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Full Length Research Paper

Phosphate solubilization of *Paenibacillus polymyxa* and *Paenibacillus macerans* from mycorrhizal and non-mycorrhizal cucumber plants

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The phosphate-solubilizing ability of sixteen *Paenibacillus* strains, which previously were isolated from rhizosphere, hyphosphere or bulk soil, respectively, from mycorrhizal and non-mycorrhizal cucumber plants, were examined. Result from this study showed that seven strains could solubilize both $Ca_3(PO_4)_2$ and $CaHPO_4$, while inositol hexaphosphate could be solubilized by two strains in National Botanical Research Institute's phosphate growth medium. However, none of the *Paenibacillus* strains produced clear solubilization halos in National Botanical Research Institute's phosphate growth medium with $AlPO_4$ or $FePO_4$ as phosphate source. Quantitative estimation of phosphate solubilization in National Botanical Research Institute's phosphate growth broth with inorganic and organic phosphate sources showed that all 16 strains solubilized $CaHPO_4$, 12 strains solubilized $Ca_3(PO_4)_2$, 6 strains solubilized $AlPO_4$, 10 strains solubilized $FePO_4$ and 7 strains solubilized inositol hexaphosphate, which showed that screening of phosphate solubilizing bacteria should be done by quantifying their potential of phosphate solubilization in liquid growth medium. Overall, our results show that the *Paenibacillus* strains have a potential in phosphate solubilization, however, the efficacy of phosphate solubilization did not seem to differ between strains of *Paenibacillus* originating from either mycorrhizal or non-mycorrhizal systems.

Key words: Arbuscular mycorrhiza, inorganic phosphate, organic phosphate, *Paenibacillus*, phosphate solubilization.

INTRODUCTION

Phosphorus (P) is one of the major plant nutrients limiting plant growth. Most of the essential plant nutrients, in particular P, remain in insoluble form in soil. Up to 75% of the soluble P fertilizers added to crops may be converted to sparingly soluble forms by reacting with the free Fe^{3+} or Al^{3+} in low pH soils or with Ca^{2+} ions in high pH soils (Alikhani et al., 2006). Organic P represents from 50 to 80% of the total soil P, and most plants are unable to utilize these P sources (Richardson, 2001). Thus, one area of increasing interest is the use of microorganisms with the ability to solubilize mineral and organic P (Nautiyal, 1999; Alikhani et al., 2006).

Arbuscular mycorrhizal (AM) fungi has been known to play an important role in effecting the availability of soil P to plant roots, and increasing P mobilization in soil (Toro et al., 1997; Smith and Read, 2008). However, bacteria in the mycorrhizosphere have been suggested to be involved in establishment of mycorrhizal fungi (Hildebrandt et al., 2006), and even specific bacterial

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strains have been known to function as mycorrhization helper bacteria (Frey-Klett et al., 2007). Using AM fungi for P uptake has received considerable attention, but the importance of the interactions between bacteria and the mycorrhizal symbiosis in the P solubilization, is far from fully understood (Artursson et al., 2006).

Bacteria from the genus *Paenibacillus* have been found to be closely associated with the external mycelium of AM fungi (Mansfeld-Giese et al., 2002; Toljander et al., 2006). Furthermore, several studies revealed that these bacteria are not only interested in relation to their mycorrhiza helper features (Li et al., 2008a), but they are also known as plant growth promoters (Artursson et al., 2006; Algam et al., 2010), and biocontrol agents (Timmusk et al., 2009; Li et al., 2007, 2010, 2011a, b). These results revealed that bacteria from the genus *Paenibacillus* played an important role in the complex interactions between AM fungi, plants, and soilborne plant pathogens.

The aim of this study was to examine the effect of bacteria from the genus *Paenibacillus* on the P solubilization. In addition, the P solubilization of *Paenibacillus* originating from AM or non-mycorrhizal cucumber rhizosphere were compared.

MATERIALS AND METHODS

Bacterial strains and AM fungi

Ten arbuscular mycorrhiza-associated bacteria were obtained from different sample fractions: rhizosphere soil (AM/R), root-free soil or sand (AM/H), and mycelium extracted from the sand by wet-sieving (AM/M) of *Glomus intraradices* BEG87-cucumber symbiosis, while six non-AM-associated bacteria were obtained from the rhizosphere (Non-AM/R) and bulk soil (Non-AM/B) of non-mycorrhizal cucumber plants (Table 1). These bacteria were all isolated in a previous study (Mansfeld-Giese et al., 2002). All chemicals in this study were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Phosphate solubilization in solid media

Phosphate solubilization activities were tested by plate assay using National Botanical Research Institute's phosphate (NBRIP) growth medium (Nautiyal, 1999). The medium was supplemented with 5 g/L $Ca_3(PO_4)_2$ or $CaHPO_4$ or $FePO_4$ or $AlPO_4$ or 5 ml/L inositol hexaphosphate as P source, respectively. The pH of the medium was adjusted to 7.0 before autoclaving. The medium were distributed in Petri plates and marked in four equal parts after solidification. Four strains per plate were stabbed using sterile toothpicks. All tests were performed with four replications. Inoculated plates were incubated in dark at 30°C and the diameter of clear zone (halo) surrounding the bacterial growth as well as the diameter of colony were measured after 4 weeks.

Phosphate solubilization in liquid medium

Phosphate solubilization activities were further tested in NBRIP broth supplemented with 5 g/L $Ca_3(PO_4)_2$ or $CaHPO_4$ or $FePO_4$ or $AlPO_4$ or 5 ml/L inositol hexaphosphate, respectively. Erlenmeyer flasks (50 ml) containing 20 ml of NBRIP broth supplemented with different P sources were inoculated with bacterial suspension to give a final bacterial concentration of 10^7 cfu/mL and then the flask was incubated at 28°C on a rotary shaker (Hualida Company, Taicang, China) at 130 r/min. Autocleaved uninoculated broth served as control. All tests were performed with four replications. After 3 d of incubation, the cultures were harvested by centrifugation at 10000 r/min for 10 min. Phosphate in culture supernatant was estimated using the molybdenum blue spectrophotometric method (Yu, 2002).

Dynamics of phosphate solubilization

Among the 16 *Paenibacillus* strains, only *P. macerans* MB02-992 was able to solubilize phosphate from five different sources of P in broth culture. In particular, strain MB02-992 showed the highest P solubilization with $Ca_3(PO_4)_2$ in both solid and liquid media. Therefore, strain MB02-992 was selected for evaluation of dynamics of P solubilization by examining the effect of incubation time on the bacterial growth and their P solubilization ability in NBRIP broth supplemented with 5 g/L $Ca_3(PO_4)_2$. Quantitative estimation of P solubilization by strain MB02-992 was carried out in 7 days according to the method described earlier. In addition, the cell numbers in NBRIP broth was determined according to the plate count method (Li et al., 2008b).

Data analysis

The software STATGRAPHICS Plus, version 4.0 (Copyright manugistics Inc., Rockville, Md., USA) was used to perform the statistical analyses. Levels of significance ($P<0.05$) of main treatments and their interactions were calculated by analysis of variance (ANOVA) after testing for normality and variance homogeneity.

RESULTS AND DISCUSSION

Among the 16 *Paenibacillus* strains, *P. polymyxa* MB02-226, MB02-376, MB02-428, MB02-1007, MB02-1172, and MB02-1265 and *P. macerans* MB02-992 were able to solubilize $Ca_3(PO_4)_2$ and $CaHPO_4$ while *P. polymyxa* MB02-1007 and *P. macerans* MB02-992 could solubilize inositol hexaphosphate on NBRIP medium (Table 1). In general, the degree of P solubilization varied with the *Paenibacillus* strains. In particular, *P. macerans* MB02-992 was found to be superior to all other strains in both inorganic and organic P solubilization. However, no *Paenibacillus* strain could produce clear solubilization halos in NBRIP medium with $AlPO_4$ or $FePO_4$ as P source.

All *Paenibacillus* strains except four strains (*P. polymyxa* MB02-428, MB02-1007, MB02-1172 and *P. macerans* MB02-454) significantly increased the solubilization of $Ca_3(PO_4)_2$ on NBRIP broth compared to the control (Figure 1a). In agreement with the result of plate assay, *P. macerans* MB02-992 caused a maximal increase of P solubilization more than 12-fold compared to the control. Interestingly, *P. macerans* MB02-429, MB02-513, and MB02-523 isolated from the mycelium of AM were able to solubilize $Ca_3(PO_4)_2$ in liquid medium (Figure 1a).

All 16 strains of *Paenibacillus* significantly increased

Table 1. Phosphate solubilization by bacteria from the genus *Paenibacillus* on National Botanical Research Institute's phosphate growth medium supplemented with inorganic and organic phosphorus.

Strain number	Species identity	Bacterial origin	Phosphate-solubilization halo (mm)				
			$Ca_3(PO_4)_2$	$CaHPO_4$	$AlPO_4$	$FePO_4$	IHP
MB02-226	*P. polymyxa*	Non-AM/B	3.5 ± 0.55	2.3 ± 0.40	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-376	*P. polymyxa*	Non-AM/B	0.6 ± 0.13	1.1 ± 0.15	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-428	*P. polymyxa*	AM/M	0.9 ± 0.13	1.4 ± 0.17	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-1007	*P. polymyxa*	Non-AM/B	3.4 ± 0.38	1.0 ± 0.09	0.0 ± 0.00	0.0 ± 0.00	3.8 ± 0.44
MB02-1172	*P. polymyxa*	AM/R	0.5 ± 0.00	1.5 ± 0.46	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-1202	*P. polymyxa*	AM/R	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-1265	*P. polymyxa*	Non-AM/R	0.5 ± 0.00	2.1 ± 0.48	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-167	*P. macerans*	AM/H	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-429	*P. macerans*	AM/M	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-454	*P. macerans*	Non-AM/B	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-513	*P. macerans*	AM/M	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-523	*P. macerans*	AM/M	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-727	*P. macerans*	AM/H	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-992	*P. macerans*	Non-AM/B	3.6 ± 0.29	5.1 ± 0.77	0.0 ± 0.00	0.0 ± 0.00	7.5 ± 0.51
MB02-1125	*P. macerans*	AM/R	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00
MB02-1180	*P. macerans*	AM/R	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00	0.0 ± 0.00

IHP: inositol hexaphosphate; Non-AM: Non-arbuscular mycorrhiza; AM: Arbsucular mycorrhiza; B: Bulk soil; M: mycelium; R: Rhizosphere; H: Hyphosphere. The data were shown as means ± standard error and this experiment was conducted twice.

the solubilization of $CaHPO_4$ in NBRIP broth compared to the control (Figure 1b). In general, the production of soluble P by the *Paenibacillus* strains with $CaHPO_4$ was significantly higher compared to other inorganic P sources. In addition, *P. polymyxa* MB02-428 isolated from the mycelium of AM caused a maximal increase of P solubilization and released about 118.6 µg/ml of soluble P from $CaHPO_4$. The quantity was more than 4.5-fold higher than the 26.1 µg/ml of soluble P in the control treatment.

P. polymyxa MB02-1007 and MB02-1265 and *P. macerans* MB02-429, MB02-454, MB02-513, and MB02-992 significantly increased the solubilization of $AlPO_4$ in NBRIP broth compared to the control (Figure 1c). In particular, *P. macerans* MB02-429 and MB02-513 were isolated from the mycelium of AM. In addition, *P. macerans* MB02-992 caused a maximal increase of P solubilization and released about 23.9 µg/ml of soluble P from $AlPO_4$. The quantity was about 1.4-fold higher than the 17.5 µg/ml of soluble P in the control treatment.

P. polymyxa MB02-376, MB02-428, MB02-1007, MB02-1172, and MB02-1202 and *P. macerans* MB02-167, MB02-429, MB02-727, MB02-992, and MB02-1125 significantly increased the solubilization of $FePO_4$ in NBRIP broth compared to the control (Figure 1d). In addition, *P. polymyxa* MB02-428 and *P. macerans* MB02-429 were isolated from the mycelium of AM. Interestingly, *P. polymyxa* MB02-428 caused a maximal increase of P solubilization and released about 58.6 µg/ml of soluble P from $FePO_4$. The quantity was about 4.0-fold higher than the 14.4 µg/ml of soluble P in the control treatment.

P. polymyxa MB02-226, MB02-376, MB02-428, MB02-1007, MB02-1172, and MB02-1265 and *P. macerans* MB02-992 significantly increased the solubilization of inositol hexaphosphate in NBRIP broth compared to the control (Figure 1e). In addition, *P. polymyxa* MB02-428 was isolated from the mycelium of AM. *P. polymyxa* MB02-1172 caused a maximal increase of organic P solubilization and released about 314.6 µg/ml of soluble P from inositol hexaphosphate. The quantity was about 2.5-fold higher than the 126.9 µg/ml of soluble P in the control treatment.

Dynamics of P solubilization indicated that the soluble P production from $Ca_3(PO4)_2$ was enhanced by *P. macerans* MB02-992 with increasing incubation time up to 5 d and remained constant to 7 d (Figure 2a). This data is different from the result of Son et al. (2006), who found that longer incubation periods decreased soluble P concentration. The maximal concentration of soluble P released by *P. macerans* MB02-992 from $Ca_3(PO4)_2$ was 58.6 µg/ml after 5 d of incubation. In addition, the level of soluble P in NBRIP broth was suddenly increased to 54.9 µg/mL after 3 d of incubation, which was about 2.5-fold higher than the 21.8 µg/ml of soluble P after 2 d of incubation. Interestingly, cells growing in NBRIP broth containing $Ca_3(PO4)_2$ as the sole P source reached the maximal number after 2 d of incubation, which may explain why the greatest increase in P solubilization was obtained after 3 d of incubation. The cell numbers increased 2.4 log10 cfu/ml compared to the initial value.

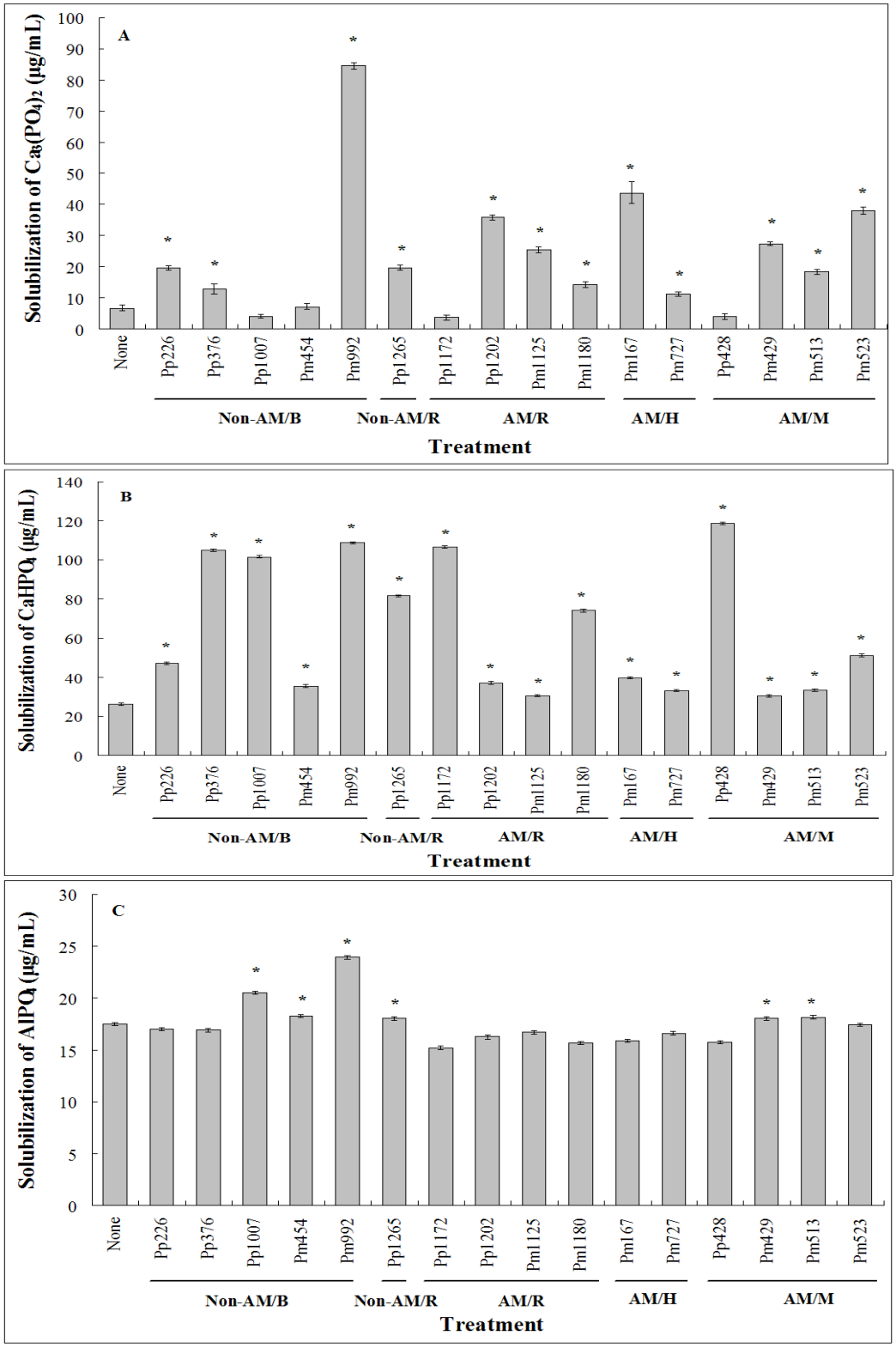

Figure 1. Phosphate solubilization by bacteria from the genus *Paenibacillus* on National Botanical Research Institute's phosphate growth broth supplemented with inorganic and organic phosphorus (a) $Ca_3(PO_4)_2$; (b) $CaHPO_4$; (c) $AlPO_4$; (d) $FePO_4$; (e) Inositol hexaphosphate. Pp: *P. polymyxa*; Pm: *P. macerans*. Error bars represent the standard error of the mean and columns marked with * are significantly different from the control without bacteria according to Fisher's LSD ($P < 0.05$). This experiment was conducted twice with four replicates each.

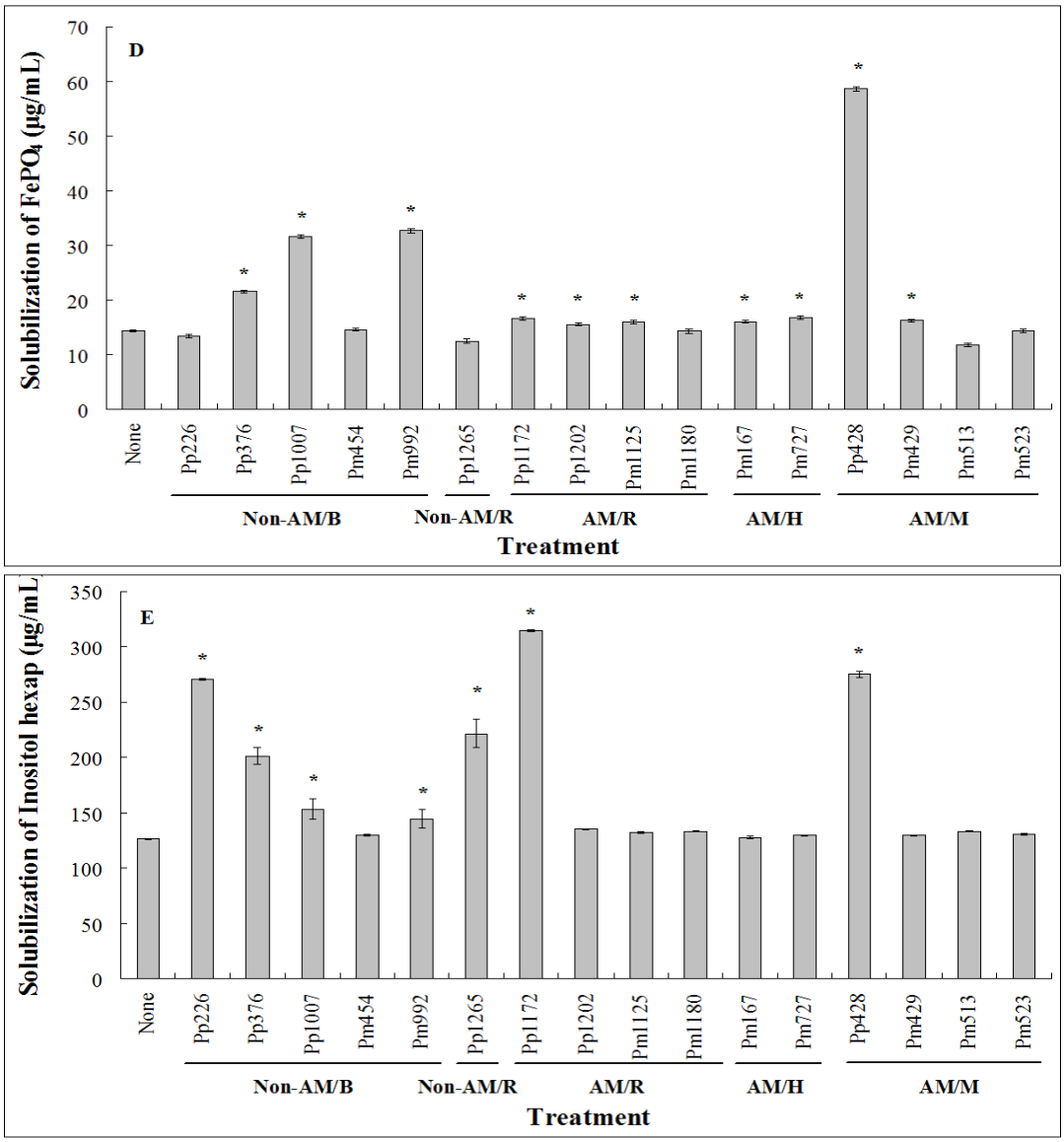

Figure 1. Contd.

As incubation time increased, the cell numbers decreased slightly to 7.66 log10 cfu/ml in NBRIP broth (Figure 2b).

Results from this study revealed that the *Paenibacillus* strains from mycorrhizal and non-mycorrhizal cucumber plants were able to at least partially solubilize inorganic and organic P, which is in agreement with the result Alonso et al. (2008), who found that both yeast isolates obtained from *Glomus mosseae* spores solubilized nonsoluble P. In addition, the results indicated that P-solubilizing bacteria should be screened in NBRIP broth assay for many strains which did not show any clear zone on agar plates solubilized insoluble P in liquid broth. However, several strains in particular *P. polymyxa* MB02-1007 produced a clear halo in NBRIP medium, but was unable to solubilize $Ca_3(PO_4)_2$ in NBRIP broth. The contrary result may be directly correlated with the acids produced (Alikhani et al., 2006; Son et al., 2006). The acids produced were obviously able to affect the growth of bacteria by altering the pH of NBRIP broth, which conversely affect bacterial P solubilization.

Though, no direct correlation could be established between *in vitro* solubilization of P, plant P accumulation and available soil P, the results of this study make these isolates attractive as P solubilizers. In developing inoculants that improve plant P nutrition and allow plants to use soil stocks of organic and inorganic P, bacteria from *Paenibacillus* may present many advantages, such

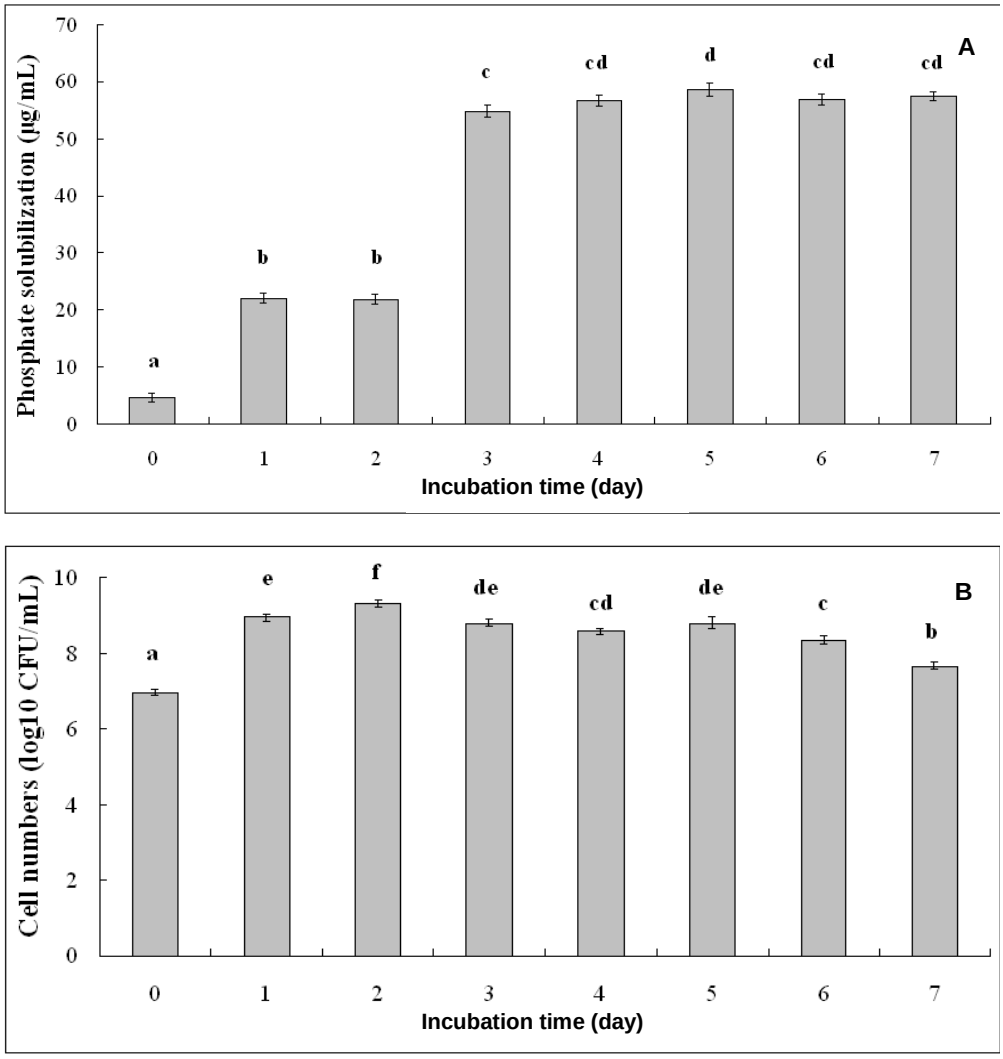

Figure 2. Growth of *Paenibacillus macerans* MB02-992 and solubilization of $Ca_3(PO_4)_2$ in National Botanical Research Institute's phosphate growth broth when cocultured in different incubation time. (a) Phosphate solubilization; (b) Cell numbers. Columns with the same letters are not significantly different (*P<0.05*). Error bars represent the standard error of the mean. This experiment was conducted twice with four replicates each.

as high temperature resistance. In addition, Vassilev et al. (2006) has given a review on the simultaneous P-solubilizing and biocontrol activity of microorganisms. Interestingly, most of the *Paenibacillus* strains all have a potential in biocontrol of *Pythium* damping-off of cucumber in our previous study (Li et al., 2007). Therefore, we can expect their application through simultaneous P-solubilizing and biocontrol activity.

The P uptakes by plants inoculated with P-solubilizing bacteria are more pronounced when coinoculated with AM fungi, which form beneficial symbioses with the plant roots (Smith and Read, 2008). Interestingly, in our previous studies (Li et al., 2008a), the *Paenibacillus* strains have showed differential effects on AM colonization. Furthermore, Larsen et al. (2009) revealed the interactions between the AM fungus *G. intraradices* and the plant growth promoting rhizobacteria *P. polymyxa* and *P. macerans* in the mycorrhizosphere of cucumber. These data, together with the effect of the *Paenibacillus* strains on P solubilization, may support the triple/tripartite interactions between AM fungi, plants, and associated bacteria. Therefore, it could be suggested that the availability of soil P to plant roots induced by AM fungi maybe due, in part, to its associated bacteria by both solubilizing P directly and improving P solubilization of AM indirectly.

Co-inoculations of plant beneficial bacteria and AM fungi in the rhizosphere are gaining increased attention in sustainable agro ecosystems (Akhtar and Siddiqui, 2007; Akhtar and Siddiqui, 2008). Indeed, synergistic

interactions between P-solubilizing bacteria and AM fungi have been revealed, which showed that the treatments inoculated with both AM fungi and bacteria did not only significantly increased plant biomass and P accumulation in plant tissues, but also the bacteria promoted mycorrhizal establishment whereas the mycorrhizal symbiosis increased the size of the P-solubilizing bacterial population (Toro et al., 1997). Therefore, dual inoculation with AM fungi and potential P-solubilizing bacteria from the genus *Paenibacillus* can be expected as the combinations of two such partners with complementary mechanisms might increase biocontrol and P uptake as well as plant-growth-promoting efficacy.

In conclusion, our results demonstrate that bacteria from the genus *Paenibacillus* possess strong solubilization activity of sparingly soluble inorganic and organic sources of P, while these paenibacilli play an active role in the complex interactions between AM fungi, plants, and soilborne plant pathogens in our previous studies. Therefore, it could be suggested that the *Paenibacillus* strains perhaps could increase the availability of soil P to plant roots by both solubilizing P directly and improving P uptake of AM indirectly.

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