P sorbing soils were represented by three Andisols. The Andisols were fertilized with Hulla rock phosphate (RP) (at 300 mg P/kg) but no RP was added to the Mollisol. Inoculation of soil with Mortierella sp. alone was effective in increasing plant P uptake and growth in the Mollisol, but not in the three Andisols. Plant P uptake and growth of L. leucocephala was further increased by inoculation of the Mollisol with G. fistulosum compared to inoculation with the PSF alone. In contrast, L. leucocephala only exhibited a significantly positive but low level of response to inoculation with AMF in one of the three Andisols (Guarne, La Selva, and Naranjal: high Pi sorbing soils). Inoculation of the Neira and La Selva soils with both fungi led to significant synergistic effects on growth and nutrient uptake of Leucaena. The effect noted in the Neira soil was significantly higher than that observed in the La Selva soil.
None was observed in the other soils. The effect of inoculation of soil with Mortierella sp. with or without AMF was largely determined by the soil P sorption capacity.

INTRODUCTION

Phosphate (Pi) sorption is a serious problem in agricultural soils, particularly in those formed from volcanic ash (Andisols) (Shoji et al., 1993). In these soils, Pi is strongly sorbed by humus-Al/Fe complexes, allophane, ferrithydrate, and goethite (Jackman et al., 1997; Parfitt, 1989; Schwertmann and Herbillon, 1992). As a result, soil Pi availability is very low and limits plant growth and development (Buol et al., 1997). High rates of soluble Pi fertilizers are required to achieve high crop yields (Brady and Weil, 1999), and the cost associated with it is prohibitive to farmers in many developing countries (Randhawa et al., 2006; Yusdar et al., 2007; Havlin et al., 1999).

The use of locally available rock phosphates (RP) is highly recommended for these soils. However the low solubility of RP limits their effectiveness (Yusdar et al., 2007; Msolla et al., 2005). Mycorrhizal fungi can help increase the agronomic effectiveness of RP (Satter et al., 2006; Lange and Vleck, 2000; Ba and Guissou, 1996; Manjunath et al., 1989), because their hypha are more extensive than root hairs and can capture more Pi than non-mycorrhizal roots (Marschner, 1997; Mosse, 1981). However, mycorrhizal fungi do not solubilize RP, but merely absorb efficiently the Pi released from the RP dissolution (Lange and Vleck, 2000). The extent to which they can enhance plant P uptake is limited by factors
other than plant uptake that contribute to the removal of Pi from the soil solution. Many researchers have observed synergistic effects on plant P uptake and growth if mycorrhizal fungi and phosphate solubilizing microorganisms (PSM) are concurrently inoculated (Barea et al., 2002; Osorio and Habte, 2001; Whitelaw, 2000; Kim et al., 1998; Kucey, 1987). Phosphate solubilizing microorganisms dissolve RP by releasing (i) protons that attack the structure of RP (Vyas et al., 2007; Duponnois et al., 2006; Kim et al., 1998; Azcon and Barea, 1996) and (ii) organics acids/anions that form stable complex with Ca²⁺ (Welch et al., 2002). These mechanisms combined with the more efficient removal of Pi from the soil solution by mycorrhizal hypha favor the dissolution of RP further which can be illustrated through the help of the solubility product of hydroxyapatite described by Lindsay (2001):

\[
\text{Ca}_6(\text{PO}_4)_3\text{OH} + 7\text{H}^+ \leftrightarrow 3\text{H}_2\text{PO}_4^{2-} + 5\text{Ca}^{2+} + \text{H}_2\text{O} \quad K=10^{14.6}
\]

The removal of Ca²⁺ by complexation with organic anions and the removal of Pi by AMF further enhance the dissolution process by shifting the equation to the right.

Most of the research on PSM has been conducted in less weathered soils that exhibit a low P sorption capacity (Mollisols, sandy soils) (Peix et al., 2001; Omar, 1998; Kucey, 1983, 1987, 1988; Asea et al., 1988; Kucey and Legget, 1989; Gleddie, 1993). The behavior of PSM with or without AMF in highly weathered soils, especially in Andisols is poorly understood. Moreover, researchers of PSM have focused their attention on plant response to inoculation...
while neglecting soil variables that regulate soil solution P status. Tinker (1980) and Bolan (1991) questioned the validity of using PSM to enhance plant P uptake on the ground that the Pi released can be re-fixed by soil components as rapidly as it was released.

My hypothesis is that soil Pi sorption and mycorrhizal association control PSF effectiveness in enhancing plant Pi uptake and growth. In low Pi sorbing soils (e.g., Mollisols), PSF inoculation can increase plant P uptake of non-mycorrhizal plants, but not in very high P sorbing (e.g., Andisols). In Andisols, PSF can be effective only in the presence of mycorrhizal association.

The objective of the present study was to determine the synergistic effect of the PSF Mortierella sp. and the mycorrhizal fungus Glomus fistulosum to enhance plant growth and Pi uptake of Leucaena leucocephala (Lam.) in soils widely differing in their P fixing capacity.

MATERIALS AND METHODS

Soil preparation

Subsurface samples (15-30 cm) of four Colombian soils (Neira, Naranjal, La Selva, and Guarne) were chosen based on their differences in their capacity to sorb Pi and the type and amount of soil phosphate they contained (Appendix D and E). Subsurface soil samples were used to reduce the influence of soil organic P. The Neira soil is a Mollisol (Typic Haplustoll; IGAC, 1988) chosen for its low P fixing capacity and its richness in calcium phosphate (Ca-P). The soil
was collected from an alluvial terrace under sugarcane cultivation in Neira town (Caldas, Colombia). The other soils were collected from the Andean mountains and classified as Andisols; among them the Guarne soil had the highest Pi-sorbing capacity, followed by La Selva and Naranjal. The Naranjal soil was classified as an Acruadoxic Melanudand (Suarez et al., 1994) and was obtained from the experimental station “Naranjal” (Chinchina, Colombia) which belongs to the Colombian federation of coffee growers (Federación Nacional de Cafeteros de Colombia). Professor Daniel Jaramillo of the National University of Colombia classified the La Selva soil as an Endoaquand. Samples of this soil were collected from the experimental station of the Colombian Institute of Agriculture “La Selva” (Rionegro, Colombia). The Guarne soil was classified as a Typic Melanudand (Guarne, Colombia) by N.W. Osorio and was collected from a grassland in a forest reserve of the Experimental Station of Piedras Blancas which belongs to the National University of Colombia.

The soils were air-dried and passed through a sieve with a 4-mm diameter aperture. Phosphate sorption isotherms were constructed for each soil samples (0.5< diameter< 2 mm) following the method proposed by Fox and Kamprath (1970). Soil analysis was carried out in the Soil Fertility Lab of the National University of Colombia at Medellin (Appendix A). Other relevant information for this experiment are provided in Table 9.1. Soil pH was adjusted to 6.0 with Ca(OH)₂ following the procedure of Uchida and Hue (2000) (Appendix C).
The soil was then autoclaved twice (120°C, 0.1 MPa for 1 hour) and 900 cm³ portions of the air-dry soil were transferred into styrofoam pots measuring 11.5-cm in diameter and 15.5 cm in height.

At planting (March 10th, 2006), nutrients were applied according to soil test results (Table 9.2). With the exception of the Neira soil, the soils were fertilized with Huila RP at the rate of 2307 mg/kg after passing it through a 0.5-mm aperture sieve. The Huila RP had a P content of 130 mg/kg, and its empirical formula is, \( \text{Ca}_{0.69}\text{Na}_{0.22}\text{Mg}_{0.06}(\text{PO}_4)_{5.14}(\text{CO}_3)_{0.88}\text{F}_{2.34} \) (Chien and Hammond, 1978).

**Inoculum preparation**

*Glomus fistulosum* Skou and Jakobsen was obtained from the Soil Microbiology Lab at the National University of Colombia. It was multiplied in a mixture of sorghum (*Sorghum bicolor*) and kudzu (*Pueraria phaseoloides*) grown in a substrate composed of soil and quartzitic sand (2:1, by weight) for 4 months as described by Habte and Osorio (2001). The inoculum consisted of spores, hyphal fragments, and pieces of mycorrhizal roots dispersed in the soil-quartz matrix. The crude inoculum contained 40 infective propagules per g which was determined by employing the most probable number (MPN) technique (Porter, 1979).

*Mortierella* sp. was originally isolated from an Andisol of Hawaii (Osorio and Habte, 2001) and has been maintained in yeast manitol agar (YMA) slants at 4°C. For this study, the fungus was multiplied in Petri dishes on YMA medium for
three days at 28°C. Then its mycelium was removed with a sterile loop and suspended in sterile deionized water and shaken by hand until the clumps were dispersed. Plate counting on YMA medium indicated that there were $5.8 \times 10^6$ colony-forming-units/cm$^3$.

**Host plant**

Seeds of *L. leucocephala* cv. K-11 was scarified in concentrated H$_2$SO$_4$ for 30 minutes, rinsed six times with sterile and distilled water and then allowed to germinate on sterile moist paper towels at 28 °C for two days. Two germinated seeds were planted into each pot, and one was removed a week later.

**Treatments**

At planting, potted soil (900 cm$^3$ per pot) was either not inoculated or inoculated with 34 g of a crude inoculum of *Glomus fistulosum* Skou and Jakobsen consisting of sand, spores, hyphal fragments, and pieces of mycorrhizal roots per pot. The crude inoculum contained 40 mycorrhizal infective propagules per g, as determined by the MPN technique (Porter, 1979). The mycorrhizal inoculum was uniformly mixed with the soil. Pots containing soil not inoculated with AMF received 34 g of the sterilized soil-sand mixture along with 10 cm$^3$ of washings from the crude inoculum after removal of mycorrhizal propagules with Whatman No. 1 filter paper.
Soils were also not inoculated or inoculated with 10 cm$^3$ of the fungal suspension of Mortierella sp., which was directly pipetted into the planting holes (2-cm diameter, 3-cm depth) prior to planting.

The plants were grown under natural light in the greenhouse of the Soil Microbiology Lab, National University of Colombia, Medellin, Colombia (6° 15´N, 75° 35´W, and 1495 m altitude). Pots were watered to maintain the soil at 50-60% of maximum water holding capacity. Twenty-five cm$^3$ of P-free Hoagland’s solution was applied per pot once a week. The plants were growing for 45 days and harvested on April 24th, 2006.

**Measured variables**

Phosphorus status of L. leucocephala was determined as a function of time by monitoring P content of the 4th pinnule (counting from the base of the pinna) of the youngest fully expanded leaf starting on the 15th day after planting (Habte et al. 1987) and at 10-day intervals thereafter. At harvesting and their shoots were dried at 70 °C for 48 hours and then shoot dry weight was measured. Shoot P content was determined colorimetrically by the molybdate-blue method (Murphy and Riley 1962) after dry-ashing of samples at 500 °C for 3 hours in a muffle furnace, dissolving the ash in one-mL of 0.1 M HCl and then bringing it up to 10 mL with deionized water.

Mycorrhizal colonization of roots was determined after clearing the roots roots with 10% KOH and staining them with 0.15 % acid fuchsin in lactic acid
(Habte et al., 1987) and then estimating the proportion of root length colonized by AMF by the grid-line intersection method (Giovannetti and Mosse, 1980).

Colony forming units of Mortierella sp. were counted in a selective YMA-medium (0.5 g KH₂PO₄, 0.2 g MgSO₄·7H₂O, 0.1 g NaCl, 10 g manitol, 1 g yeast extract, and 15 g agar per liter) containing streptomycin sulfate (500 μg/mL), benomyl (75 μg/mL), and cycloheximide (100 μg/mL) (Appendix G). Twenty root fragments of 1 cm length were taken at random from the root system and transferred into petri-dishes that contained the sterilized selective medium. The development of hyphae around the root was considered as evidence of colonization of the roots by Mortierella sp.

**Experimental design**

The treatments consisted of a factorial combination of four soil types x two levels of mycorrhizal inoculation (with or without) x two levels of PSF inoculation (with or without), which were arranged in a completely randomized design with four replicates per treatment. Data were subjected to analysis of variance (ANOVA) and LSD tests for mean separation (P-value ≤0.05) using the software Statgraphics Plus, version 4.0 (Statpoint, Inc.; Herdon, Virginia).

**RESULTS**

Mycorrhizal colonization was detected only in the roots of plants grown in soil inoculated with G. fistulosum, and this was significantly higher in the Neira soil
than in the other soils (Figure 9.1.A). Inoculation with Mortierella sp. significantly reduced the extent of mycorrhizal colonization in all soils from a mean value of 47 to 27% (Figure 9.1.B). Significant interaction effects were noted between soil type and AMF inoculation ($P<0.0001$) and between AMF inoculation and PSF inoculation ($P<0.0001$) for the variable (Table 9.3).

Mortierella sp. was detected only in root samples of plants grown in soil inoculated with the fungus. The fungus was more abundant in roots of plants grown in the Neira soil (66%) than in the roots of plants grown in the other three soils (mean of 32%) (Figures 9.2.A). Likewise, inoculation with G. fistulosum significantly decreased the presence on the rhizoplane of Mortierella sp. in all soils. In mycorrhiza-free roots, the Mortierella sp. was detected in 53% of the root segments incubated on the selective medium, while in mycorrhizal roots the value decreased to 29% (Figure 9.2.B).

The presence of Mortierella sp. in the rhizoplane of leucaena was significantly affected by the interactions Soil x PSF and AMF x PSF (Table 9.3).

The P content of pinnules of leucaena was significantly affected by the three-way interaction at some sampling dates, particularly at 35 and 45 days after planting (Table 9.3). In the uninoculated Andisols (-AMF-PSF), the pinnule P content decreased drastically (Figure 9.3) and typical symptoms of P deficiency such as chlorosis and defoliation (Habte and Manjunath, 1987; Smith et al., 1992) were evident 25 days after planting. At harvest, the pinnule P content of these plants was around 1.5 μg/pinnule and pinnule P concentration was lower
than 0.1% (Figure 9.4). A similar pattern was observed if Mortierella sp. and G. fistulosum were inoculated separately or concurrently (Figure 9.3).

By contrast, plants grown in the Neira soil responded positively to inoculation with PSF, AMF, or to dual inoculation (Figure 9.3). For instance, in the Neira soil not inoculated with fungus, the maximum pinnule P content value observed was increased with time after an initial decline, reaching a peak value (7.2 µg P/pinnule) on day 35. The peak value observed was 9.4 µg P/pinnule if soil was inoculated with Mortierella sp alone (Figures 9.3 and 9.4) compared to a peak value of 9.8 µg P/pinnule observed if soil was inoculated with G. fistulosum alone (Figure 9.3), and to a peak value of 10.5 µg P/pinnule if soil was inoculated with both fungi. Pinnule P contents of plants grown in the presence of AMF or PSF were not significantly different from each other, and the effect of dual inoculation was superior to inoculation with PSF but not to inoculation with AMF.

At harvest, shoot dry weight was significantly affected by the three-way interaction between soil type, AMF inoculation, and PSF inoculation (Table 9.3). The uninoculated plants grown in the three Andisols exhibited low shoot dry weights ranging between 0.24 and 0.30 g/pot, which did not differ significantly from each other (Figure 9.5). Inoculation of soil with either fungus or with both did not increase shoot dry weight of leucaena significantly in these soils, except in the La Selva in which positive response was detected with AMF inoculation although the overall growth of the plants was poor (Figure 9.5). In contrast, in the Neira soil the shoot dry weight of uninoculated leucaena was 0.73 g/pot, which
was significantly superior to that observed in the Andisols. In the former soil, inoculation with Mortierella sp. led to a 59% increase in shoot dry wt. The increase in shoot dry wt was significantly higher than this value if soil was inoculated with G. fistulosum (86%), and dual inoculation led to a 103% increase in yield, which was significantly higher than that observed in the presence of G. fistulosum alone (Figure 9.5).

Shoot P content exhibited a pattern similar to that observed for shoot dry weight. In the Andisols, shoot P content was very low (0.23 mg) and inoculation with AMF or PSF had no influence on the variable. However, dual inoculation of the La Selva soil led to a significant increase in shoot P content (Figure 9.6). In contrast, plants grown on the Mollisol had higher shoot P content than those grown in the Andisols, even if they were not inoculated with fungi. Shoot P content was further and progressively enhanced significantly if the former soil was inoculated with AMF, PSF, or both, the respective values being 2.73, 3.34, and 4.22 mg/pot (Figure 9.6).

**DISCUSSION**

The results of the present study indicate that the effectiveness of Mortierella sp. in increasing plant P uptake and growth is tightly controlled by soil P sorption capacity. Hence, the Mollisol with a very low P sorption capacity ($P_{0.2} < 100$ mg/kg) had a lesser influence on the variables measured while the three Andisols with very high P sorption capacity ($P_{0.2} > 1000$ mg/kg) (Juo and Fox, 1977) exerted greater influence on the variables. Inoculation of the Mollisol with
Mortierella sp. alone led to a significantly large increase in plant P uptake and growth of leucaena (Figures 9.4 and 9.5). While the effect of inoculating the Andisols with PSF on P uptake and growth of the legume was very limited, there was variation in the response of leucaena to inoculation of the different Andisols which also was explained by variation in the P sorption capacity of the soils.

The magnitude of host response to PSF inoculation observed in the Mollisol is comparable to those obtained in other low P-fixing soils (Table 9.3). Other investigators (Muthkumar et al., 2001; Toro et al., 1996) have also noted the kind of synergistic interaction observed here between the PSF and the AMF tested in the current study (Figures 9.5 and 9.6) even though they used a different PSM. Leucaena leucocephala has a root system that is inefficient in taking up Pi from soil, because of its coarse root system and very few and short root hairs (Manjunath and Habte, 1991). The synergistic effect observed between AMF and PSF suggests that the Pi released by the PSF was rapidly absorbed by the mycorrhizal network, which is much more efficient than the unaided root in taking up Pi. Barea et al. (2002) inoculated Enterobacter sp. in a RP-fertilized soil with or without AMF and observed the highest Pi uptake by plants when the soil was inoculated with both the PSM and AMF; the intermediate and the lowest levels of response if the soil was inoculated with the AMF or the PSM alone, respectively. Furthermore, without the RP addition Enterobacter sp. was capable of increasing plant P uptake of mycorrhizal plants but not of mycorrhiza-free plants.

It is evident that Mortierella sp. solublized P from the soil Ca-P fraction, which was the most abundant P form in this soil (Table 9.1). This is the soil P
fraction susceptible to solubilization by rhizosphere acidification through the release of protons and oxalic acid (Appendix H) (Trolove et al., 1996, 2003). However, inoculation of the Andisols with Mortierella sp. did not yield results similar to those observed in the Mollisol despite their relatively high soil Ca-P content (Table 9.1). The limited effect of Mortierella sp. to stimulate plant P uptake and growth of leucaena in these soils thus cannot be due to an insufficiency of Ca-P. Even if Ca-P was exogenously supplied to these soils as RP (600 mg P/kg), inoculating them with Mortierella sp. was not effective in stimulating P uptake by plants grown on them (Appendix F). However, this additional amount of RP significantly ($P < 0.05$) stimulated mycorrhizal activity in the Guarene and La Selva soils, but not in the Naranjal soil.

Given the very-high P sorption capacity of Andisols the ineffectiveness of Mortierella to stimulate Pi uptake and growth of associated plants should not be surprising. Once Pi is released by a PSM from a native Ca-P or an applied RP source, Pi is subject to adsorption or precipitation by soil constituents, which clearly limit its diffusion rate toward the root surface (Barber, 1995). These phenomena can explain the limited effectiveness and unpredictability of PSM inoculation to enhance non-mycorrhized plant Pi uptake in subtropical-tropical acidic soils (Table 9.4).

The high P sorption capacity of Andisols is caused by the presence of short-order-range materials (e.g., Allophanes). I have demonstrated under in-vitro conditions (chapter 4), that the presence of allophane in suspension can obscure the RP solubilizing activity of Mortierella sp. by adsorbing the Pi released on the
very large surface area of the mineral. The Pi sorbed becomes strongly held and is not available for uptake by roots or other organisms. In contrast, in the presence of Montmorillonite, the clay mineral that is most likely dominant in the Mollisol (based on soil classification), the Pi released due to solubilization of RP or soil Ca-P by a PSM can remain in the soil solution for a longer duration than would be possible in the presence of Allophanes.

Mycorrhizal inoculation thus seems to be more critical in highly Pi sorbing soils in order to rapidly intercept Pi released by PSF, and thereby increase plant Pi uptake. However, in the current study, even mycorrhizal activity was restricted under this condition. In the Guarne and Naranjal soils, the concentrations of Pi in the soil solution were quite low (0.003 and 0.005 mg/L) (Table 9.1) thereby limiting the effectiveness of mycorrhizal fungi in increasing plant P uptake. Habte and Manjuhath (1987) found that a soil solution P of 0.02 mg/L was optimal for mycorrhizal activity in highly mycorrhizal dependent host species such as Leucaena. Among the Andisols, only La Selva exhibited a soil solution P concentration (0.016 mg/L) approximating the optimum value. This could explain the relatively higher shoot dry weight of leucaena observed if the soil was inoculated with G. fistulosum than if it was not inoculated (Figure 9.5). Moreover, shoot P content was significantly increased if the AMF was concurrently inoculated with the PSF (Figure 9.6). Nevertheless, despite these significant increases in shoot P content, plants grew poorly in the soil.

In summary, the effectiveness of Mortierella sp. in increasing plant P uptake and growth was controlled by soil P sorption capacity, the effectiveness of the
microorganism being significantly higher in soil with low P sorption capacity and even further increasing in the presence of mycorrhizal association. In contrast, in very highly P sorbing soils Mortierella sp. was ineffective by itself, its effectiveness improving if co-inoculated with AMF at least in one of the three Andisols that had an initial solution Pi concentration that was conducive to mycorrhizal activity. The synergistic effect between the AMF and the PSF tested seems to be limited by the low soil solution P concentrations that are characteristic of these soils. It is recommended that future studies evaluate the effectiveness of Mortierella sp. in long-term experiments and at different concentrations of soil solution P that favor the mycorrhizal activity, particularly in Andisols, and hence, the synergistic effect with both AMF and PSF.
Table 9.1. Soil P_{0.2} value, calcium phosphate (Ca-P) content, and soil available P.

<table>
<thead>
<tr>
<th>Soil</th>
<th>P_{0.2} value * (mg/kg)</th>
<th>Soil Ca-P (mg/kg)</th>
<th>0.01 M CaCl2-P (mg/L)</th>
<th>Bray II-P (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neira</td>
<td>45</td>
<td>142.8</td>
<td>0.089</td>
<td>82</td>
</tr>
<tr>
<td>Naranjal</td>
<td>1429</td>
<td>5.7</td>
<td>0.009</td>
<td>1</td>
</tr>
<tr>
<td>La Selva</td>
<td>2222</td>
<td>59.4</td>
<td>0.016</td>
<td>10</td>
</tr>
<tr>
<td>Guame</td>
<td>4000</td>
<td>19.9</td>
<td>0.005</td>
<td>2</td>
</tr>
</tbody>
</table>

* P_{0.2} = amount of P required to achieve 0.2 mg L^{-1} in the soil solution.

Table 9.2. Nutrients added to soils (mg/pot).

<table>
<thead>
<tr>
<th>Soil</th>
<th>Ca(OH)\textsubscript{2}</th>
<th>MgO</th>
<th>K\textsubscript{2}SO\textsubscript{4}</th>
<th>(NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}</th>
<th>Maximal Water holding capacity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neira</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>430</td>
<td>43.0</td>
</tr>
<tr>
<td>Naranjal</td>
<td>690</td>
<td>230</td>
<td>114</td>
<td>430</td>
<td>93.9</td>
</tr>
<tr>
<td>La Selva</td>
<td>-</td>
<td>314</td>
<td>-</td>
<td>430</td>
<td>99.3</td>
</tr>
<tr>
<td>Guame</td>
<td>862</td>
<td>256</td>
<td>160</td>
<td>430</td>
<td>92.2</td>
</tr>
</tbody>
</table>

Table 9.3. Significant P-values of ANOVA test.

<table>
<thead>
<tr>
<th>Source</th>
<th>Pinnule P content (µg) (days after transplanting)</th>
<th>Pinnule P concentration at harvest (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15</td>
<td>25</td>
</tr>
<tr>
<td>Treatments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil (A)</td>
<td>NS</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AMF (B)</td>
<td>0.0058</td>
<td>0.0228</td>
</tr>
<tr>
<td>PSM (C)</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AxB</td>
<td>NS</td>
<td>0.0022</td>
</tr>
<tr>
<td>AxC</td>
<td>NS</td>
<td>0.0203</td>
</tr>
<tr>
<td>BxC</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>AxBxC</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 9.3. (Continued). Significant \( P \)-values of ANOVA test.

<table>
<thead>
<tr>
<th>Source</th>
<th>SDW</th>
<th>SPC</th>
<th>MC</th>
<th>PSFR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatments</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soil (A)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AMF (B)</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PSF (C)</td>
<td>0.0020</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AxB</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>NS</td>
</tr>
<tr>
<td>AxC</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>NS</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BxC</td>
<td>0.0022</td>
<td>NS</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>AxBxBxC</td>
<td>0.0027</td>
<td>0.0222</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

SDW= shoot dry weight; SPC= shoot P content; MC= mycorrhizal colonization; PSFR= presence of *Mortierella* sp. on the rhizoplane.

Table 9.4. Increases in plant P uptake due to the sole inoculation with PSM.

<table>
<thead>
<tr>
<th>PSM</th>
<th>Soil/substrate</th>
<th>Plant</th>
<th>Increase in P uptake due to PSM (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Low P sorbing soils</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Arthrobotrys oligospora</em></td>
<td>Sandy soil of Senegal Unfertilized/ RP fertilization</td>
<td><em>Acacia holoserica</em></td>
<td>56 / 74</td>
<td>Dupponois et al., 2006</td>
</tr>
<tr>
<td><em>Pencillus radicium</em></td>
<td>Sandy soils of Australia with neutral to alkaline soil reactivity</td>
<td><em>Wheat</em></td>
<td>34 to 76</td>
<td>Wakelin et al., 2004</td>
</tr>
<tr>
<td><em>Mesorhizobium mediterraneum</em></td>
<td>Calcareous Alfisol of Spain (fertilized with tricalcium phosphate)</td>
<td><em>Chickpea</em></td>
<td>100</td>
<td>Peix et al., 2001</td>
</tr>
<tr>
<td><em>Enterobacter sp.</em></td>
<td>Calcareous soil of Spain</td>
<td><em>Barley</em></td>
<td>125</td>
<td></td>
</tr>
<tr>
<td><em>Penicillium thomii</em></td>
<td>Vermiculite-perlite substrate fertilized with RP</td>
<td><em>Mint</em></td>
<td>200</td>
<td>Cabello et al. 2005</td>
</tr>
<tr>
<td><strong>Medium-High P sorbing soils</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Penicillium radicum</em></td>
<td>Ultisol</td>
<td><em>Wheat</em></td>
<td>8</td>
<td>Whitelaw et al., 1997</td>
</tr>
<tr>
<td><em>Mortierella</em> sp.</td>
<td>Oxisol</td>
<td><em>Leucaena</em></td>
<td>13</td>
<td>Osorio and Habte, 2001</td>
</tr>
<tr>
<td>Unknown</td>
<td>Acidic soils of Taiwan</td>
<td><em>Leucaena</em></td>
<td>20-24</td>
<td>Young et al., 1980</td>
</tr>
</tbody>
</table>

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Figure 9.1. Mycorrhizal colonization of leucaena roots as a function of the interaction between (A) soil and inoculation with *Glomus fistulosum* (with: +AMF; without: -AMF) and (B) soil and inoculation with *Mortierella* sp. (with: +PSF; without: -PSF) 45 days after transplanting.
A

Mycorrhizal colonization (%)

LSD0.05 = 3.2

GUARNE  LA SELVA  NARANJAL  NEIRA

Soil x AMF

B

Mycorrhizal colonization (%)

LSD0.05 = 2.3

-AMF  +AMF

AMF x PSF

-PSF  +PSF
Figure 9.2. Presence of *Mortierella* sp. on the rhizoplane of leucaena roots as a function of the interactions between (A) soil x *Mortierella* sp. inoculation (with: +PSF; without: -PSF) and (B) *Glomus fistulosum* (with: +AMF; without: -AMF) x *Mortierella* sp. inoculations, 45 days after transplanting.
A

![Graph A](image)

B

![Graph B](image)
Figure 9.3. Pinnule P content (μg/pinnule) of *Leucaena leucocephala* as a function of the three-way interaction among the four soils, inoculation with *Glomus fistulosum* (with: +AMF; without: -AMF), and inoculation with *Mortierella* sp. (with: + PSM; without: - PSM) at different sampling dates after transplanting.
Figure 9.4. Pinnule P content (µg/pinnule) (A) and Pinnule P concentration (%) of *Leucaena leucocephala* as a function of the three-way interaction among the four soils, *Glomus fistulosum* inoculation (with: +AMF; without: -AMF), and *Mortierella* sp. inoculation (with: +PSF; without: -PSF) at 45 days after transplanting.
Figure 9.5. Shoot dry weight (SDW) of *Leucaena leucocephala* as a function of the three-way interaction among the four soils, the inoculation with *Glomus fistulosum* (with: +AMF; without: -AMF), and the inoculation with *Mortierella* sp. (with: + PSF; without: - PSF) 45 days after transplanting.
Figure 9.6 Shoot P content of *Leucaena leucocephala* as a function of the three-way interaction among the four soils, the inoculation with *Glomus fistulosum* (with: +AMF; without: -AMF), and the inoculation with *Mortierella* sp. (with: +PSF; without: -PSF) at 45 days after transplanting.
LSD_{0.05} = 0.322

CV = 24.56%
LITERATURE CITED


Toro, M., R. Azcon, and J. M. Barea. 1998. The use of isotopic dilution techniques to evaluate the interactive effects of rhizobium genotypes, mycorrhizal fungi, phosphate-solubilizing rhizobacteria and rock phosphate


CHAPTER TEN
GENERAL DISCUSSION

The effectiveness of Mortierella sp. in increasing the concentration of available P in the soil solution, plant P uptake, and growth is controlled by several interacting factors that include: soil P sorption, mycorrhizal association, soil calcium-phosphate (Ca-P) content, ammonium supply, and rock phosphate (RP) addition.

The inverse relationship between soil pH and solution P concentration under in vitro conditions clearly suggests that acid production is the major mechanism for RP dissolution shown by Mortierella sp. as amply demonstrated in other PSMs (Kim et al., 1998; Bar-Yosef et al., 1999; Wakelin et al., 2004).

However, the capacity of Mortierella sp. in dissolving RP cannot be explained by the release of protons alone. The concomitant release of oxalic acid/oxalate by this fungus is also very important in this respect as explained by Welch et al.(2002). Based on the solubility product principle, illustrated by the dissolution of francolite (Robinson and Syers, 1990; Robinson et al., 1992), oxalic acid can chelate calcium (Log \(K_{Ca}=3.44\)) favoring the RP dissolution.

\[
Ca_{10}(PO_4)_{6-x}(CO_3)_xF_{2+0.4x} + 12H^+ \leftrightarrow 10Ca^{2+} + (6-x)H_2(PO_4)^- + (2+0.4x)F^- + xH_2O
\]
This reaction can also be applicable to the microbial dissolution of native Ca-P as observed mainly in the Mollisol I studied and other Mollisol studies by Kucey (1987, 1988).

The results of the current investigation suggest that P solubilization in soil by Mortierella sp. can also be achieved by the desorption of Pi from the surfaces of soil minerals, likely through anion exchange with oxalate. However, the increase of soluble Pi by Mortierella sp. via Pi desorption is controlled by the soil Pi sorption capacity. The extent of the Pi desorption induced by the fungus is in the order of Mollisol > Oxisol > Ultisol > Andisol. This desorption is in turn controlled by the dominant minerals in the soils, and the extent of this control is in the order Allophane > Goethite > Kaolinite > Montmorillonite. The P_{0.2} value thus seems to be a good predictor of microbial desorption of Pi. It is expected that the effectiveness of PSM in desorbing Pi is low when the P_{0.2} value increases; when the P_{0.2} reaches a value above 1000 mg/kg (commonly with Andisols) indicates a limited Pi desorption. Also, reductions of the values of P_{0.2} in minerals and soils after a desorption-incubation period with Mortierella sp. (chapter 5) indicate that the oxalic acid/oxalate released by the fungus could displace Pi from sorption sites. Hue (1991) and Bolan et al. (1994) demonstrated that tricarboxylic and dicarboxylic organic acids (including oxalate) are efficient in desorbing Pi from the surfaces of soil of diverse mineralogies (Andisols, Ultisols, Oxisols, and Vertisols).

However, the capacity of Mortierella sp. to dissolve native Ca-P or added RP does not guarantee an increase in the concentration of available Pi in the soil.
Once Pi is released by the PSF into solution, it is subject to re-
adsorption on soil surfaces precipitation by Al or Fe species (Barber, 1995;
Lindsay, 2001). These Pi removal processes constitute serious limitations on the
effectiveness of the PSF tested in increasing available P, particularly in soils with
very-high Pi sorption capacity (P0.2 > 1000 mg/kg). The degree to which soil
minerals and soils limited the increase of soluble P followed the same order as
for minerals and soils indicated above. Another constraint to the amelioration of
Pi in solution is the microbial immobilization of Pi by the PSF itself, which was
particularly prominent if the fungus was grown in the presence of very-high Pi
sorbing soils (Andisols) that rapidly removed soluble P, especially if the fungus
was supplied with NO3− as the only source of N.

The role of the nitrogen (N) form in the dissolution of RP by PSM has been
previously reported by Whitelaw (2000) and Fransson et al. (2004). In the current
study supplying the PSF with NH4+ as the sole source of N led to a greater
depression of pH than supplying it with NO3− or a mixture of both N forms. This
decrease in pH was accompanied by increases in the dissolution of RP.
However, in the presence of NO3− as the sole source of N, Mortierella sp. not only
solubilized RP to a lesser extent but also tended to immobilize into its mycelium
most of the Pi that it released into solution. The effect of NH4+ in increasing acid
production by plant roots is very well reported, but its significance to rhizosphere
microorganisms is not well understood.

The limitations on Mortierella sp. effectiveness imposed by the soil Pi sorption
could be overcome by the presence of mycorrhizal association (as schematically

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illustrated in Figure 10.1). The mycorrhizal network is far more efficient to remove Pi from the soil solution than the root alone (Barber, 1995; Azcon and Barea, 1996), thereby allowing the associated plant to have access to Pi release by the action of Mortierella sp. before it is re-adsorbed on the surface of soil minerals. This phenomenon explains the relevance of the synergistic effect of inoculation with PSF and AMF in enhancing plant Pi uptake and growth of leucaena observed in the two Oxisols studied (Wahiawa and Carimagua) (Figure 10.2). Since the Pi sorption capacity in these Oxisols was intermediate (100 < P0.2 < 500 mg/kg; Juo and Fox, 1977), the impact of Mortierella sp. in maintaining soil solution Pi concentration at levels near the optimal for mycorrhizal activity in L. leucocephala (0.02 mg/L; Habte and Manjunath, 1987) is quite evident (chapter 4). On the other hand, the effectiveness of the fungus to increase soil solution Pi concentration was markedly curtailed in the Andisols tested, because of their very high Pi sorption capacity, which maintained soil solution Pi concentration well below that which is optimal for effective mycorrhizal functioning. Therefore, no synergistic effects were detected if leucaena was grown in the presence of Mortierella sp. and G. fistulosum in the Andisols studied.

In the Mollisol tested, Mortierella sp. exhibited high effectiveness in increasing soil solution Pi concentration and hence in enhancing plant Pi uptake even in the absence of mycorrhizal association because of the very low Pi sorption capacity of the soil. However, the effectiveness of the PSF in stimulating plant Pi uptake and growth was further enhanced by the presence Glomus fistulosum (Figure 10.2 A, B). It is also worth noting that the
effectiveness exhibited by *Mortierella* sp. in this soil was not dependent on the amendment of the soil with RP, suggesting that the fungus solubilized native Ca-P which was abundantly found in the soil.

In contrast, in the Oxisols studied, the activity of *Mortierella* sp. was significantly limited by their low Ca-P content, and it was necessary to amend the soils with RP before *Mortierella* sp. became appreciably effective in ameliorating soil solution Pi concentration (Figures 8.5 and 8.6). This was particularly evident in the Carimagua soil since more RP was required to enhance *Mortierella* sp. activity in it than was required in the Wahlawa soil. This increase in RP was required in order to increase the Ca-P content of the soil, this is considered the P fraction of soil susceptible to dissolution by the activity of PSF. At the end of the experiment, the rhizosphere of leucaena inoculated with *Mortierella* sp. had lower Ca-P than that which was not inoculated. Other soil P-fractions (Al-P and Fe-P) although present more abundantly in the unfertilized soil did not appear to contribute to the effectiveness of the fungus in increasing plant Pi uptake. However, the behavior of *Mortierella* sp. in the Andisols, where there were high levels of Ca-P but low effectiveness of the fungus, suggest that the effectiveness of the fungus was constrained more by soil Pi sorption capacity than by Ca-P content.

The results obtained in the current investigation indicate that PSM can play a significant role in the alleviation of P deficiency in highly weathered Oxisols by increasing RP dissolution and it is also likely by desorbing sorbed Pi. The suitability of *Mortierella* sp. for use in the acid soils of the tropics lies not only in
its effectiveness in increasing PI uptake and growth of mycorrhizal plants but also in its tolerance to Al$^{3+}$ (Figure 7.4).

More investigations are needed to evaluate the effectiveness of this PSF in a wider range of soils and plant species and at different levels of soil solution P concentrations. It is also necessary to identify the organic acids by means of which the fungus solubilizes Ca-P.
Figure 10.1. Diagram showing the microbial solubilization of soil and added P by *Mortierella* sp. and the mycorrhizal Pi uptake (Nelson W. Osorio, original drawing).
Mycorrhiza

Soil solution

\( \text{H}_3\text{PO}_4^- \)

Dissolution

Desorption

Immobilization

\( \text{Pi} \) uptake

\( \text{Pi} \) sorption

Soil minerals

\( \text{NH}_4^+ \)

Oxalic acid

Rhizodeposition

Cartoon

Soil solution

\( \text{PSF} \)

\( \text{Pi} \) sorption

\( \text{Pi} \) uptake

Added \( \text{Pi} \)

\( \text{Native Ca-P} \)

\( \text{Sorbed Pi} \)
Figure 10.2. Shoot P content (A) and shoot dry weight (B) of *Leucaena leucocephala* grown in soils inoculated or not inoculated with an AMF and with *Mortierella* sp. In parenthesis are the amount of P added (mg/kg) using Rock phosphate as P source.


Enterobacter agglomerans and cloned Escherichia coli in culture medium. Biol.

Kucey, R.M.N. 1987. Increased phosphorus uptake by wheat and field beans
inoculated with a phosphorus solubilising Penicillium biliae strain and with

Kucey, R.M.N. 1988. Effect of Penicillium biliae on the solubility and uptake of P and


Lindsay, W.L. 2001. Chemical equilibria in soils. The Blackburn Press, Caldwell,
New Jersey.


Robinson, J.S., J.K. Syers, and N.S. Bolan. 1992. Importance of proton supply and
calcium-sink size in the dissolution of phosphate rock materials of different

solubilization by Penicillium spp. closely associated with wheat roots. Biol.
Fertil. Soils 40:36-43.

Welch, S., A.E. Taunton, and J.F. Banfiled. 2002. Effect of microorganisms and

Whitelaw, M.A. 2000. Growth Promotion of plants inoculated with phosphate-
### Appendix A. Soil chemical and physical properties, soil classification, and geographic location of soil samples used.

<table>
<thead>
<tr>
<th>Soil</th>
<th>Sand</th>
<th>Silt</th>
<th>Clay</th>
<th>Texture</th>
<th>Soil pH</th>
<th>O.M.</th>
<th>Al</th>
<th>Ca</th>
<th>Mg</th>
<th>K</th>
<th>ECEC</th>
<th>TCEC</th>
<th>P</th>
<th>S</th>
<th>Fe</th>
<th>Mn</th>
<th>Cu</th>
<th>Zn</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guane</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5.4</td>
<td>8.9</td>
<td>0.3</td>
<td>0.3</td>
<td>0.04</td>
<td>0.04</td>
<td>0.7</td>
<td>70.2</td>
<td>2</td>
<td>59</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>La Salva</td>
<td>No dispersion was achieved</td>
<td>5.8</td>
<td>3.8</td>
<td>9.5</td>
<td>0.80</td>
<td>0.78</td>
<td>11.1</td>
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<td>0</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>&lt;0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Naranjal</td>
<td>5.7</td>
<td>2.6</td>
<td>8.0</td>
<td>0.08</td>
<td>0.07</td>
<td>0.86</td>
<td>35.9</td>
<td>1</td>
<td>28</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>&lt;0.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasus</td>
<td>24</td>
<td>10</td>
<td>66</td>
<td>Clay</td>
<td>4.3</td>
<td>0.9</td>
<td>13.7</td>
<td>0.2</td>
<td>0.20</td>
<td>0.08</td>
<td>14.2</td>
<td>26.0</td>
<td>1</td>
<td>48</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>Ceramagua</td>
<td>20</td>
<td>50</td>
<td>30</td>
<td>Clay loam</td>
<td>4.9</td>
<td>3.5</td>
<td>3.1</td>
<td>0.1</td>
<td>0.10</td>
<td>0.05</td>
<td>3.4</td>
<td>13.9</td>
<td>2</td>
<td>5</td>
<td>188</td>
<td>2</td>
<td>1</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Neira</td>
<td>56</td>
<td>30</td>
<td>14</td>
<td>Sandy loam</td>
<td>5.4</td>
<td>4.0</td>
<td>0.3</td>
<td>4.7</td>
<td>1.10</td>
<td>0.34</td>
<td>6.4</td>
<td>15.0</td>
<td>82</td>
<td>4</td>
<td>163</td>
<td>6</td>
<td>1</td>
<td>3</td>
<td>0.3</td>
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<tr>
<td>Letras</td>
<td>94</td>
<td>4</td>
<td>2</td>
<td>Sand</td>
<td>5.4</td>
<td>0.9</td>
<td>0.7</td>
<td>0.12</td>
<td>0.03</td>
<td>0.02</td>
<td>0.9</td>
<td>8.2</td>
<td>54</td>
<td>29</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.2</td>
<td></td>
</tr>
</tbody>
</table>

### Appendix A. (Continued).

<table>
<thead>
<tr>
<th>Soil</th>
<th>Soil taxonomy</th>
<th>Geographic position</th>
<th>Soil MWHC</th>
<th>NO₃⁻</th>
<th>NH₄⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guane</td>
<td>Melanudand</td>
<td>6°15'N, 75°30'W</td>
<td>92.2</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td>La Salva</td>
<td>Endoaquand</td>
<td>6°08'N, 75°25'W</td>
<td>83.5</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>Naranjal</td>
<td>Acruadox Melanudand</td>
<td>4°58'N, 75°39'W</td>
<td>93.9</td>
<td>10</td>
<td>25</td>
</tr>
<tr>
<td>Caucasus</td>
<td>Paleudult</td>
<td>8°03'N, 75°07'W</td>
<td>88.0</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Ceramagua</td>
<td>Haplustox</td>
<td>4°34'N, 71°20'W</td>
<td>61.6</td>
<td>1</td>
<td>54</td>
</tr>
<tr>
<td>Neira</td>
<td>Typic Haplustoll</td>
<td>5°08'N, 75°35'W</td>
<td>43.0</td>
<td>10</td>
<td>16</td>
</tr>
<tr>
<td>Letras</td>
<td>Vitrand (Umbric Vitrandept)</td>
<td>5°02'N, 75°22'W</td>
<td>33.3</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>Wahliwa</td>
<td>Haplustox</td>
<td>medial over pumiceous or cindery, fenthofirydcll,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tantalus</td>
<td>loameric Typic Hapludand</td>
<td>21°19'N, 157°49'W</td>
<td>68.8</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Keana</td>
<td>very fine, amectitic, loameric Typic Natraquert</td>
<td>21°17'N, 157°48'W</td>
<td>72.5</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Soil tests were carried out in the soil lab of the National University of Colombia at Medellin. Methods: Soil texture: by the Bouyoucos method; Soil pH: in water (1:1, volume) (pH-meter); Soil organic matter content: Walkley & Black method; Exchangeable Al: 1M KCl; Exchangeable Ca, Mg, K: 1M ammonium acetate (pH 7); ECEC: sum of Al, Ca, Mg, and K; Total CEC: 1M ammonium acetate (pH 7); P: Bray II; Fe, Mn, Cu, Zn: Olsen-EDTA; B: hot water; NO₃⁻: 0.025 M Al sulfate; NH₄⁺: 1M KCl. ND: Not determined.
Appendix B. Origin of clay minerals used. These clay minerals were supplied by Dr. Mitiku Habte and originally obtained by Rollin Jones, Soil Mineralogy Laboratory, University of Hawaii at Manoa.

<table>
<thead>
<tr>
<th>Clay mineral</th>
<th>Origin</th>
<th>Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allophane</td>
<td>Fine earth</td>
<td>University of Hawaii at Manoa</td>
</tr>
<tr>
<td>Bauxite</td>
<td>Nadine, Georgia</td>
<td>Ward's Natural Science Establishment Inc., Rochester, New York</td>
</tr>
<tr>
<td>Goethite</td>
<td>Biwabik, Minnesota</td>
<td>University of Hawaii at Manoa</td>
</tr>
<tr>
<td>Kaolinite</td>
<td>Dixie Rubber pit; Bath, South Carolina</td>
<td>Ward's Natural Science Establishment Inc., Rochester, New York</td>
</tr>
<tr>
<td>Montmorillonite</td>
<td>Columbia, Missouri</td>
<td>Department of Geology, University of Missouri</td>
</tr>
</tbody>
</table>
Appendix C. Lime requirement curves for five Colombian soils.

Guame

$y = -0.0121x^2 + 0.3385x + 5.3996$

La Selva

$y = -0.0088x^2 + 0.2576x + 5.965$
Naranjal

\[ y = -0.0108x^2 + 0.2897x + 5.6823 \]

\[ \text{Ca(OH)_2 (g/kg)} \]

Caucasla

\[ y = 0.0048x^2 + 0.0571x + 4.3904 \]

\[ \text{Ca(OH)_2 (g/kg)} \]

Canfragueu

\[ y = 0.0024x^2 - 0.0792x^2 + 0.8359x + 6.225 \]

\[ \text{Ca(OH)_2 (g/kg)} \]
Appendix D. Soil and soil mineral P\textsubscript{i} sorption isotherms.

![Graph of soil and soil mineral P\textsubscript{i} sorption isotherms.](image)

<table>
<thead>
<tr>
<th>Soil</th>
<th>$P_{0.2}$ value (mg/kg)</th>
<th>Pi sorption category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guama</td>
<td>4000</td>
<td>Very High</td>
</tr>
<tr>
<td>La Selva</td>
<td>2222</td>
<td>Very High</td>
</tr>
<tr>
<td>Naranjal</td>
<td>1429</td>
<td>Very High</td>
</tr>
<tr>
<td>Caucasia</td>
<td>714</td>
<td>High</td>
</tr>
<tr>
<td>Carimagua</td>
<td>417</td>
<td>Medium</td>
</tr>
<tr>
<td>Letras</td>
<td>123</td>
<td>Medium</td>
</tr>
<tr>
<td>Neira</td>
<td>45</td>
<td>Low</td>
</tr>
</tbody>
</table>
CLAY MINERALS

Soil mineral P sorption categories based on the $P_{0.2}$ value.

<table>
<thead>
<tr>
<th>Clay mineral</th>
<th>$P_{0.2}$ (mg/kg)</th>
<th>Pi sorption category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allophane</td>
<td>4146</td>
<td>Very High</td>
</tr>
<tr>
<td>Bauxite</td>
<td>580</td>
<td>High</td>
</tr>
<tr>
<td>Illite</td>
<td>552</td>
<td>High</td>
</tr>
<tr>
<td>Montmorillonite</td>
<td>340</td>
<td>Medium</td>
</tr>
<tr>
<td>Goethite</td>
<td>224</td>
<td>Medium</td>
</tr>
<tr>
<td>Kaolinite</td>
<td>94</td>
<td>Low</td>
</tr>
</tbody>
</table>
Appendix E. Soil P fractionation according to the method of Kuo (1996).

<table>
<thead>
<tr>
<th>SOIL</th>
<th>Labile-P *</th>
<th>Al-P</th>
<th>Fe-P</th>
<th>Ca-P</th>
<th>Mineral-P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guarne</td>
<td>0.3</td>
<td>54.7</td>
<td>1.8</td>
<td>19.9</td>
<td>76.8</td>
</tr>
<tr>
<td>La Selva</td>
<td>1.2</td>
<td>217.2</td>
<td>28.0</td>
<td>59.4</td>
<td>305.8</td>
</tr>
<tr>
<td>Naranjal</td>
<td>0.5</td>
<td>38.9</td>
<td>3.6</td>
<td>5.7</td>
<td>48.7</td>
</tr>
<tr>
<td>Caucasia</td>
<td>0.6</td>
<td>21.2</td>
<td>217.7</td>
<td>0.1</td>
<td>239.5</td>
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<tr>
<td>Carimagua</td>
<td>0.3</td>
<td>2.0</td>
<td>22.4</td>
<td>1.3</td>
<td>26.0</td>
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<tr>
<td>Wahiawa</td>
<td>1.0</td>
<td>51.5</td>
<td>145.3</td>
<td>16.5</td>
<td>214.3</td>
</tr>
<tr>
<td>Neira</td>
<td>1.7</td>
<td>95.7</td>
<td>30.0</td>
<td>142.8</td>
<td>270.1</td>
</tr>
<tr>
<td>Letras</td>
<td>1.4</td>
<td>53.0</td>
<td>2.0</td>
<td>44.1</td>
<td>100.5</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>SOIL</th>
<th>Labile-P</th>
<th>Al-P</th>
<th>Fe-P</th>
<th>Ca-P</th>
<th>Mineral-P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guarne</td>
<td>0.4</td>
<td>71.3</td>
<td>2.4</td>
<td>25.9</td>
<td>100</td>
</tr>
<tr>
<td>La Selva</td>
<td>0.4</td>
<td>71.0</td>
<td>9.2</td>
<td>19.4</td>
<td>100</td>
</tr>
<tr>
<td>Naranjal</td>
<td>1.0</td>
<td>79.7</td>
<td>7.5</td>
<td>11.8</td>
<td>100</td>
</tr>
<tr>
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<td>8.8</td>
<td>90.9</td>
<td>0.0</td>
<td>100</td>
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<tr>
<td>Carimagua</td>
<td>0.3</td>
<td>10.3</td>
<td>87.6</td>
<td>1.8</td>
<td>100</td>
</tr>
<tr>
<td>Wahiawa</td>
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<td>67.8</td>
<td>7.7</td>
<td>100</td>
</tr>
<tr>
<td>Neira</td>
<td>0.6</td>
<td>35.4</td>
<td>11.1</td>
<td>52.9</td>
<td>100</td>
</tr>
<tr>
<td>Letras</td>
<td>1.3</td>
<td>52.7</td>
<td>2.0</td>
<td>43.9</td>
<td>100</td>
</tr>
</tbody>
</table>

* Labile P= soluble and loosely bound.
Appendix F. Pinnule P content of Leucaena leucocephala as a function of the inoculation with Glomus fistulosum (with: +AMF, Without -AMF) and Mortierella sp. (with: +PSF, Without -PSF) in three Andisols (A: Guane; B: La Selva; C: Naranjal) at different days of sampling (12, 26, 36, and 46 days after transplanting). (D) Shoot dry weight at harvest. All experimental conditions were similar to those reported for the experiment of chapter nine, except that all soils received P at a rate of 600 mg/kg, Hulla RP was used as the source of P. Mortierella sp. was ineffective in increasing plant P uptake and growth of mycorrhizal and non-mycorrhizal plants. At harvest, the AMF inoculation significantly (P≤0.05) increased the shoot dry weight (CV = 19.9%) in Guane only and the pinnule P content (CV= 15.1%) in Guane and in a lesser extent in La Selva (P≤0.05), but not in the Naranjal (CV= 15.1%).
APPENDIX G.

DEVELOPMENT OF A SELECTIVE MEDIUM FOR RECOVERING POPULATIONS OF Mortierella sp. INTRODUCED INTO TROPICAL SOILS

INTRODUCTION

Although phosphate-solubilizing microorganisms (PSM) have been known to exist for a long time, interest in the microorganisms has intensified over the past decade (Whitelaw, 2000; Barea et al., 2002; Bashan et al., 2007). We screened several bacterial and fungal isolates from highly weathered and volcanic ash soils of Hawaii and noted a fungal isolate identified as Mortierella sp. that was the most active PSM (Osorio and Habte, 2001). Since then, a series of experiments have demonstrated that the fungus can stimulate P uptake and growth of mycorrhizal Leucaena leucocephala in highly weathered soils. At present, there is not a selective medium that can allow researchers to detect and quantify populations of the fungus subsequent to its inoculation into soil.

The aim of the current investigation was to develop a selective medium based on the tolerance of Mortierella sp. to two fungicides and the sensitivity of background bacteria to streptomycin, using rock phosphate as a sole P source.

MATERIALS AND METHODS

This work was conducted in the soil microbiology labs of the University of Hawaii (Honolulu) and the National University of Colombia (Medellin). The phosphate solubilizing fungus (PSF), Mortierella sp., was isolated from the rhizosphere of L. leucocephala grown in the Tantalus soil series (medial over pumiceous or cindery, ferrhydritic, isothermic Typic Hapludand) and has been maintained on yeast manitol agar (YMA) slants at 4°C. The YMA used consisted of 0.5 g KH$_2$PO$_4$, 0.2 g MgSO$_4$.7H$_2$O, 0.1 g NaCl, 10 g manitol, 1 g yeast extract, and 15 g agar per liter of deionized water. The medium was sterilized in an
autoclave at 120°C and 0.1 MPa. Yeast mannitol agar plates were used for cultivating Mortierella sp. for seven days in the dark at 32°C. At the end of the growth period, the fungal colonies were aseptically suspended in 250 mL sterile deionized water. The suspension was manually shaken and stored at 4°C for further experiments.

Determination of tolerance to benomyl

Serial dilutions (10^{-3} to 10^{-7}) of the initial suspension of Mortierella sp. (1 day-old) were prepared using sterile deionized water. One-mL of the suspension from each dilution was aseptically transferred into petri dishes and approximately 30 mL portions of YMA medium amended with streptomycin sulfate (500 µg/mL) to suppress background bacterial populations and different concentrations of benomyl (0, 25, 75, 100 µg/mL) was poured into the petri dishes. The content of the dishes were homogenized by swirling the dishes 10 times in the right direction and 10 times in the left direction. The medium was sterilized by autoclaving for 30 minutes at 120°C and 0.1 MPa. Streptomycin was obtained from Sigma Chemical Company, St.Louis, MO. A sterile stock solution of the antibacterial agent was prepared by dissolving the material in 70%ethyl alcohol and storing it at 4°C for 24 hours before use. Benomyl [methyl 1 (butylcarbamoyl) benzimidazole carbamate] was supplied as a wettable powder (50% active ingredient) by E.I. du Pont de Nemours and Co., Midland, Mich. Sterile stock suspension of benomyl was obtained by suspending the fungicide in 70% ethanol and incubating it at 4°C for 24 hours before use. Benomyl suspension and streptomycin solution were introduced into melted YMA at 45-50°C immediately before the medium was poured into Petri plates. The Petri dishes were incubated for 36 hours in the dark at 32°C. Treatments were arranged in a completely randomized design with three replicates per treatment. At the end of the incubation period, colonies forming unit (CFU) were counted, and mean colony diameter (MCD) and total surface area (TSA) occupied by fungal colonies.
estimated as follows: \( TSAC = N \pi r^2 \). Where, \( N \) = number of CFU per Petri dish, \( \pi = 3.1416 \), \( r \) = mean colony radius (cm).

**Combined effects of benomyl and cycloheximide**

Serial dilutions (10\(^{-5}\) and 10\(^{-6}\)) of Mortierella sp. were prepared as described above with a 3-days old suspension. One-mL of the suspension from each dilution was transferred into petri dishes. Yeast mannitol agar contained streptomycin sulfate (500 \( \mu \)g/mL), benomyl (75 \( \mu \)g/mL), and cycloheximide (100 \( \mu \)g/mL). Cycloheximide was obtained from Sigma Chemical Co., St. Louis, MO. and was disinfected as in streptomycin and benomyl. Petri dishes without any fungicide were used as controls. Petri dishes were incubated for 36 hours in the dark at 32°C. Each treatment had three replicates. Colony forming units were counted and colony diameter and fungal surface area determined as above. Treatments were arranged in a completely randomized design with three replicates per treatment.

**Rock phosphate and bromothymol addition**

A further refinement of the selective medium was sought by including bromothymol blue (BTB) (Vincent, 1970) and using rock phosphate as the sole source of P. Serial dilutions of the test fungus were prepared and one mL of the 5 day-old Mortierella sp. suspension from the 10\(^{-5}\) dilution was transferred into the Petri plates. Melted YMA containing BTB (25 \( \mu \)g/mL) as a pH indicator, streptomycin sulfate (500 \( \mu \)g/mL), benomyl (75 \( \mu \)g/mL), cycloheximide (100 \( \mu \)g/mL) with either KH\(_2\)PO\(_4\) or North Caroline rock phosphate (NCRP) as a P source was poured into the Petri plates. Plating and incubation conditions were as described above. Colony forming units were counted and colony diameter and fungal surface area determined as above. Treatments were arranged in a completely randomized design with three replicates per treatment.
Suppression of soil fungi by fungicides in YMA

Surface soil samples (0-15 cm) of two Hawaiian soils [Tantalus (Andisol) and Molokai (Oxisol)] and three Colombian soils [Guame (Andisol), Niquia (Ultisol), and Carimagua (Oxisol)] were passed through a sieve with 2-mm aperture size and were maintained at 50% of maximum water holding capacity (MWHC). One-mL of the suspension from the $10^{-3}$ soil dilution was transferred to Petri dishes. Yeast mannitol agar containing KH$_2$PO$_4$ as P source, streptomycin sulfate (500 $\mu$g/mL), and benomyl (75 $\mu$g/mL), cycloheximide (100 $\mu$g/mL) or both fungicides together, with or without BTB (25 $\mu$g/mL) was poured into the Petri plates. Petri dishes without any fungicide served as controls. Plating and incubation conditions were as described above except that the incubation period was 114 hours. After that time CFU were enumerated and MCD and TSAC were estimated. Treatments were arranged in completely randomized design with three replicates per treatment.

Recovery of Mortierella sp. inoculated into Molokai soil

Twenty g (dry basis) of a surface Molokai soil sample was passed through a 2-mm aperture sieve and was maintained at 50% of the MWHC in sterile Petri dishes. Soil samples were either inoculated or not inoculated with 5 mL of an aqueous suspension containing $1.6 \times 10^7$ CFU of Mortierella sp. per mL (5 days-old), homogenized, and incubated for 72 hours at 28°C. After this incubation period, one-mL of the suspension from each of the $10^{-2}$ and $10^{-3}$ soil dilution was separately transferred into Petri dishes. Melted YMA with KH$_2$PO$_4$ as P source containing streptomycin sulfate (500 $\mu$g/mL), benomyl (75 $\mu$g/mL), cycloheximide (100 $\mu$g/mL) with or without BTB (25 $\mu$g/mL) was poured into the plates and homogenized with the soil dilution suspension as previously describe above. After 86 hours of incubation at 32°C, CFU and MCD of Mortierella sp. were determined. Treatments were arranged in a completely randomized design with three replicates per treatment.
Recovery of Mortierella sp. inoculated into Niquia soil

Surface Niquia soil sample was passed through a 4-mm aperture sieve. Styrofoam pots measuring 7.5-cm in diameter and 10 cm depth were filled with 0.2 kg (dry weight basis). The potted soil was either not inoculated or inoculated with 5 mL of a 3 days-old suspension of Mortierella sp. containing 9x10⁵ CFU/mL and uniformly mixed after. Then, germinated seeds of L. leucocephala cv. K-11 were either planted or non-planted in the moistened soil. Pots were incubated in a greenhouse of the National University of Colombia at Medellin (6° 15’N, 75° 35’W, and 1495 m altitude). Pots were watered to maintain the soil at 50-60% of maximum water holding capacity.

Subsamples were collected from the rhizosphere of leucaena or from the bulk soil in the unplanted pots (3-g, dry weight basis) at 10, 18, 22, 26, and 30 days after planting. Serial dilutions were prepared for plating, as described above, with YMA containing streptomycin sulfate (500 μg/mL), benomyl (75 μg/mL), cycloheximide (100 μg/mL). Petri dishes were incubated for six days at 32°C after which time CFU of Mortierella sp. were counted. Treatments were arranged in a completely randomized design with three replicates per treatment.

The data of all experiments were statistically analyzed with the software Statgraphics Plus, version 4.0 (Statpoint, Inc.; Herdon, Virginia) employing a regression analysis or a F-test for the analysis of variance and Duncan test for mean separation (P≤ 0.05) as appropriated.

RESULTS AND DISCUSSION

Tolerance to benomyl

The growth of Mortierella sp. was significantly lowered by the increase of benomyl concentration in the culture medium (Table 1). The increase in the benomyl concentration produced a reduction in the diameter of the fungal
colonies; consequently, the total surface area occupied by the fungus on the medium was also significantly reduced. The rate of change in MCD and TSAC with the levels of the fungicide was properly represented by the following regression models:

\[
MCD = 0.71 - 0.0025x(\text{benomyl, } \mu\text{g/mL}), \ r^2 = 0.77 \\
TSAC = 50.14 - 0.3025x(\text{benomyl, } \mu\text{g/mL}), \ r^2 = 0.79
\]

However, there were not significant changes in the number of CFU of Mortierella sp. with the increasing concentration of benomyl. These results indicate that Mortierella sp. exhibited sensitivity to benomyl, but the presence of the fungicide did not reduce the fungal population counted. For that reason, the use of benomyl in a selective medium for culturing Mortierella sp. seems to be suitable.

Table 1. Sensitivity of Mortierella sp. to benomyl indicated in terms of number of colony forming units (CFU), mean colony diameter (MCD) in mm, and total surface area of colonies (TSAC) with the 10^{-5} dilution.

<table>
<thead>
<tr>
<th>Benomyl concentration (\mu g/mL)</th>
<th>CFU</th>
<th>MCD (mm)</th>
<th>TSAC (cm^2/plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>128</td>
<td>7</td>
<td>49.1</td>
</tr>
<tr>
<td>25</td>
<td>131</td>
<td>6</td>
<td>42.1</td>
</tr>
<tr>
<td>75</td>
<td>117</td>
<td>6</td>
<td>33.0</td>
</tr>
<tr>
<td>100</td>
<td>128</td>
<td>4</td>
<td>5.9</td>
</tr>
</tbody>
</table>

Regression analysis P-value
NS: not significant.

**Combined effects of benomyl and cycloheximide**

The growth of Mortierella sp. was significantly lowered by the presence of benomyl or cycloheximide in the culture medium (Table 2). The fungus growth was significantly slower in the presence of both fungicides than in the presence of each alone. However, the number of CFU was not significantly affected by the
individual or combined presence of these fungicides. The use of both fungicides seems to be adequate for a selective medium for culturing Mortierella sp.

Table 2. Sensitivity of Mortierella sp. to benomyl and cycloheximide indicated in terms of number of colony forming units (CFU), mean colony diameter (MCD) in mm, and total surface area of colonies (TSAC) with the $10^6$ dilution.

<table>
<thead>
<tr>
<th>Fungicide</th>
<th>CFU</th>
<th>MCD (mm)</th>
<th>TSAC (cm²/plate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>149</td>
<td>7a</td>
<td>57.5 a</td>
</tr>
<tr>
<td>Benomyl</td>
<td>156</td>
<td>6b</td>
<td>49.1 b</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>117</td>
<td>3c</td>
<td>10.2 c</td>
</tr>
<tr>
<td>Benomyl + Cycloheximide</td>
<td>128</td>
<td>2d</td>
<td>5.3 d</td>
</tr>
</tbody>
</table>

Anova $P$-value: NS <0.001 <0.001

NS: not significant. Means followed by the same lower case letter are not significantly different from each other, Duncan test ($P<0.05$).

**Rock phosphate and bromothymol blue addition**

The number of CFU of Mortierella sp. was not significantly affected by the presence of both fungicides and the additional presence of BTB (Table 3). Similarly, the number of CFU was not significantly affected by the use of NCRP instead of KH₂PO₄ in the culture medium. However, the rate of fungal growth was lowered by the additional presence of BTB, but not by the use of NCRP respect to KH₂PO₄. Unfortunately, the culture medium was very cloudy when NCRP was used, which made difficult the detection and identification of CFU in the culture. The presence of BTB facilitated the detection of Mortierella sp. colonies because the fungus formed a yellow halo around it as a results of the acid production that characterized this fungus (Osorio and Habte, 2001).
Table 3. Mortierella sp. colony forming units (CFU), mean colony diameter (MCD) in mm, and total surface area of colonies (TSAC) as affected by the presence of KH₂PO₄, North Carolina rock phosphate (NCRP), bromothymol blue (BTB), and fungicides (benomyl and cycloheximide) in the growth medium. Duration of incubation is given in parenthesis.

<table>
<thead>
<tr>
<th>YMA amendment</th>
<th>CFU x 10⁶</th>
<th>MCD (mm)</th>
<th>TSA (cm²/plate)</th>
<th>Appearance of medium in plates</th>
</tr>
</thead>
<tbody>
<tr>
<td>YMA (KH₂PO₄)</td>
<td>167</td>
<td>5 (24 h)</td>
<td>32.9</td>
<td>Clear</td>
</tr>
<tr>
<td>YMA (KH₂PO₄)+ benomyl + cycloheximide</td>
<td>168</td>
<td>3 (72 h)</td>
<td>15.0</td>
<td>Clear</td>
</tr>
<tr>
<td>YMA (KH₂PO₄) + benomyl + cycloheximide + BTB</td>
<td>152</td>
<td>2 (105 h)</td>
<td>4.8</td>
<td>Clear</td>
</tr>
<tr>
<td>YMA (NCRP)</td>
<td>142</td>
<td>5 (24 h)</td>
<td>27.9</td>
<td>Cloudy</td>
</tr>
<tr>
<td>YMA (NCRP) + benomyl + cycloheximide</td>
<td>173</td>
<td>2 (72 h)</td>
<td>4.6</td>
<td>Cloudy</td>
</tr>
<tr>
<td>YMA (NCRP) + benomyl + cycloheximide + BTB</td>
<td>147</td>
<td>2 (105 h)</td>
<td>4.6</td>
<td>Cloudy</td>
</tr>
</tbody>
</table>

Anova P-value  NS  -  -

NS: not significant, Duncan test (P<0.05).

**Suppression of soil fungi by fungicides in YMA**

The presence of benomyl in the culture medium was more effective to suppress the growth of soil fungi than cycloheximide. Although a few fungi grew in the presence of either benomyl or cycloheximide, no fungus tolerated the presence of both fungicides together in the culture medium (Table 4). It means that the tolerance of soil fungi to both fungicides is hardly found in nature. The soils used are diverse (two Andisols, two Oxisols, and one Ultisol) and are representative of tropical environments although they are collected from location far away from each other.
Table 4. Number of colony forming units (CFU) of soil fungi at $10^{-3}$ dilution as affected by benomyl with or without cycloheximide and/or bromothymol blue (BTB). Duration of incubation was 114 h or as indicated in parenthesis.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Molokai</th>
<th>Tantalus</th>
<th>Carimagua</th>
<th>Niquia</th>
<th>Guane</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>71a (36 h)</td>
<td>91a (36 h)</td>
<td>48 a (36 h)</td>
<td>16a (36 h)</td>
<td>54a (36 h)</td>
</tr>
<tr>
<td>Benomyl</td>
<td>1b (36 h)</td>
<td>2c (36 h)</td>
<td>3c</td>
<td>2b</td>
<td>2b</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>55a (60 h)</td>
<td>18b (60 h)</td>
<td>18b</td>
<td>4b</td>
<td>9b</td>
</tr>
<tr>
<td>Benomyl+Cycloheximide</td>
<td>0b</td>
<td>0c</td>
<td>0c</td>
<td>0c</td>
<td>0c</td>
</tr>
<tr>
<td>Benomyl+Cycloheximide+BTB</td>
<td>0b</td>
<td>0c</td>
<td>0c</td>
<td>0b</td>
<td>0b</td>
</tr>
</tbody>
</table>

ANOVA $P$-value  

- 0.001  
- 0.001  
- 0.001  
- 0.001  
- 0.001

Means followed by the same lower case letter are not significantly different from each other, Duncan test ($P<0.05$).

Recovery of Mortierella sp. inoculated into Molokai soil

Table 5 showed that Mortierella sp. was recovered from the Molokai soil after 72 hours after being introduced in that soil. No other soil fungi grew in the medium.

Recovery of Mortierella sp. inoculated into Niquia soil

Figure 1 illustrates that the proposed culture medium allowed the recovery of Mortierella sp. introduced in the Niquia soil. Although the CFU of Mortierella sp.
decreased over time, the fungus was significantly more abundant in the rhizosphere of leucaena than in the bulk soil.

Figure 1. Recovery of Mortierella sp. following its introduction into the Niquia soil and planted or not-planted with Leucaena leucocephala cv. K-11. CFU of Mortierella sp. were recovered on YMA containing streptomycin sulfate (500 µg/mL), benomyl (75 µg/mL), and cycloheximide (100 µg/mL). Petri dishes were incubated for 6 days prior to the counting of CFU's.

LITERATURE CITED


Rodríguez, H., R. Fraga, T. Gonzalez, and Y. Bashan. 2007. Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting


Appendix H. DETECTION DE ORGANIC ACIDS/ANIONS IN LIQUID MEDIUM INCUBATED WITH *Mortierella* sp.

One-mL of a *Mortierella* sp. suspension containing 5.9x10^5 CFU was aseptically transferred in triplicate into 250-mL Erlenmeyer flasks that containing 75-mL of an autoclaved (30 min., at 120°C and 0.1 MPa) liquid medium. The medium consisted of 1.0 g NaCl, 1.0 g NH₄NO₃, 1.87 g KCl, 0.2 g CaCl₂.2H₂O, 0.4 g MgSO₄.7H₂O, 28 mg Fe-EDTA, 28 mg Cu-EDTA, 28 mg Mn-EDTA, 14 mg Zn-EDTA, 10.0 g glucose, and 3.5 g of Huila RP per liter. The empirical formula of this RP was Ca₉.₉₉Na₀.₂₂Mg₀.₀₉(PO₄)₆.₁₄(CO₃)₀.₈₈F₂.₃₄ (Chien and Hammond, 1978). The RP was passed through a 0.5-mm aperture sieve and its P content was 130 g kg⁻¹. The initial solution pH was adjusted with 0.1 M NaOH to pH 7.6. Flasks were continuously shaken at 150 rpm on an orbital shaker (model Innova 4400, New Brunswzc Scientific Co., Inc., Edison, NJ) at 25°C for seven days.

After the period of incubation, 30 mL of of the medium were centrifuged at 5000xg for 15 minutes. Then, the samples were passed through Whatman No. 42 filter paper and through a membrane filter with pore of 0.45 μm od diameter.

Aliquots of the liquid samples were injected through a fine capilar (CE Standard Capillary, 150 μm, 104 cm) into a Hewlett Packard 3D-CE instrument of Capillary Electrophoresis. For this purpose a pH 8 buffer solution was employed, which contained 5 mM sodium chromate, 16 mM potassium phtalate acid, 20 mM hidroxy-methyl-amino-methane, and 0.5 mM cetil-trimethyl-ammonium-bromure (CTAB). The absorbance of the sample, standard organic acids and fluoride was measured during 45 min using a wavelenght of 350 nm.
Initially, five organic acids (formic acid, citric acid, acetic acid, propionic acid, and butyric acid) were injected into the capilar (March 15, 2008). Later, oxalic acid and fluoride were injected (March 27, 2008) The results are shown in the figures 1 and 2.

Figure 1. Absorbance detected with a sample of the liquid medium where Mortierella sp. was grown and with some organic acids using Capillary Electrophoresis (Hewlett Packard 3D-CE).

The results of the Figure 1 indicates that citric acid, formic acid, acetic acid, propionic acid, and butyric acid were not detected in the sample.

When oxalic acid (at 80 mg/L) was used there was a peak at 11.5 minutes of migration time, similar to that detected in the samples of the liquid medium where Mortierella sp. was grown (Figure 2). Then, oxalic acid was added at 40 mg/L to the sample of the liquid medium and again, the absorbance peak was similar to the original samples. This result indicates that Mortierella sp. produced oxalic
acid and its conjugated base (oxalate) are responsible of rock phosphate solubilization, Pi desorption from soil minerals, and formation of an Al complex as seen in the previous chapters. These activities of oxalic acid have been reported by several authors.

Figure 2. Absorbance detected with oxalic acid and fluoride (above), sample of the liquid medium where Mortierella sp. was grown (below), sample of the liquid medium plus oxalic acid at 40 mg/L. Capillary Electrophoresis (Hewlett Packard 3D-CE).

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