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Rock phosphate solubilization by the ectomycorrhizal fungus *Laccaria fraterna* and its associated mycorrhizal helper bacterial strains

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Phosphorus (P) is mostly present in the form of insoluble phosphates in soil and so plants cannot utilize it. Plant roots are often associated with mycorrhizal fungi and other microorganisms that effectively can absorb P and other elements from the soil. In the present study, few mycorrhizal helper bacterial (MHB) strains such as *Pseudomonas putida*, *Erwinia herbicola* and *Bacillus subtilis* were isolated and identified from surface sterilized basidiomata and mycorrhizosphere of an ectomycorrhizal fungus *Laccaria fraterna* association with *Eucalyptus globulus*. To evaluate phosphate solubilization of the fungus and its MHB strains, they were grown on rock phosphate (RP) and tricalcium phosphate as sole P sources in Pikovskaya's liquid medium. The fungus and its MHB showed varying levels of phosphate solubilization activity in the presence of different carbon and nitrogen sources. These fungus and MHB had the potential to solubilize these phosphates by decreasing the pH and confirmed that phosphate solubilization is accompanied by acid production. Thus, the evidence that fungus and MHB strains do convert insoluble phosphates such as RP into soluble forms, may not only replace expensive phosphorous fertilizer in agriculture field but also lead to better mobilization of the fertilizers added to soils.

Key words: Ectomycorrhizal fungi, *Laccaria fraterna*, mycorrhizal helper bacteria, phosphate solubilization, rock phosphate.

INTRODUCTION

Phosphorus (P) is the second major nutrient next to nitrogen required by plants. However, many soils throughout the world are P deficient while nearly 95 to 99% of soil P is present in the form of insoluble phosphates (Vassileva et al., 1998) and only 1 to 5% is available in the form of soluble, plant-available form (Molla et al., 1984). To overcome the deficiency, large amount of chemical P fertilizers is being applied to soil. Despite this, a large proportion (75 to 90%) of fertilizer P is quickly transformed into the insoluble form (Omar, 1998), thus making a continuous application necessary (Abd Alla, 1994). Moreover, widespread phosphorus

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deficiencies throughout the world's soil make use of phosphorus fertilizers an expensive necessity for agricultural crops worldwide.

Many soil fungi and bacteria are known to solubilize inorganic phosphates (Asea et al., 1998). Phosphates solubilized by these microorganisms are absorbed more efficiently by plants through mycorrhizal-association that occurs between the plant roots and surrounding soil (Landeweert et al., 2001). Ectomycorrhizal fungi (EMF) that live in symbiotic association with root of most terrestrial plants play an important role in forest ecosystems. Ectomycorrhizal symbioses are mostly formed by basidiomycetes and some by ascomycetes. The extramatrical hyphae of the fungus explore the soil, travelling long distances beyond the nutrient depletion zone. Minerals like N, P, K, Ca, S, Zn, Cu and Sr are absorbed from soils and translocated to the host plant. In turn, the plant supplies the fungus with carbohydrate necessary for its growth (Chellappan et al., 2002). The EMF *Laccaria fraterna* is widespread throughout the world, where *Eucalyptus* and other ectomycorrhizal associated plants are grown (AnithaChristy et al., 2014). *L. fraterna* is an early colonizing fungus that produces large number of basidiomes and dominates disturbed land and farm sites (Tommerup et al., 1991). Studies showed that many bacterial strains were able to promote ectomycorrhizal symbioses and these bacterial strains have been called as mycorrhizal helper bacteria (MHB) (Duponnois, 2006).

In EMF, mostly basidiomycetes positively interact with MHB except in EM ascomycete fungi *Tuber melanosporum*, where Mamoun and Olivier (1992) reported the presence of soil pseudomonads. The MHB strains that have been identified thus far belong to bacteria of the gram-negative and gram positive groups such as *Pseudomonas, Bacillus, Rhizobium, Burkholderia, Achromobacter, Agrobacterium, Micrococcus, Aereobacter, Flavobacterium* and *Erwinia* beside some *actinomycetes*. Application of these microorganisms along with EMF enhances the availability of scarce nutrients such as N, P and micronutrients through nitrogen fixation, phosphate solubilization or as biological disease suppression. Thus, they might be reducing the use of more P fertilizer and increased crop yield (Aspray et al., 2006). MHB interactions in *L. fraterna* have not been adequately explored. So in the present study, the RP solubilization ability of MHB and the ectomycorrhizal fungus *L. fraterna* were investigated.

MATERIALS AND METHODS

Microorganisms

The ectomycorrhizal fungus *L. fraterna* was isolated from the basidiomata associated with *Eucalyptus globulus* plantations in the Nilgiri hills of the state of Tamilnadu in Southern India. The sporocarp and mantle of *L. fraterna* were surface sterilized using sterile distilled water, chopped, homogenized in 10 ml sterile distilled water using a homogenizer. A 1.0 ml aliquot of this was then plated onto Tryptic Soy Agar containing cycloheximide (75 mg/L) and incubated at 25°C for three to five days.

For isolation of bacteria, soil samples each weighing 1.0 g were taken from the mycorrhizosphere of the *E. globulus* roots. The soil samples were suspended in one-quarter strength Ringer's solution. The suspension was then diluted to 10^{-4} dilutions and 200 µL of diluted soil samples were spread on Tryptic-Soy agar medium (TSA) for estimation of total number of bacterial colonies (Kirchner et al., 1993) and on P1 medium (Katoh and Itoh, 1983) for fluorescent pseudomonads. Plates were incubated at 25°C. Bacterial colony forming units (cfu) were counted after 3 days for the fast growing bacterial colonies and after 5 days for the slower-growing colonies.

Bacterial strains were identified as *P. putida*, *E. herbicola* and *B. subtilis* using phenotypic and physiological tests. They were further confirmed using partial 16S rRNA gene amplication (data not shown) (Bending et al., 2002). These strains were maintained on TB agar medium as well as in glycerol stock (Sambrook et al., 1989).

Media and growth conditions

Fungal cultures were maintained on Modified Melin-Norkrans (MMN) (Marx, 1969) medium at 25 ± 2°C in the dark. Fungal mycelial discs of 1.0 mm were grown on MMN medium for 10 days (Marx and Bryan, 1975), then transferred to Pikovskaya's (PKS) medium (Pikovskaya, 1948) with tricalcium phosphate (Ca_3 $(PO_4)_5$) (TCP) as sole P source.

Single colony of each bacterium was picked up and suspended into 10% TS broth. After 48 h at 25°C, culture was centrifuged (3 300 *g*, 25°C, 10 min), washed once, and resuspended in deionized water to obtain an OD of ~ 0.7 at A600 nm. Flasks inoculated with 10 days old mycelia discs of the fungus and 50 µL of each bacterial suspension, were incubated at 25 ± 2°C in the dark for 20 days (Deveau et al., 2007). Phosphate solubilizing ability of the fungus and MHB strains were tested on six different liquid media with 0.5% TCP as sole P source after 20 days (Table 1). Further experiments were performed on PKS medium with rock phosphate (RP-140) as sole P source.

Effect of different carbon sources such as cellulose, cellobiose, carboxyl methyl cellulose (CMC), lactose, maltose and fructose instead of glucose (Table 2) were also checked for P solubilization. For determining the effect of different nitrogen sources on P solubilization, $(NH_4)_2SO_4$ was replaced with 0.05% of different nitrogen salts such as Urea, KNO_3, $NaNO_3$, NH_4Cl, $NaNO_2$ (Figure 2). Phospholytic activity of fungus along with MHB strains without any carbon and nitrogen sources acted as control and measurements were made after 20 days at 25 ± 2°C in the dark

Solubilization of phosphorus from rock phosphate

The RP -140 samples which contained ~18.9% of P_2O_5 was used for all the experiments. Quantitative estimation of RP solubilization was carried out in PKS medium amended with 0.25% (w/v) of RP as sole P source for duration of 20 days.

Estimation of phosphorus

Cultures were checked after 20 days to see the drop in pH and increase in concentration of P released in the medium. After centrifugation at 12 000 rpm for 20 min, the pH of the culture medium was measured with a pH meter. Dissolved phosphate concentration in the culture filtrate was measured using Murphy and Riley's (1962) method. Measurement of phosphate was expressed as µg/ml of phosphorus released into culture medium.

Table 1. Composition of different media used for this study.

Component g/L	Medium 1	Medium 2	Medium 3	Medium 4	Medium 5	Medium 6
Glucose	20	10	100	10	10	10
$(NH_4)_2SO_4$	1.0	-	-	0.5	0.5	0.1
$MgSO_4.7H_2O$	0.5	0.4	0.2	0.1	0.1	0.25
Yeast extract	0.2	0.5	-	0.5	-	0.5
KCL	-	-	-	0.2	0.2	0.2
NaCl	-	1.0	-	0.2	0.2	-
$FeCl_3$	Trace	-	-	-	-	-
$FeSO_4.7H_2O$	-	-	-	0.002	0.002	-
$MnSO_4.H_2O$	Trace	-	-	0.002	0.002	-
$MgCl_2.6H_2O$	-	-	-	-	-	5.0
$CaCl_2$	-	0.2	-	-	-	-
NH_4NO_3	-	1.5	0.5	-	-	-
$ZnSO_4$	-	-	0.004	-	-	-
$Ca_3(PO_4)_5$	5.0	5.0	5.0	5.0	5.0	5.0
pH	6.8	7.0	5.0	7.0	7.0	7.0

Medium 1, AYG: Halder et al. (1991); medium 2, Kim et al. (1997); medium 3, Vassilev et al. (1998); medium 4, PKS; Pikovskaya (1948); medium 5 and 6, NBRIY; Nautiyal (1999).

Table 2. Effect of different Carbon and Nitrogen sources.

Carbon source	P concentration (µg/ml)	Final pH	Nitrogen sources	P concentration (µg/ml)	Final pH
Glucose	528	4.8	$(NH_4)_2SO_4$	622	4.2
Fructose	399	4.7	Urea	484	6.5
Lactose	380	4.65	KNO_3	300	5.08
Cellobiose	482	4.8	$NaNO_3$	382	4.5
Maltose	280	5.4	NH_4Cl	398	4.3
Cellulose	228	5.3	$NaNO_2$	322	5.4
Carboxyl Methyl Cellulose	200	5.3	Control	82	6.18
Starch	180	5.35			
Control	85	6.11			

Statistical analysis

All experiments have been conducted in triplicates and average mean values were estimated.

RESULTS AND DISCUSSION

Solubilization of insoluble phosphates

To check the phosphorus solubilizing ability of the fungus *L. fraterna* and all MHB strains such as *P. putida*, *E. herbicola* and *B. subtilis*, we used TCP as sole P source and used six different media (Table 1) to figure out which medium supported best results. We noticed among six different media (Figure 1), that PKS medium and AYG medium (Halder et al., 1991) showed maximum solubilization. PKS medium showed maximum P solubilization at the rate of 722 µg/ml of P from 0.5% TCP with decrease in pH from 7.00 to 4.53 when used with 10% glucose and 0.5% $(NH_4)_2SO_4$. While AYG medium with 20% glucose and 1% $(NH_4)_2SO_4$ released 916 µg/ml of P from 0.5% TCP with decrease in pH from 6.88 to 4.05 was observed in culture filtrate after 20 days and in remaining other three media low level of P solubilization was observed.

Among these six different media, PKS medium proved to be most cost effective based upon the amount of glucose utilization and corresponding efficacy of P solubilization (Pradhan and Shukla, 2005). Recent results with *L. fraterna* (AnithaChristy et al., 2014) and other fungi (Pradhan and Shukla, 2005) have also confirmed that PKS medium was most cost effective on the basis of P solubilization efficacy. Therefore for further studies PKS medium was used.

Phosphate solubilization was usually accompanied by a decrease in the pH of the medium by all these strains.

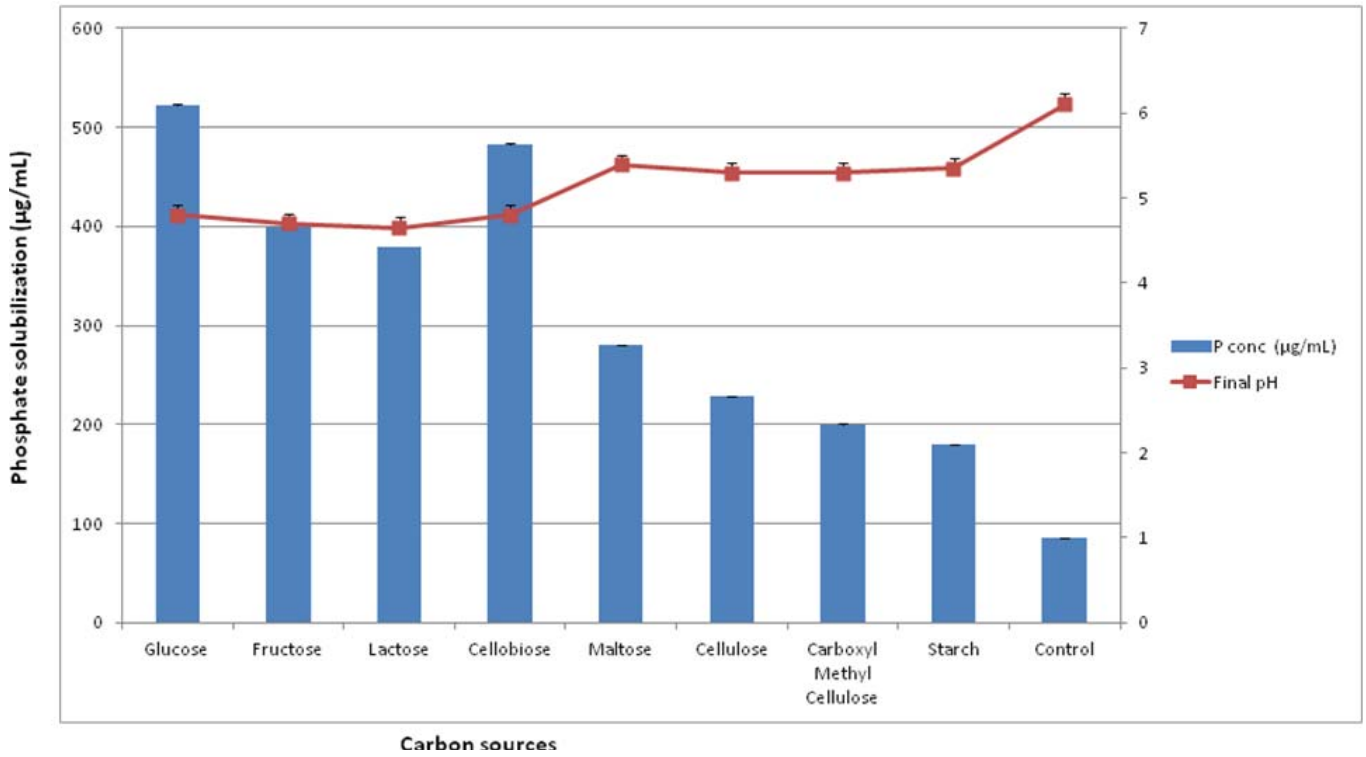

Figure 1. Effect of carbon sources on 'P' solubilization using *L. fraternal* and its MHB strains.

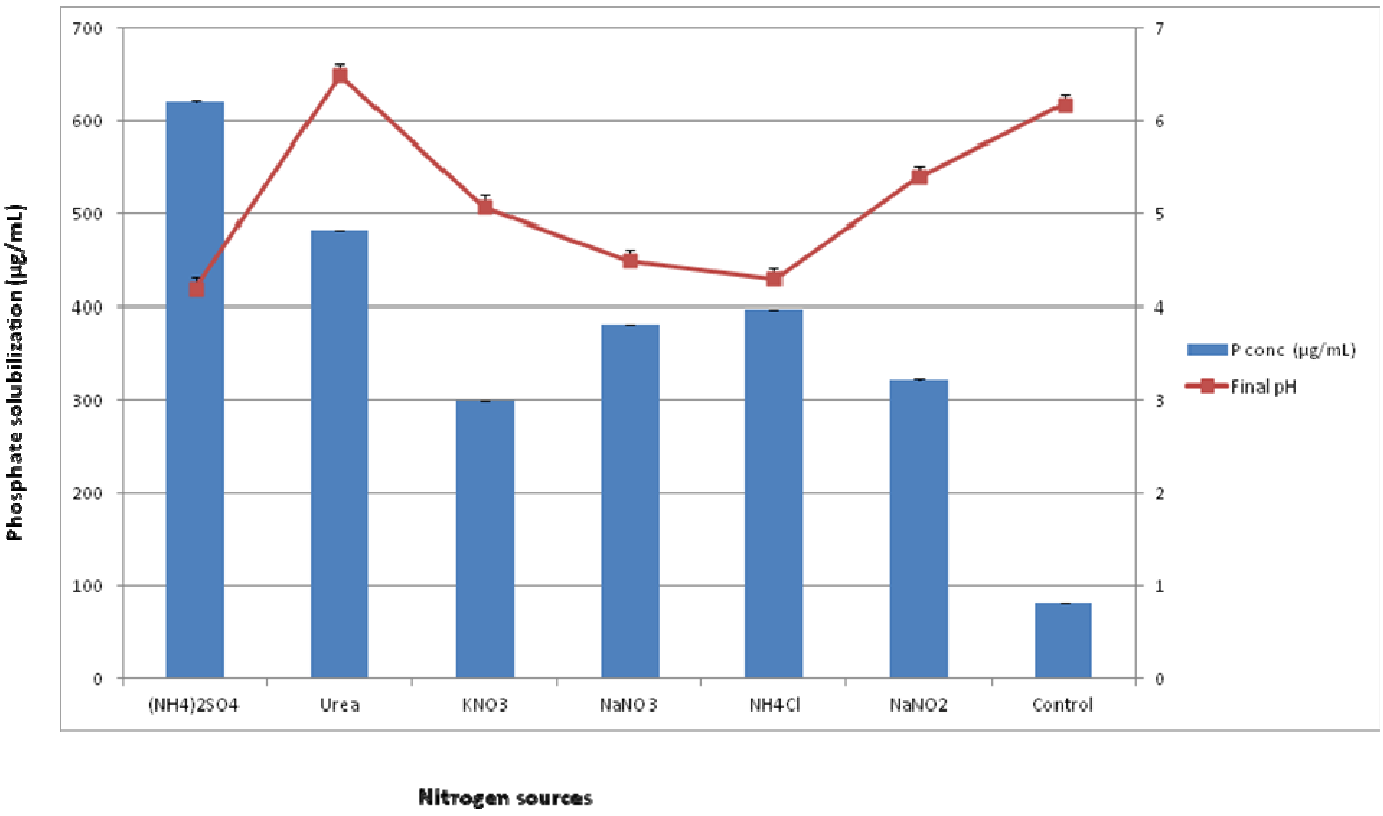

Figure 2. Effect of nitrogen sources on 'P' solubilization using *L. fraternal* and its MHB strains.

L. fraterna solubilized 116 µg/ml of phosphorus from 0.5% TCP and caused a decrease in pH from 7.0 to 4.2 in 20 days. When RP was used as sole P source, *L. fraterna* released 85 µg/ml of P in PKS medium after 20 days of incubation. In comparison, an *Aspergillus sp.* isolated from rice field soils of Orissa, India released 58 µg/mL of P after seven days of incubation in PKS medium with RP as sole P source (Pradhan and Shukla, 2005). *L. fraterna* and the other MHB strains solubilized 928 µg/ml of phosphorus in 20 days with a reduction in pH reduced from 7.0 to 4.16. It was lower when compared to TCP as sole P source because of the complexity of RP's structure. Similar finding was also observed by Pradhan and Shukla (2005).

Phosphorus solubilizing microorganisms mostly dissolved insoluble phosphates by the production of organic acids (Khan et al., 2010) such as acetate, lactate, oxalate, tartarate, succinate, citrate, gluconate, ketogluconate, glycolate and others to decrease the pH. These organic acids solubilized RP using acidification, chelation, and exchange reactions (Bashan et al., 2013b, Omar, 1998). The reduction in pH and the extent of soluble phosphate formed was negatively correlated.

Earlier reports states that the soluble or insoluble phosphates are dissolved using acidification while showing some phosphorus solubilizing activity. AnithaChristy et al. (2014) also observed that oxalic acid was secreted by *L. fraterna* to solubilize RP. In most soils, proton substitution reactions are driven by microbial production of organic acids, which is represented generically by the following equation:

$$(Ca^{2}+)\ m\ (PO4^{3-})\ n + (HA) = (H^{+})\ (PO4^{3-}) + (Ca^{2+})\ (A^{-})$$

There is no stoichiometry in the equation, because of the complexity of CaP chemistry, release of microbial produced organic acids (HAs) or differing numbers of dissociable protons released to their surroundings (Goldstein, 1986). The organic acids secreted directly dissolve the mineral phosphate either as a result of anion exchange of PO_4^{2-} by acid anion or by chelating both Fe and Al ions associated with phosphate molecule (Gyaneshwar et al., 2002).

Factors affecting solubilization of phosphorus

The role of carbon source is important in mineral phosphate solubilization as the carbon source determined the type of organic acid to be secreted which played an important role in solubilization (Di Simine, 1998). Thus production of acid greatly influenced phosphate solubilization activity. Nature of acid produced during solubilization is more important than the quantity of the acid (Agnihorti, 1970). Phosphate solubilization activity of fungus and MHB strains was tested in the presence of five different carbon and seven different nitrogen sources by replacing glucose and $(NH_4)_2SO_4$, respectively of the PVK medium. The fungus and MHB strains showed different amounts of phosphate solubilization activity in the presence of different carbon sources and nitrogen sources. The fungus and MHB strains showed varying amounts of phosphate solubilization activity in the presence of various carbon sources such cellulose, cellobiose, carboxyl methyl cellulose (CMC), lactose, maltose and fructose. Glucose, cellobiose, fructose, lactose decreased the pH of the medium to a maximum extent and caused highest solubilization of phosphate, followed by maltose, cellulose, CMC and starch (Table 2).

The growth of *L. fraterna* on PKS medium with glucose as carbon source also supported maximum phosphate solubilizing activity (AnithaChristy et al., 2014). This observation is also in line with other earlier reports. *A. aculeatus* and *Aspergillus sp.,* also showed maximum phosphate solubilizing activity on glucose (Narsian and Patel, 2000; Pradhan and Shukla, 2005). Fasim et al. (2002) have reported that most bacterial isolates solubilized P well in presence of glucose, while other researchers have showed varying degrees of utilization and solubilization in presence of a wide range of carbon sources. Our results correlated with results of Nautiyal et al. (1999). Control flask even without the addition of a carbon source showed some growth due to the presence of yeast extract in the medium. It also showed a small drop in pH and P solubilization

Nitrogen salts having either an ammonium or nitrate group or both were used as nitrogen sources for this study. It is known that nitrogen sources such as ammonium nitrogen or nitrate nitrogen significantly influenced phosphate solubilization by ectomycorrhizal fungi (Lapeyrie et al., 1991). In our experiment, we observed that $(NH_4)_2SO_4$ was used efficiently in reducing the pH of the medium to 4.2 while showing also maximum solubilization of 622 µg/ml of P (Table 2). Here also the control flask showed a drop in pH and P solubilization due to the presence of yeast extract and glucose. When compared to $(NH_4)_2SO_4$ the remaining nitrogen sources showed only moderate increase in P solubilization. This finding was also confirmed by the effects of different media on P solubilization (Table 1). AYG and PKS media confirmed that *L. fraterna* and MHB strains used $(NH_4)_2SO_4$ as nitrogen source showed maximum P solubilization. Low levels of P solubilization were observed in other media containing NH_4NO_3 (Kim et al., 1997; Vassileva et al., 1998) and medium with lower concentration of $(NH_4)_2SO_4$ (NBRIY medium) as nitrogen sources (Figure 2). Previous reports on phosphorus solubilizing microorganisms (Whitelaw, 2000; Pradhan and Shukla, 2005; Khan et al., 2010; AnithaChristy et al., 2014) have also confirmed that phosphate solubilization depends upon the presence of ammonium as the nitrogen source. It has also been reported that in the presence of ammonium nitrogen, calcium phytate and calcium phosphate were easily solubilized, due to acidification (Salsac et al., 1982).

Nitrogen sources such as ammonium nitrogen or nitrate nitrogen significantly influenced phosphate solubilization which uses different mechanisms to generate of acidity in the culture (Lapeyrie et al., 1991; AnithaChristy et al., 2014). Our observation was similar. However, there is no significant correlation between the amount of phosphate solubilization and drop in pH. Pradhan and Shukla (2005) noticed that when urea was used as a nitrogen source, the pH decreased from 7.0 to 5.23. However in our study, urea decreased the pH only to 6.5 without compromising phosphate solubilization. Besides acid production there are many factors that also influenced phosphate solubilization (Whitelaw, 2000).

MHB strains and their association with mycorrhizal fungi deserve special attention because of their practical importance in crop production and their potential applications in agriculture, horticulture and forestry for nutrient mobilization from soil minerals, fixation of atmospheric nitrogen, and protection of plants against root pathogens (Frey-Klett et al., 2007). MHB strains involved in RP solubilization along with *L. fraterna* were identified as *P. putida*, *E. herbicola* and *B. subtilis*. Frey-Klett and Garbaye (2005) showed that, in a forest nursery, the proportion of phosphorus-solubilizing fluorescent pseudomonads was much higher in Douglas-fir-*L. bicolor* ectomycorrhizas than in the surrounding root-free soil. Similarly, Calvaruso et al. (2007) also observed that the oak (*Quercus sessiliflora*)-*Scleroderma citrinum* mycorrhizosphere significantly selected very efficient strains for phosphorus mobilization from the organo mineral horizon of a forest composed of various fungal and bacterial strains. When associated with plant roots, mycorrhizal fungi received up to 30% of the total carbon fixed and frequently transformed it into trehalose, a disaccharide that has been proposed to act as a carbon sink (Lopez et al., 2007). Reports showed that this trehalose was responsible for the selection of specific bacterial communities in the mycorrhizospheres of tree roots (Uroz et al., 2007). Dunstan et al. (1998) showed that the sporocarp of *L. fraterna* associated with *E. diversicolor* showed colonization with *Bacillus sp.* and *Pseudomonas sp.* increased mycorrhizal formation. They increased the formation of first order ectomycorrhizal roots (Bending et al., 2002). Most of the *Laccaria sp.* showed an association with *Pseudomonas* sp. This *Pseudomonas sp.* protects the mycorrhizal fungi against virulence of other plant-pathogenic fungal isolates (Schelkle and Peterson, 1997). It has been showed some EMF and arbuscular mycorrhizas are associated with *E. herbicola*. *E.herbicola* produced indole 3 acetic acid (IAA) (Hamil et al., 1993) an auxin required for organogenesis in EMF when secreted at lower concentrations (Kaska et al., 1999).

Conclusions

The results of the present study show the effective utilization of TCP and RP by *L. fraterna* and its MHB strains under different conditions. Thus, the use of these organisms could present a better strategy to improve plant growth than the use of expensive phosphorous fertilizers.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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