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Coimmobilization of *Azospirillum lipoferum* and *Bacillus megaterium* for Successful Phosphorus and Nitrogen Nutrition of Wheat Plants

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Summary

The efficacy of strains of *Pseudomonas fluorescens*, *Bacillus megaterium* and *Azospirillum* spp. in *in vitro* solubilization of Ca_3PO_4 was studied. *Pseudomonas fluorescens* and *Bacillus megaterium* strains were the most powerful phosphate solubilizers on Pikovskaya (PVK) plates and liquid medium. *Azospirillum lipoferum* strains showed weak zones of solubilization on the PVK plates. Phosphate solubilization by the tested organisms was accompanied with pH reduction of the culture medium. Maximum pH reduction was 2.8, 1.2 and 0.5 units for *Pseudomonas fluorescens*, *Bacillus megaterium* and *Azospirillum lipoferum* strain 137, respectively. Alginate and agar immobilization of the tested bacteria or coimmobilization of *A. lipoferum* 137 and *B. megaterium* significantly enhanced phosphorus solubilization for four consecutive 4-day cycles. In a pot experiment, phosphorus mobilization in wheat (*Triticum aestivum* L. cv. Beni Swif 1) inoculated with *B. megaterium* or *A. lipoferum* 137 as single or mixed inocula (as free or alginate immobilized beads) was studied in presence of Ca_3PO_4. Wheat inoculated with mixed inocula exhibited high shoot dry weight, total nitrogen (N) yield and the shoot phosphorus content increased by 37 and 53 % compared to the plants inoculated with *A. lipoferum* and uninoculated ones, used as control, respectively. Maximum nitrogenase activity (measured by acetylene reduction assay) was observed in mixed inoculum treatment, and was increased by 500 and 32 % compared to uninoculated and *A. lipoferum* inoculated plants. Results demonstrate the beneficial influence of coinoculation of *A. lipoferum* and *B. megaterium* for providing balanced N and P nutrition of wheat plants.

Key words: phosphate solubilization, *Azospirillum*, *Bacillus*, *Pseudomonas*, immobilization

Introduction

Nitrogen and phosphorus are essential nutrients required by both plants and microorganisms, their major physiological roles are the accumulation and release of energy during cellular metabolism (*1*). Phosphorus is generally deficient in most natural soils, because it is fixed as water-insoluble iron and aluminum phosphates in acidic soils or calcium phosphate in alkaline soils (*2*). However, calcium phosphate, which is of low solubility, can be dissolved and made available to plants by soil rhizosphere microorganisms through the production of organic acids and chelating oxo acids from sugars (*3*). Therefore, the inoculation of soil with phosphate solubilizing microorganisms may alleviate this problem (*4*,*5*).

Plant growth-promoting bacteria (PGPB) of the genus *Azospirillum* are widely distributed in the rhizosphere of tropical and subtropical grasses (*6*). The mech-

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anisms by which *Azospirillum* spp. can exert a positive effect on plant growth is probably composed of multiple effects including synthesis of phytohormones, N_2-fixation, nitrate reductase activity and enhancing minerals uptake (*7,8*). However, very few reports have indicated the P-solubilizing activity by different *Azospirillum* spp. (*3,9*). Therefore, a promising trend for increasing nitrogen and phosphorus availability to plants has been increased using combined inoculation of nitrogen fixing and P-dissolving organisms. There have been many successful attempts to improve plant development by using mixtures of *Azospirillum* and VA mycorrhiza (*10,11*). Similarly, the combined inoculation of *Azospirillum* and P-solubilizing bacteria was successfully used for plant N and P nutrition and growth yield (*12,13*).

In the last few years, several new inocula formulations have been proposed including alginate and agar immobilization inoculants (*14,15*). These carriers permit entrapment of living cells, protecting the organisms against stresses. In addition, microbial immobilization promotes slow release of bacteria into soil (*16,17*). Bashan and Gonzalez (*18*) reported that *Azospirillum* can survive in dry alginate inoculants for prolonged periods without losing effectiveness. Moreover, El-Katatny *et al.* (*19*) demonstrated that microbial immobilization gives prolonged metabolic activity when microbial cells are reused. Organisms could be immobilized separately or coimmobilized together (*20,21*).

The objectives of this study were to investigate the P-solubilizing capacity of different strains of *Azospirillum brasilense* and *Azospirillum lipoferum* as well as other rhizospheric bacterial strains as free or alginate or agar formulations. Another objective was to study the effect of inoculation with such bacterial formulations on growth and N and P nutrition of wheat plants in a pot experiment.

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains used in this study are listed in Table 1. *Azospirillum lipoferum* strains (Z1, R1, R3 and R5) and *Azospirillum brasilense* strains (Z2, Z5 and R2) were isolated from the rhizosphere of maize and rice by El-Komy (*22*). *Azospirillum* strains were identified on the basis of usual phenotypic and genetic properties (G + C content and DNA-DNA homology) according to Tarrand *et al.* (*23*). *Pseudomonas fluorescens* strain 201 and *Bacillus megaterium* strain 98 were obtained from the culture collection of Botany Department, Faculty of Science, Minia University. Bacterial isolates were maintained on nutrient agar (NA) slopes.

Production of inocula and macroencapsulation

Methods used for production of inocula and macroencapsulation were described earlier (*15,24*). Macroencapsulation was performed using 2 % alginate or agar to obtain beads of 2 mm in diameter. Fresh beads were either used directly, or kept at 4–5 °C in sealed flasks for several days. The viable population size of bacteria was determined in pellets before its use in batch cultures and pot experiments. One gram of fresh bead was dissolved in 10 mL of 0.1 M potassium-phosphate buffer (pH=6.8) by shaking vigorously for 45 min. The suspended cells were serially diluted in sterile Na-pyrophosphate (mass fraction, w=0.1 %; pH=7.0), and total bacterial counts were measured by the plate dilution method.

Table 1. Solubilization efficiency (SE) of the tested bacteria in PVK plates

Strains	Solubilization efficiency (SE)			
	t/day			
	2	4	6	8
P. fluorescens	266.6	350.0	333.0	300.0
B. megaterium	185.7	167.7	150.0	83.9
A. lipoferum 137	157.1	133.3	120.0	50.0
A. lipoferum Z1	91.6	107.6	128.7	63.6
A. lipoferum R1	88.5	130.0	43.5	20.6
A. lipoferum R3	100.0	185.7	42.8	30.8
A. lipoferum R5	90.0	65.6	30.2	25.8
A. brasilense R2	0.0	0.0	0.0	0.0
A. brasilense Z2	0.0	0.0	0.0	0.0
A. brasilense Z5	0.0	0.0	0.0	0.0

Screening for phosphate-solubilization in Petri plates

Bacterial strains were screened for their phosphate-solubilizing ability on Pikovskaya (PVK) medium (*25*) containing (in g/L): glucose 10, $Ca_3(PO_4)_2$ 5.0, $(NH_4)_2SO_4$ 0.5, NaCl 0.2, $MgSO_4 \cdot 7H_2O$ 0.1, KCl 0.2, yeast extract 0.5, $MnSO_4 \cdot H_2O$ 0.002, and $FeSO_4 \cdot 7H_2O$ 0.002, agar-agar 17.0, and the pH was adjusted to 7.0 before autoclaving. Four bacterial isolates per plate were inoculated in triplicate with sterile toothpicks. The halo and colony diameter were measured 2, 4, 6 and 8 days after the incubation of the plates at 30 °C. The results are expressed as solubilization efficiency (SE) according to Nguyen *et al.* (*26*) where

$$SE = \frac{\text{solubilization diameter (S)} \cdot 100}{\text{growth diameter}}$$

Quantitative estimation of phosphate-solubilization by immobilized or free bacterial isolates in liquid PVK medium

Experiments were carried out in Erlenmeyer flasks (100 mL) each containing 20 mL of PVK medium, pH=7.0, before autoclaving. Flasks were inoculated with either 1 mL of bacterial suspension, 3.0 g of fresh alginate or 2.7 g of agar beads containing 10^9 CFU/flask. Autoclaved, uninoculated flasks were used as controls. The flasks were incubated at 30 °C as still-surface culture. Cultures were harvested by centrifugation at 7000 x *g* for 10 min, 2, 4, 6 and 8 days after incubation, and the phosphorus in culture supernatant was estimated by the paramolybdate blue method (*27*). Phosphorus content was expressed as μg/mL and pH of the medium was recorded at the same time.

Repeated use of immobilized bacteria

The reusability of the immobilized cultures was tested by replacing the PVK-culture medium with a fresh sterile one every 4 days. Cultivation conditions, phosphorus estimation and pH recording were performed as described above.

Mobilization of phosphorus in wheat plants

A pot experiment for studying phosphorus mobilization in wheat plants was made in surface-sterilized plastic pots (500 mL) filled with steam sterilized mixed soil of sand and clay in a mass ratio of 1:2. Chemical analysis of the soil was: pH=8.0, w(organic C)=0.15 %, w(total N)=0.014 %, w(P)=4.0 ppm, w(K)=88.0 ppm, $w(NH_4^+)$=3.0 ppm and $w(NO_3^-)$=0.3 ppm. Pots, each containing 500 g of soil, were arranged in groups of 5 replications for each treatment. The experimental design was performed as follows; treatment 1: uninoculated soil (control soil); treatment 2: uninoculated soil with the addition of poorly soluble phosphate (Ca_3PO_4 0.2 %); treatment 3: uninoculated soil with the addition of soluble phosphate (K_2HPO_4 0.1% and KH_2PO_4 0.1%); treatment 4: soil inoculation with either *B. megaterium*, or *A. lipoferum* strain 137 or a mixture of both as free bacterial suspension (10^7 CFU/seed) or equivalent mass of alginate immobilized beads buried near the germinated seeds. Bacterial inoculation was applied with the addition of poorly soluble phosphate (Ca_3PO_4 0.2 %). Soluble and poorly soluble phosphate were mixed thoroughly with soil in a plastic bag before use. Five surface sterilized (in 1 % NaOCl for 3 min, then washed with H_2O) pregerminated (48 h) seeds of wheat (*Triticum aestivum* L. cv. Beni Swif 1) were transplanted in each pot at 2 cm depth. On the second week after sowing, plants were thinned down to 3 per pot and were irrigated daily as needed.

At harvest (30 days), shoot length (cm/pot), fresh and dry weight (g/pot) were estimated. Nitrogenase activity was assayed in defined fresh washed root (0.2 g) of control and inoculated plants by the acetylene reduction method as described by Turner and Gibson (*28*). Nitrogenase activity in fresh root was expressed as $n(C_2H_4)$ in nmol/(g h). Total N content of dry shoot was determined after Kjeldahl digestion and total N yield was calculated according to Rennie and Rennie (*29*). Sodium and potassium were determined by flame photometric method (*30*), and calcium and magnesium by the versine titration method (*31*).

Statistical analyses

Results of all repetitions were analyzed together by one-way analysis of variance (ANOVA) at $P \leq 0.05$ using Statistica Software (PC STAT, Ver. 1A, Copyright 1985, the University of Georgia).

Results

Phosphate solubilization on PVK plates

The results for the detection of phosphate solubilization by the tested bacterial strains on Pikovskaya medium are shown in Table 1. Solubilization efficiency (SE) was increased after 2 and 4 days of incubation, and then the solubilization stopped although the colony was still growing. Therefore, the solubilization efficiency (SE) started to decrease after 6 days of incubation. *Pseudomonas fluorescens* and *Bacillus megaterium* strains were able to solubilize phosphate effectively, and recorded higher solubilization efficiency up to 350 and 185, respectively, than different *Azospirillum* strains. While *Azospirillum lipoferum* strains showed weak zone of solubilization on PVK plates, *Azospirillum brasilense* strains did not show any clear zones and did not grow on PVK medium. *Azospirillum lipoferum* strains 137 and Z1 showed relatively high solubilization efficiency up to 157 and 128, respectively, and hence were selected for further tests.

Phosphate solubilization on PVK broth by the tested bacteria as free bacterial suspension

Pseudomonas fluorescens, Bacillus megaterium and *Azospirillum lipoferum* strains Z1 and 137 were further tested for their ability to solubilize tricalcium phosphate in PVK broth (Fig. 1). Contrary to indirect measurement of phosphate solubilization by plate assay, the direct measurement of phosphate solubilization in PVK broth resulted in more accurate results, especially for *Azospirillum lipoferum* strains. On PVK broth, the P concentration increased gradually, reached a peak on the 6th day and declined slowly afterwards (Fig. 1). *Pseudomonas fluorescens* and *Bacillus megaterium* strains showed maximum solubilization activity (γ(P)=126.6 and 106.5 μg/mL, respectively) on the 6th day, whereas *Azospirillum lipoferum* strains Z1 and 137 recorded γ(P)=86.5 and 80.0 μg/mL, respectively. pH values decreased gradually in PVK broth during early days of incubation and no revival was observed in latter days (Fig. 1). Maximum pH reductions recorded were 2.8 and 1.2 units for *Pseudomonas fluorescens* and *Bacillus megaterium* strains, respectively, whereas maximum pH reduction for *Azospirillum lipoferum* strains Z1 and 137 were 0.4 and 0.5 units, respectively.

Phosphate solubilization by agar or alginate immobilized bacteria

Data presented in Fig. 2 showed that phosphate solubilization increased significantly when bacterial strains were used as agar or alginate immobilized beads as compared with bacterial cell-suspension in PVK broth. Earlier reduction in pH values was also observed in PVK broth when bacterial strains were used in the immobilized forms. Maximum pH reductions for immobilized *Pseudomonas fluorescens* and *Azospirillum lipoferum* strains were 3.2 and 2.5 units, respectively, after 6 days of incubation. Coimmobilization of *B. megaterium* and *A. lipoferum* showed maximum phosphate solubilization (γ(P)=260 and 245 μg/mL) as alginate and agar beads, respectively, after 8 days of incubation.

Reusability of immobilized bacteria

The reusability of agar and alginate immobilized bacteria for P-solubilization was studied. Beads entrapping bacterial strains were used successfully in 4 repetitions in the presence of fresh sterile tricalcium phosphate in PVK broth in each set (Fig. 3). Nearly steady amounts of free phosphorus as well as pH reduction were obtained in sets 1, 2 and 3. However, maximum

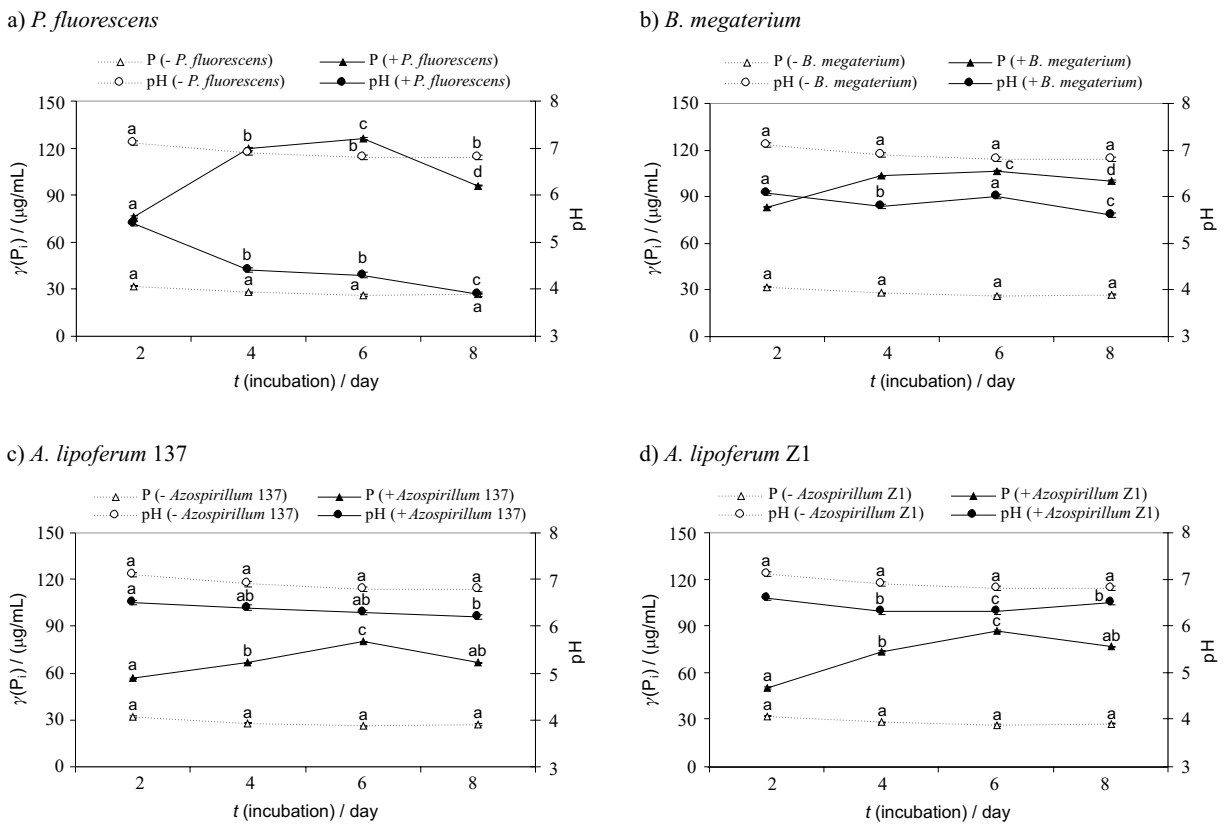

Fig. 1. Changes in pH and P concentrations in PVK broth in presence (+) or absence (-) of different bacterial isolates, a) *P. fluorescens*, b) *B. megaterium*, c) *A. lipoferum* 137, and d) *A. lipoferum* Z1. Points on each curve marked with a different letter differ significantly at $P \leq 0.05$ in one-way ANOVA. Bars represent the standard error (s.e.). When the s.e. bar is absent, the s.e. is smaller than the symbol used

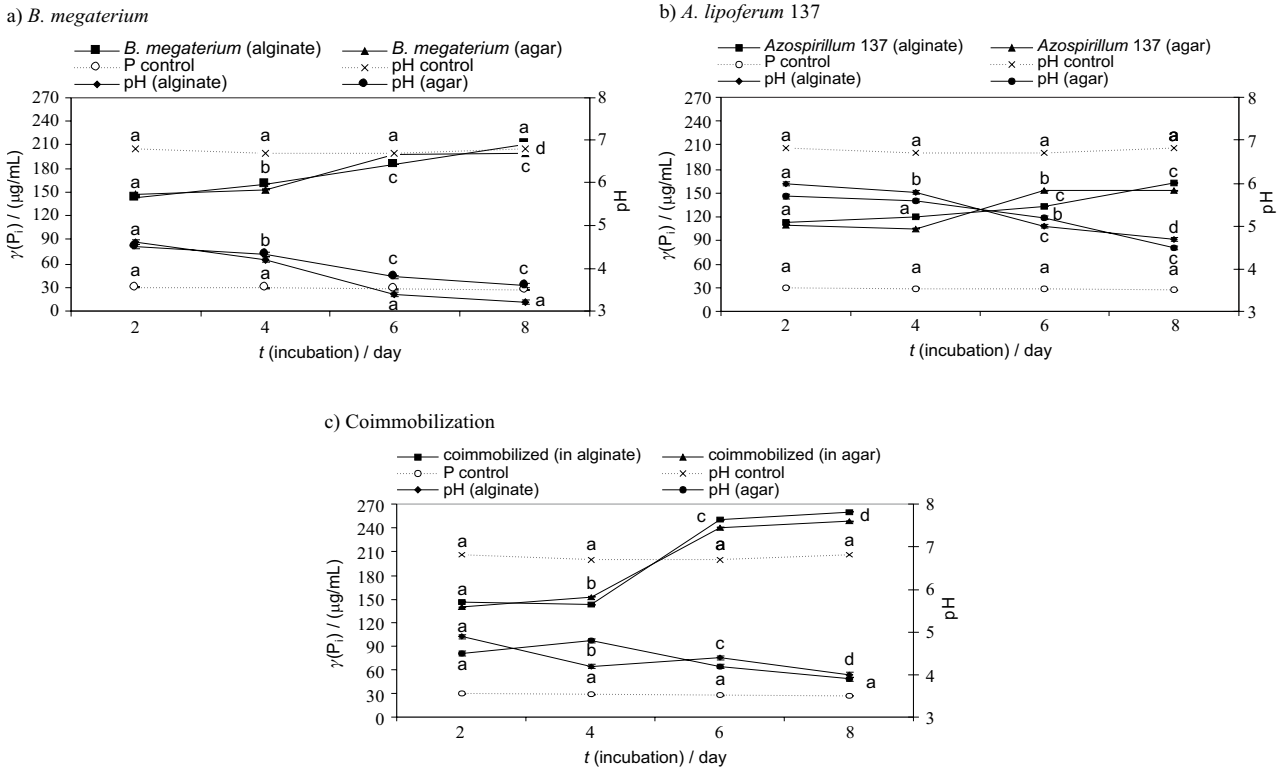

Fig. 2. Changes in pH and P concentrations in PVK broth by alginate or agar encapsulated bacterial isolates, a) *B. megaterium*, b) *A. lipoferum* 137, and c) coimmobilization. Points on each curve marked with a different letter differ significantly at $P \leq 0.05$ in one-way ANOVA. Bars represent the standard error (s.e.). When the s.e. bar is absent, the s.e. is smaller than the symbol used

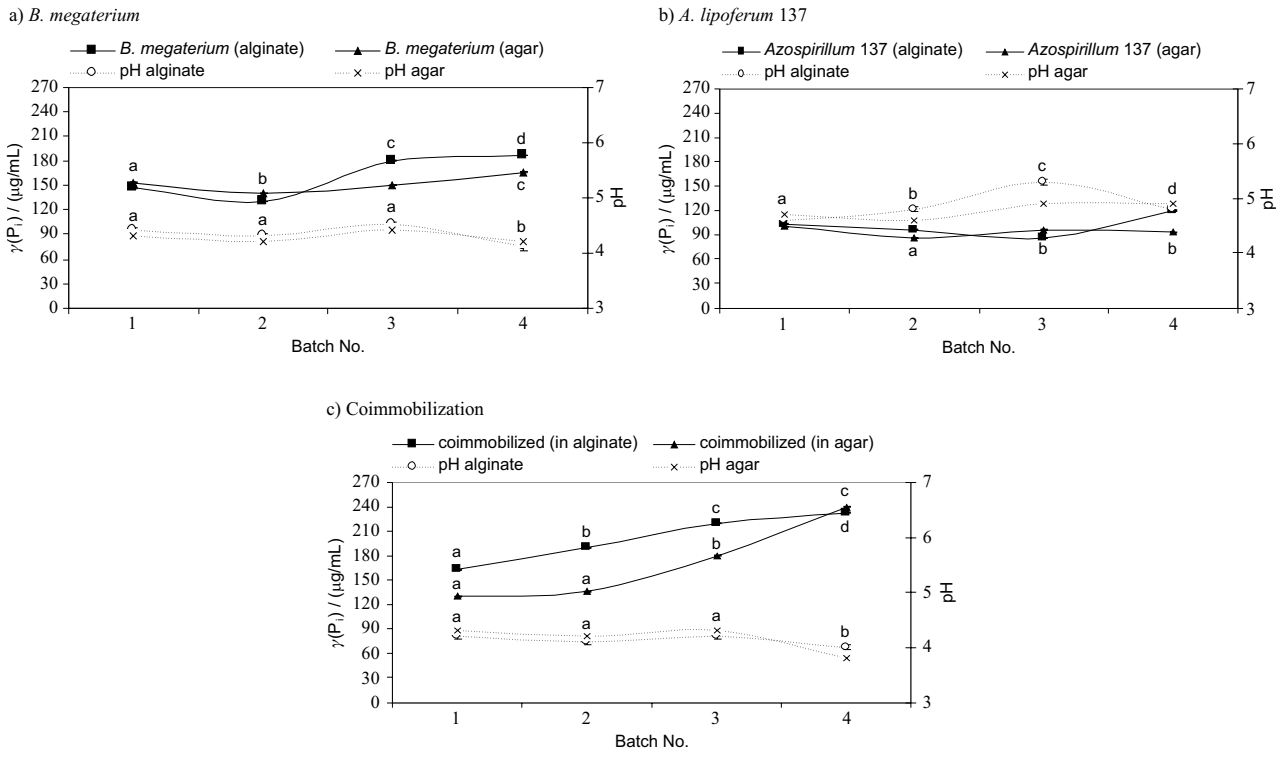

Fig. 3. Reusability of alginate or agar immobilized bacteria, a) *B. megaterium*, b) *A. lipoferum* 137, and c) coimmobilization for P-solubilization. Points on each curve marked with a different letter differ significantly at $P \leq 0.05$ in one-way ANOVA. Bars represent the standard error (s.e.). When the s.e. bar is absent, the s.e. is smaller than the symbol used

phosphate solubilization and pH reduction were reported in the 4th set (γ(P)=180, 120 and 250 μg/mL, and pH=2.7, 2.0 and 2.8) for *B. megaterium*, *A. lipoferum* 137 and coimmobilization treatments, respectively.

Mobilization of phosphorus in wheat plants

The results of the inoculation assays are shown in Tables 2 and 3. According to these results, a significant increase in most parameters measured in this study was observed when wheat inoculated with *A. lipoferum* strain 137 alone or with a mixture of *A. lipoferum* and *B. megaterium* was compared to noninoculated plants. Results also indicated that there were no significant differences in plant growth response when bacterial inocula were used either as free or alginate encapsulated beads (Table 2). Plant dry weight and total shoot N yield of wheat inoculated with mixed inocula were significantly higher than uninoculated control. The influence of inoculation on nitrogenase activity (*in situ*) was apparent at harvesting (30-day-old plants). The maximum level of acetylene reduction in fresh root ($n(C_2H_4)$=34.9 nmol/(g h))

Table 2. Effect of inoculation with *A. lipoferum* 137 and/or *B. megaterium* on wheat growth, total N yield and nitrogenase activity (*in situ*)

Treatments	Aerial height	Dry shoot mass	Total N yield	Nitrogenase activity as $n(C_2H_4)$
	cm	g/pot	Mg/pot	g h
Uninoculated soil	26.0^{a}	0.11^{a}	1.12^{a}	5.6^{a}
Uninoculated soil + Ca_3PO_4	25.0^{a}	0.09^{a}	0.99^{a}	7.1^{a}
Uninoculated soil + soluble P	29.5^{b}	0.12^{a}	1.10^{a}	8.5^{a}
Inoculated with *A. lipoferum* 137*	30.0^{b}	0.16^{b}	1.76^{b}	26.4^{b}
Inoculated with *B. megaterium**	28.3^{ab}	0.12^{a}	1.36^{c}	12.1^{ab}
Coinoculation*	31.0^{c}	0.18^{b}	1.81^{b}	34.9^{c}
Inoculated with *A. lipoferum* 137**	31.3^{c}	0.15^{ab}	1.80^{b}	24.9^{b}
Inoculated with *B. megaterium***	29.0^{b}	0.14^{ab}	1.54^{c}	14.2^{ab}
Coinoculation**	29.3^{b}	0.16^{b}	1.88^{b}	32.1^{c}

* In presence of Ca_3PO_4 and bacterial suspension inoculum
** In presence of Ca_3PO_4 and alginate encapsulated inoculum
Readings marked with a different letter differ significantly at $P \leq 0.05$ in one-way ANOVA

Table 3. Effect of wheat inoculation with *A. lipoferum* 137 and/or *B. megaterium* on shoot mineral content (mg/g)

Treatments	P	Na	K	Ca	Mg
Uninoculated soil	2.51[a]	13.40[a]	10.42[a]	6.0[a]	3.0[a]
Uninoculated soil + Ca_3PO_4	2.55[a]	17.69[b]	10.97[a]	11.0[b]	3.6[b]
Uninoculated soil + soluble P	5.34[b]	17.64[b]	15.72[b]	11.0[b]	4.5[c]
Inoculated with *A. lipoferum* 137*	2.85[c]	17.19[c]	13.02[c]	11.5[b]	4.5[c]
Inoculated with *B. megaterium**	2.81[c]	16.83[d]	11.35[d]	8.0[c]	3.9[b]
Coinoculation*	3.73[d]	14.67[e]	11.44[d]	7.0[d]	4.1[c]
Inoculated with *A. lipoferum* 137**	2.62[a]	17.28[bc]	12.74[c]	7.5[d]	5.1[d]
Inoculated with *B. megaterium***	2.80[c]	15.84[f]	13.02[c]	6.0[a]	4.5[c]
Coinoculation**	3.85[d]	16.20[d]	13.21[c]	7.5[d]	4.2[c]

* In presence of Ca_3PO_4 and bacterial suspension inoculum
** In presence of Ca_3PO_4 and alginate encapsulated inoculum
Readings marked with a different letter differ significantly at $P \leq 0.05$ in one-way ANOVA

was observed after inoculation with mixed inocula, and was increased by 500 and 32 % compared to uninoculated plants used as control and inoculated only with *Azospirillum*, respectively.

Although the P-uptake by wheat plants was higher in soil treated with soluble phosphate than inoculated soil, the phosphorus content in wheat plants inoculated with mixed inoculum was significantly increased by 53 and 37 % as compared to uninoculated control and *Azospirillum* inoculated plants, respectively. Similarly, Na, K, Ca and Mg contents were higher in all inoculation treatments than in untreated control plants (Table 3).

Discussion

Most of the recent literature concerning microbial solubilization of phosphorus in soil and their potential use for enhancement of soil fertility deals with many soil bacteria (*3,5*). However, few reports have also indicated the P-solubilizing activity of some nitrogen-fixing bacteria (*4,32*). Results of this study indicate that *Pseudomonas fluorescens* and *Bacillus megaterium* strains are the most powerful phosphate solubilizers on PVK plates as well as PVK broth. These results are in accordance with previous studies of Rodriguez and Fraga (*3*) and Illmer and Schinner (*33*), especially on tricalcium phosphate and hydroxyapatite rather than rock phosphate.

Our results also indicated that *A. lipoferum* strains showed weak zones of solubilization on PVK plates, while *A. brasilense* strains, non-glucose utilizing bacteria, did not exhibit acidity in the presence of glucose in this medium. *A. lipoferum* strains 137 and Z1 recorded solubilization efficiency up to 157 and 128, respectively, on PVK plates. Similarly, *Azospirillum halopraeferans* strains recorded solubilization efficiency of 150.5–152.0 on Sperber's medium (*9*).

Results also indicated that the P concentration in PVK broth increased gradually, achieving a peak on the 6th day and then declined slowly during the later days (Fig. 1). In general, the bacterial activity was initially slow, and then increased gradually followed by a decline at the end of incubation period. Decrease in P concentration during initial stages in PVK medium can be attributed to the utilization of the existing P for growth development of the organism, in a later phase the bacteria would have started acting on the substrate for the need of nutrients, thus releasing P from poorly soluble sources (*34*).

Poorly soluble P is solubilized mainly by the production of organic acids; consequently, P_i is released from mineral phosphate by proton substitution for Ca^{2+} (*35*). In this study, pH values decreased gradually in PVK broth during early days of incubation, and no revival was observed in later days for all the tested bacterial strains (Fig. 1). This supports the major role of organic acid production in mineral phosphate solubilization (*4*).

Entrapment of microbial cells has been reported to improve their metabolic activities and enhance the production of several hydrolic enzymes (*19,36*). Also, alginate immobilization has been used as inoculant for plant growth promoting bacteria (PGPB) for over more than two decades (*16*). Results of this study showed that alginate and agar immobilization of *A. lipoferum* and/or *B. megaterium* improved phosphate-solubilization by these strains compared with free bacterial cell suspension. Moreover, coimmobilization of *A. lipoferum* and *B. megaterium* recorded higher values of phosphate solubilization than single organism alone. These results are consistent with those of de-Bashan *et al.* (*20,21*), who reported that alginate coimmobilization of the microalga *Chlorella vulgaris* with *Azospirillum brasilense* significantly enhanced the metabolic activity of the first organism to remove ammonium and phosphate ions in polluted water samples for 6 consecutive 48-hour cycles.

Results of this study also show that alginate and agar encapsulation of *Azospirillum* and/or *B. megaterium* prolongs the durability of the inoculum and retains or, in some cases, even increases the phosphate solubilization during 4 repetitions. However, it was observed that alginate beads become weak and breakable (fragile) before the last cycle of reuse. This might explain why phosphate solubilization and pH reduction were at their maximum values in the last cycle, since the fragile beads allowed the release of more bacterial cells supporting higher

solubilization activity. The degradation of beads has been reported to be due to the presence of free phosphate ions in the medium acting as calcium ion trapping, thus affecting the stability of the gel (*37*). In order to overcome this problem, aluminum ion, strontium ion, or several other divalent metal ions can be used instead of calcium ion. Moreover, the treatment of calcium alginate gel with a cationic polymer such as polyethylene imine can improve the stability of the gel in the presence of phosphate (*38*).

B. megaterium and *A. lipoferum* 137, as it has been pointed out, were the best phosphate solubilizers in plates and liquid-medium assays. These strains were therefore, selected for studies of phosphorus mobilization in wheat plants. According to the obtained results, although plants inoculated with *A. lipoferum* 137 and Ca_3PO_4 have lower phosphorus content than those fertilized with soluble phosphates, they have higher dry weight and total N yield. These results and those reported by other authors confirm that plant growth response to *Azospirillum* inoculation is probably composed of multiple mechanisms including nitrogen fixation, hormonal effect, nitrate reductase activity and enhancing soil nitrogen and mineral uptake (*6*,*8*).

An alternative approach for the use of phosphate-solubilizing bacteria as microbial inoculants is the use of mixed cultures or coinoculation with other microorganisms. The results of this study showed that wheat inoculated with mixed inocula of *A. lipoferum* 137 and *B. megaterium* (either as bacterial suspension or alginate beads) in the presence of Ca_3PO_4 exhibited high shoot dry weight and total N yield. Also, the phosphorus content increased by 53 and 37 % as compared to uninoculated and *Azospirillum* inoculated plants, respectively. Similarly, coinoculation of *Pseudomonas striata* and *Bacillus polymyxa* strains, showing phosphate-solubilizing ability with a strain of *Azospirillum brasilense*, resulted in a significant improvement of grain and dry matter yields, with a concomitant increase in N and P uptake, compared to separate inoculations with each strain (*12*). Rojas *et al.* (*39*) recorded enhancements in nitrogen fixation, total nitrogen content and root colonization of black mangrove seedlings by the nitrogen fixing bacteria *Phylobacterium* sp. when coinoculated with the P-solubilizing bacterium *Bacillus licheniformis*. Thus, the results of this study and several investigations demonstrate the beneficial influence of combined inoculation of phosphate-solubilizing bacteria and *Azospirillum* or *Azotobacter* on yield, as well as on N and P accumulation in different crops (*13*,*40*).

In conclusion, coinoculation of wheat with *Azospirillum lipoferum* strain 137 and *Bacillus megaterium* provided more balanced nutrition for the plants, and the improvement in N and P uptake is the major mechanism of PGPB and phosphate-solubilizing bacteria.

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Koimobilizacija *Azospirillum lipoferum* i *Bacillus megaterium* za uspješnu ishranu pšenice fosforom i dušikom

Sažetak

Proučavana je učinkovitost sojeva *Pseudomonas fluorescens*, *Bacillus megaterium* i *Azospirillum* spp. da otapaju kalcijev fosfat *in vitro.* Sojevi *Pseudomonas fluorescens* i *Bacillus megaterium* najviše su otapali fosfate na pločama Pikovskaja (PVK) i u tekućem mediju. Na PVK-pločama sojevi *Azospirillum lipoferum* pokazivali su najslabiju zonu otapanja. Otapanje fosfata testiranim organizmima popraćeno je snizivanjem pH u podlozi. Maksimalno snizivanje pH iznosilo je za *Pseudomonas fluorescens* 2,8, za *Bacillus megaterium* 1,2, a za soj *Azospirillum lipoferum* 0,5 jedinica. Imobilizacija ispitivanih bakterija alginatom i agarom ili koimobilizacija *A. lipoferum* 137 i *B. megaterium* bitno je povećala otapanje fosfora tijekom 4 uzastopna četverodnevna ciklusa. U pokusima provedenim u loncu mobilizacija fosfora u pšenici (*Triticum aestivum* L. cv. Beni Swif 1), inokuliranoj samo s *B. megaterium* i *A. lipofe-*

rum 137 proučavana je u prisutnosti kalcijeva fosfata. Isti je pokus proveden i s miješanim inokulumom (*B. megaterium* i *A. lipoferum* 137) u obliku slobodnih ili alginatom imobiliziranih zrnaca. Pšenica inokulirana s miješanim inokulima imala je u mladicama veliki udjel suhe tvari i ukupnog dušika, a udjel fosfora u mladicama povećan je za 37 odnosno 53 % u usporedbi s biljkama inokuliranim s *Azospirillum lipoferum* ili neinokuliranim, uzetim kao kontrolni uzorak. Maksimalna nitrogenazna aktivnost (mjerena redukcijom acetilena) opažena je u mladicama pšenice s miješanim inokulumom, a povećanje je iznosilo 500 odnosno 32 % u usporedbi s neinokuliranim uzorkom ili pšenicom inokuliranom s *A. lipoferum*. Rezultati pokazuju povoljni utjecaj koinokulacije s *A. lipoferum* i *B. megaterium* za uravnoteženu ishranu pšenice dušikom i fosforom.