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PROMOTING COMMON BEAN GROWTH AND NITROGEN FIXATION BY THE CO-INOCULATION OF *RHIZOBIUM* AND *PSEUDOMONAS FLUORESCENS* ISOLATES

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Abstract

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The efficiency of five *Rhizobium* and two *Pseudomonas fluorescens* isolates in solubilizing mineral phosphate under in vitro condition, and their effects on Common bean growth and nutrient uptake and nitrogenase activity of rhizobia isolates under greenhouse conditions, were tested in this study. Results indicated that not only the mineral phosphate solubilizing ability varied between the rhizobacteria, but also some of them showed this ability more slowly than the others. Seed bacterizing with the assessed bacterial isolates improved common bean growth indices. It was also observed that the improvement of P and N uptake as the result of rhizobacterial dual application was significantly correlated with root growth factors and both root and shoot data, respectively. Besides, although both *Pseudomonas* isolates showed negative effects on RH7 nitrogenase activity, the interactions of tested *Rhizobia* × *Pseudomonas* isolates increased this ability in most cases. Therefore, it can be concluded that *Pseudomonas* and *Rhizobium* isolates can improve common bean growth and yield production.

Key words: nitrogenase activity, phosphate solubilizing, biofertilizer, sustainable agriculture

Introduction

Nitrogen is a vital element in plant growth which is usually absorbed as nitrate or ammonium, taking part in proteins, enzymes and chlorophyll structure (Malakooti and Tabatabayee, 2005). Nitrogen is provided to agricultural lands by the application of urea and ammonium nitrate chemical fertilizers, but the harmful effects of these chemical inputs have encouraged researchers to develop the sustainable agriculture practices, for example by applying the biological fertilizers. *Rhizobium* inoculum is a biofertilizer, which meets some part of legumes nitrogen requirement through the biological nitrogen fixation. This biological nitrogen fixation system can fix 70 - 85 million ton of nitrogen annually, which is about 50% of the world fixed nitrogen, and equivalent to all chemical fertilizer factories production (Saleh-Rastin, 1992; Afshari Aliabad, 1996). Nowadays, many researchers are attracted to the nitrogen fixation and the factors affecting that, because of the importance of N cycle in the fecundity of agricultural lands (Postgate, 1998).

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Phosphorus is also a highly required nutrient for plant growth, and is the most limiting factor following nitrogen. Phosphorus has an important role in the energy transfer, photosynthesis, changing sugar to starch and caring genetic traits (Kim et al., 1989). The mobility of P in soil is too low and cannot be quickly absorbed by plants. Moreover, a large part of P in chemical fertilizers changes to insoluble form after applied in soil, becoming unavailable to plants. Researches showed that the mentioned problem could be overcome by the application of phosphate solubilizing microorganisms as a biological fertilizer (Richardson, 2001). The bacteria from *Pseudomonas*, *Rhizobium* and *Bacillus* are the most important examples of phosphate solubilizing microorganisms (Rodriguez and Fraga, 1999; Sridevi and Mallaiah, 2007).

Although many experiments have studied the effects of the mentioned microorganisms on plant nutrition and N and P availability, but considerably lower attention has been paid to their interactions in the rhizosphere. These interactions are of a high importance as they stimulate or inhibit the activity of each microorganism. Studies conducted by Dashti et al. (1998) represented that soybean nodulation and nitrogen fixation by the nitrogen-fixing bacteria were increased at the presence of plant growth promoting rhizobacteria (PGPR). Parmar and Dadarwal (1999) reported that co-inoculation of *Pseudomonas* and *Bacillus* isolates along with *Rhizobium* strains enhanced nodules weight, root length, shoot biomass and the total nitrogen content in pea plants. In another research, Tilak et al. (2006) concluded that growth, nodulation and enzymes activity were significantly increased in plants co-inoculated with *Pseudomonas putida*, *P. fluorescens* and *Bacillus cereus*, compared with those inoculated only with *Rhizobium*. The results also indicated that the number of nodules occupied by *Rhizobium* was higher at the presence of *P. putida* (85% vs. 50%). Perveen et al. (2002) also represented that co-application of *Rhizobium* isolates and phosphate solubilizing microorganisms in soils with low available phosphorus improved yield production. Finally, Remans et al. (2007) reported an increased ability of *Rhizobium* isolates nodulation on bean plants as the result of phosphate solubilizing PGPR co-application.

The mentioned bacteria improve the efficiency of *Rhizobium* isolates in nodulation and nitrogen fixation; the effect depends on the applied strains, so this experiment was conducted to (1) study the mineral phosphate solubilizing ability of Iranian strains of *Rhizobium* and *P. fluorescens*, (2) evaluate the effect of their interaction on nitrogenase activity and (3) assess the effect of all bacteria isolates individually, on the improvement of common bean growth factors, in order to find the two most compatible isolates for application in sustainable agricultural production systems.

Materials and Methods

Two laboratory and greenhouse experiments were conducted in 2008 at the College of Agriculture and Natural Resources of Tehran university, Alborz province, Iran, to study the phosphate solubilizing ability of five *Rhizobium* isolates from *R. etli* (RH5) and *R. leguminosarum* bv. *phaseoli* (RH3, RH4, RH6, RH7) and also two *Pseudomonas fluorescens* isolates (UTPF68 and UTPF109), and their effects on common bean (*Phaseolus vulgaris* L.) growth. The isolates were selected based on the results of a previous experiment conducted by the author (Samavat et al., 2008). Plant and geographic sources of the bacterial isolates are listed in Table 1.

Table 1
Plant and geographic sources of the selected bacterial isolates

Bacterial isolate	Geographic source	Plant source
Rhizobium leguminosarum (RH3)	Tehran, Iran	*Phaseolus vulgaris* root nodules
R. leguminosarum (RH4)	Tehran, Iran	*Phaseolus vulgaris* root nodules
R. etli (RH5)	Zanjan, Iran	*Phaseolus vulgaris* root nodules
R. leguminosarum (RH6)	Tehran, Iran	*Phaseolus vulgaris* root nodules
R. leguminosarum (RH7)	Tehran, Iran	*Phaseolus vulgaris* root nodules
Pseudomonas fluorescens (UTPF68)	Mazandaran, Iran	*Brassica napus* rhizosphere
P. fluorescens (UTPF109)	Semnan, Iran	*Rosmarinus officinalis* rhizosphere

The laboratory experiment was conducted in a completely randomized design with three replications to test the phosphate solubilizing ability of the selected microorganisms. To do this, the Sperber method (Sperber, 1958) was used and bacteria were cultured in a spotted manner on the Sperber medium (glucose: 10 g; $MgSO_4$ $7H_2O$: 0.25 g; yeast extract: 0.5 g; Ca_3 $(PO_4)_2$: 2.5 g; $CaCl_2$: 0.1 g; agar: 15 g; distilled water: 1000 ml). Petri dishes were then placed in the dark incubator (26°C) and the ratio of transparent halo diameter / bacterial colony diameter was measured once an every 48 h for 8 days. Means were compared using Duncan's Multiple Range test (P<0.01).

The greenhouse experiment was conducted in a completely randomized design with three replications to evaluate the selected microorganism's effects on common bean growth. The greenhouse studies were carried out using Goli cultivar of common bean, obtained from the Department of Agronomy and Plant Breeding, University of Tehran. Bean seeds were surface-sterilized by washing with 96% ethanol for 30 seconds and 2.5% sodium hypochlorite for 3 minutes, and then rinsed four times with sterile, distilled water. The seeds were put on water agar (WA) (Technical agar, 15 g/l) to germinate in dark condition at 26ºC in the incubator. After 48h, seedlings were treated with *P. fluorescens* (UTPF68 or UTPF109) and rhizobia (RH3-RH7). Cell suspensions were cultured in King's B medium and Yeast extract Mannitol Broth (YMB) respectively, or the dual application of *Rhizobium* and *Pseudomonas* isolates by the method of Weller and Cook (1986) with some modifications. A loop of *P. fluorescens* (UTPF68 or UTPF109) or *Rhizobium* spp. (RH3-RH7) was grown for 48 h on King's B agar or Yeast extract Mannitol Agar (YMA), respectively in a petri dish, then scraped from the surface of the medium, and suspended in 1.0% methylcellulose to reach the equal population of the bacterial isolates. For co-inoculations, the *Pseudomonas* and *Rhizobium* suspensions were equally mixed together and added to seedlings. Then, the seedlings were put on an orbital shaker at 120 rpm for 1 to 3 h under a stream of filtered air. This method resulted in $1\text{-}5\times10^8$ colony forming units (cfu) $seed^{-1}$ at planting. Control treatments consisted of non-treated seedlings, which were only coated with 1.0% methylcellulose. After that, three seedlings were transplanted into each pot, containing 300 g sterile soil. The soil used here was passed through a 3mm sieve, air-dried and stored in plastic bags at 4ºC. Microbe-free soil was obtained by treating the soil with live steam (121ºC) for 30 min (Tarpero-Casas et al., 1990). Other soil properties are listed in Table 2.

The plants were grown in greenhouse under natural light supplemented with artificial light (80 µMs-1m-2; 16-h day, 8-h night). The daytime temperature ranged from 22 to 27°C, and at night, the temperature was 19ºC. All plants were harvested 40 days after planting. Plant growth measurements included root and shoot length/ dry weight, number of root nodules, leaves chlorophyll content, N and P absorption and the nitrogenase activity (acetylene reduction assay).

To measure leaves chlorophyll content at the three leaves stage, the chlorophyll meter (CCM-200 model by Opti-Sciences, USA) was used. Plant N and P absorption were also measured by Kjeldahl and Yellow methods (Tandon, 1998).

Nitrogenase activity can was accurately measured by acetylene reduction assay (ARA) according to Denison et al. (1983) with some modifications. Briefly, plants were harvested at flowering stage (40 days after planting) during the peak nitrogen fixation. Then, 1 g of the removed roots including rhizobial nodules was placed in 10 cc test tube and sealed with a lid tightly. Using a 2 cc syringe, 10% of the tube's air volume was sucked out and the same amount of C_2H_2 was then injected into the sample tube. The root system was allowed to remain in the tube with C_2H_2 for 15 min, after the whole gas content was withdrawn from the tube, and injected into a 10 cc vacutainer tube. From this 10 cc tube, 1 cc aliquot was later injected into the gas chromatography (14B Shimadzu, Japan) equipped with a flame ionization detector (FID), Propak-Q column, and CR4A processor. The carrier gas was N_2, Column temperature

Table 2
Soil properties

Texture	Zn, mg/kg	Fe, mg/kg	K, mg/kg	P, mg/kg	T.N.V	N, %	O.C, %	pH	EC, ds/m
Loam	0.8	3	180	9	12	0.05	0.6	7.6	1.4

100°C, injector temperature 110ºC and FID detector temperature 120ºC.

Finally, data were analyzed by MSTAT-C, and means were compared using the Least Significant Difference (LSD) at $P<0.01$ for the laboratory experiments and at $P<0.05$ for the greenhouse experiments. Correlation coefficients were determined by SPSS. All traits containing a zero value were subjected to $\sqrt{x+0.5}$.

Results

Mineral phosphate solubilizing ability of the isolates. Results indicated that all *Rhizobium* and *Pseudomonas* isolates were able of solubilizing mineral phosphate and showed this ability after 48 h except for RH5, which showed the ability after 6 days. After 8 days of growth in the Sperber culture medium, RH6 (2.33) and RH5 (1.55) were the best and the worst *Rhizobium* isolates respectively; UTPF68 (2.26) was also better than UTPF109 (2.06) (Figure 1).

The effect of co-inoculation on common bean growth factors. Studying the growth factors such as plant height, root length, root biomass and shoot biomass revealed the significant effect of all bacterial isolates compared with the control ($P<0.01$). In most cases, RH3+UTPF109 had the best effect on the measured traits and after that, RH6+UTPF68 was the most effective treatment, which increased the measured traits compared with both RH6 and UTPF68 individual application (Table 3).

Leaf chlorophyll content. Results indicated that all bacterial treatments significantly improved leaves chlorophyll content compared with the control ($P<0.01$). Co-inoculation of RH3+UTPF109 had the most improving effect on this trait. RH6+UTPF68 was also the second most effective treatment (Table 3).

The number of nodules in plant. All *Rhizobium* isolates and their co-application with *Pseudomonas* isolate colonized plant root and induced nodulation, but their potential differed. Nodulation also varied between individual applications of *Rhizobium* isolates (3.67 to 12.67 nodules/plant) and their co-application with *Pseudomonas* isolates (11.34 to 36.33 nodules/plant). RH6 was the best treatment among *Rhizobium* isolates and the co-inoculation of RH6+UTPF68 also resulted in the highest number of nodules; significantly

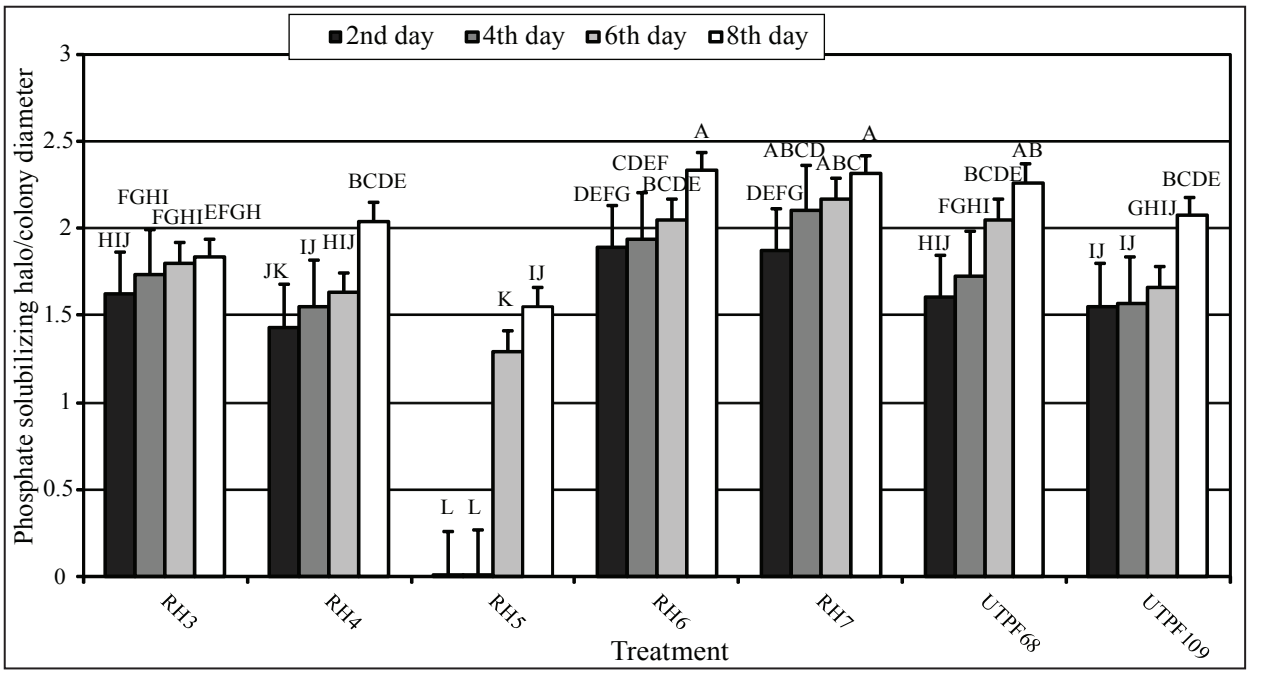

Fig. 1. The effect of time on the mineral phosphate solubilizing ability of *Rhizobium* (RH3 to RH7) and *Pseudomonas* (UTPF68-UTPF109) isolates, under laboratory conditions

The same letters represent no significant difference at $P<0.01$.

different from other treatments (P<0.05). Generally, it can be concluded that the co-application of *Rhizobium* and *Pseudomonas* isolates excelled their individual application and improved the nodulation better (Table 3).

Phosphorus absorption by the plant. Among the bacterial treatments of this experiment, only RH6 and RH7+UTPF68 had no significant effect on the plant P absorption. Under greenhouse conditions, RH5+UTPF68 and RH3+UTPF68 resulted in the highest P absorption compared with the other treatments (Figure 2). P absorption was also significantly correlated to plant root length and root biomass (Table 4).

Plant nitrogen content. Measuring plant nitrogen content showed the significant effect of all bacterial treatments on this trait (P<0.05). RH3+UTPF109 and RH6+UTPF68 had the highest increasing effect on the plant N content. All co-applications had better effect compared with their *Rhizobium* and *Pseudomonas* isolates individual application (Figure 3). Plant N content was significantly correlated to all growth factors of common bean (Table 4).

Nitrogenase activity. Results of gas chromatography indicated the highest rate of nitrogenase activity in RH7, among individual applications, and in RH3+UTPF109 and RH6+UTPF68, among co-applications. The co-

Table 4
Correlation coefficient between common bean's growth factors with N and P absorption

	Plant height	Root length	Shoot dry weight	Root dry weight	Leaf chlorophyll content	Nodule / plant
N absorption	0.575*	0.498*	0.589*	0.524*	0.747**	0.554*
P absorption	0.417ns	0.539*	0.300ns	0.489*	0.137ns	0.044ns

NS, non-significant; *, significant at P<0.05; **, significant at P<0.01.

Table 3
The effects of *Rhizobium* (RH3 to RH7) and *Pseudomonas* (UTPF68-UTPF109) isolates individual or co-application on growth factors of the plant

Treatments	Root dry weight, g	Shoot dry weight, g	Root length, cm	Plant height, cm	Leaf chlorophyll content	Nodule/ plant
RH3	0.323±0.12gh	0.623±0.05ef	18.45±0.22fgh	38.44±1.92hi	22.20±2.12gh	5.00±1.0f
RH3+UTPF68	0.457±0.11cd	0.830±0.10c	18.92±1.3efg	47.33±1.85d	29.59±2.14def	14.00±2.0de
RH3+UTPF109	0.573±0.07b	1.493±0.07a	29.83±1.26a	63.12±2.88a	39.26±3.08a	20.00±3.0c
RH4	0.463±0.06cd	0.853±0.09c	25.22±0.98c	47.06±1.31d	21.95±1.15i	7.33±1.15f
RH4+UTPF68	0.413±0.09def	0.733±0.12d	18.28±1.34fgh	38.70±1.21h	29.75±1.26de	12.67±2.08de
RH4+UTPF109	0.507±0.13c	0.850±0.08c	26.47±0.71bc	52.55±1.75c	31.03±2.35d	26.33±2.08b
RH5	0.283±0.10h	0.545±0.04fg	18.44±0.97fgh	40.27±1.13gh	23.11±1.97i	11.67±1.15e
RH5+UTPF68	0.513±0.12c	0.737±0.11d	23.58±1.18d	45.52±1.0de	26.33±2.56h	24.00±4.0b
RH5+UTPF109	0.467±0.17cd	0.653±0.16e	20.62±0.54e	42.56±0.75fg	32.95±3.17c	18.00±2.64c
RH6	0.397±0.04ef	0.823±0.05c	17.16±0.46ghi	45.33±1.2de	22.42±2.19i	12.67±1.15de
RH6+UTPF68	0.640±0.06a	1.383±0.08b	27.13±0.95b	56.47±1.45b	36.58±1.86b	36.33±2.52a
RH6+UTPF109	0.437±0.05de	0.837±0.06c	19.83±1.26ef	47.59±0.59d	25.97±1.47h	24.33±1.53b
RH7	0.187±0..07i	0.590±0.13efg	16.36±1.21ij	36.14±0.48ij	27.16±2.22gh	3.67±0.58f
RH7+UTPF68	0.127±0.07j	0.533±0.09g	15.18±0.93j	31.00±1.2k	28.26±2.04fg	11.34±1.53e
RH7+UTPF109	0.077±0.09jk	0.400±0.03h	12.93±0.84k	29.28±0.63k	25.90±1.25h	16.33±1.65cd
UTPF68	0.360±0.14fg	0.653±0.15	16.82±0.72hij	34.78±1.5j	28.56±1.63efg	-
UTPF109	0.433±0.12de	0.747±0.09d	19.65±0.59ef	44.28±1.3ef	30.95±3.03d	-
Control	0.060±0.005k	0.213±0.17i	11.15±0.86L	24.93±1.72L	19.55±2.10j	-

Means in a column followed by the same letter are not significantly different at P<0.01.

application of *Pseudomonas* and *Rhizobium* isolates significantly increased this feature compared with the control (P<0.05). The only exception here was RH7, which resulted in the highest nitrogenase activity when applied individually, but reduced it when applied along with *Pseudomonas* isolates (Figure 4).

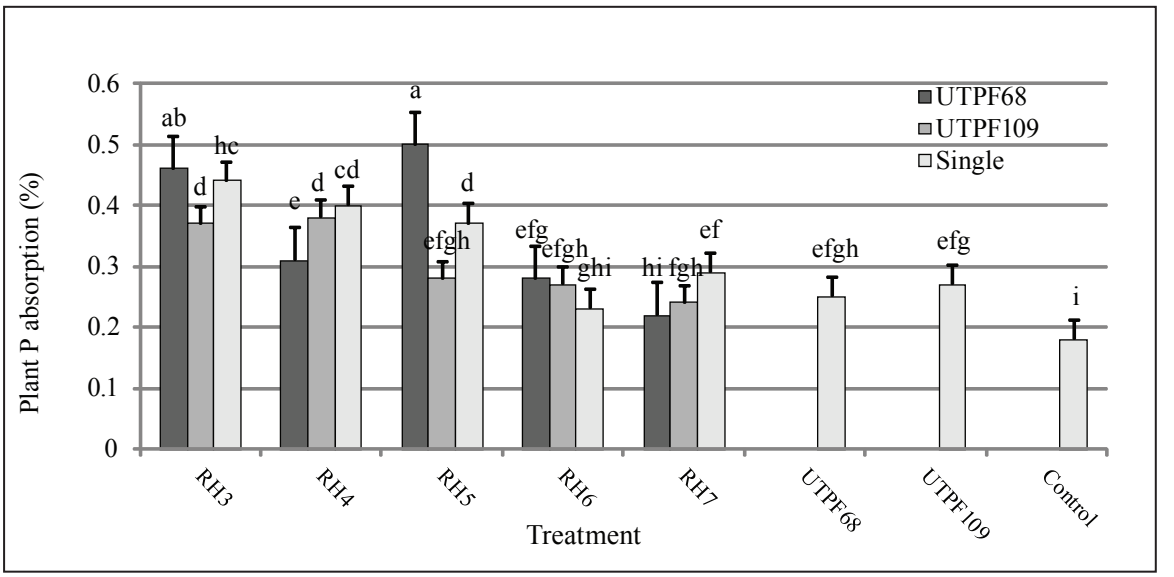

Fig. 2. The effect of *Rhizobium* (RH3 to RH7) and *Pseudomonas* (UTPF68-UTPF109) isolates interaction on the plant P absorption

The same letters represent no significant difference at P<0.01

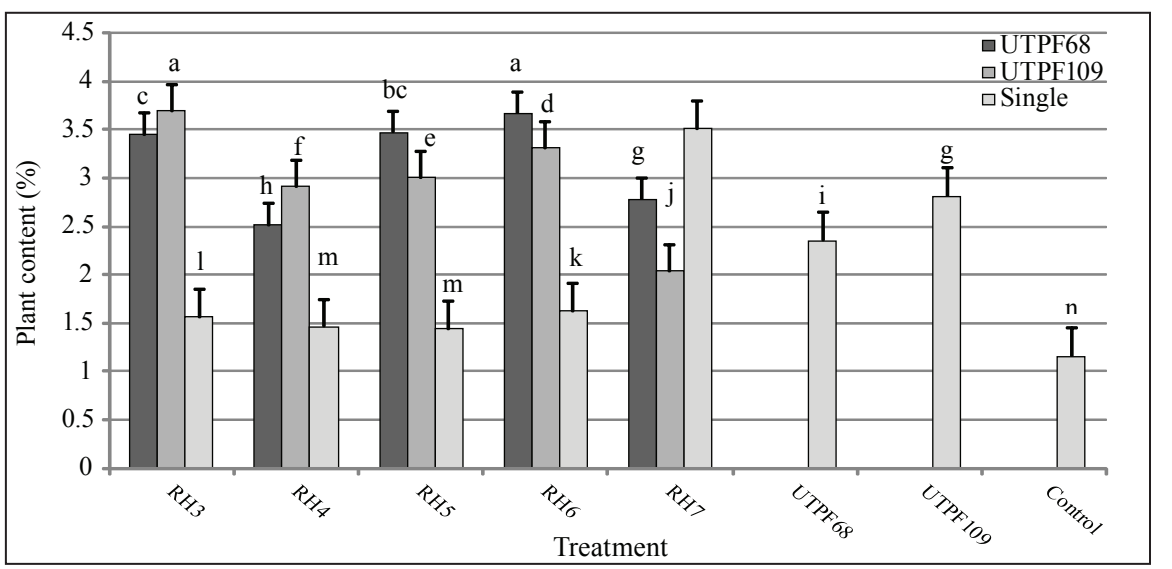

Fig. 3. The effect of *Rhizobium* (RH3 to RH7) and *Pseudomonas* (UTPF68-UTPF109) isolates interaction on the plant N absorption

The same letters represent no significant difference at P<0.01

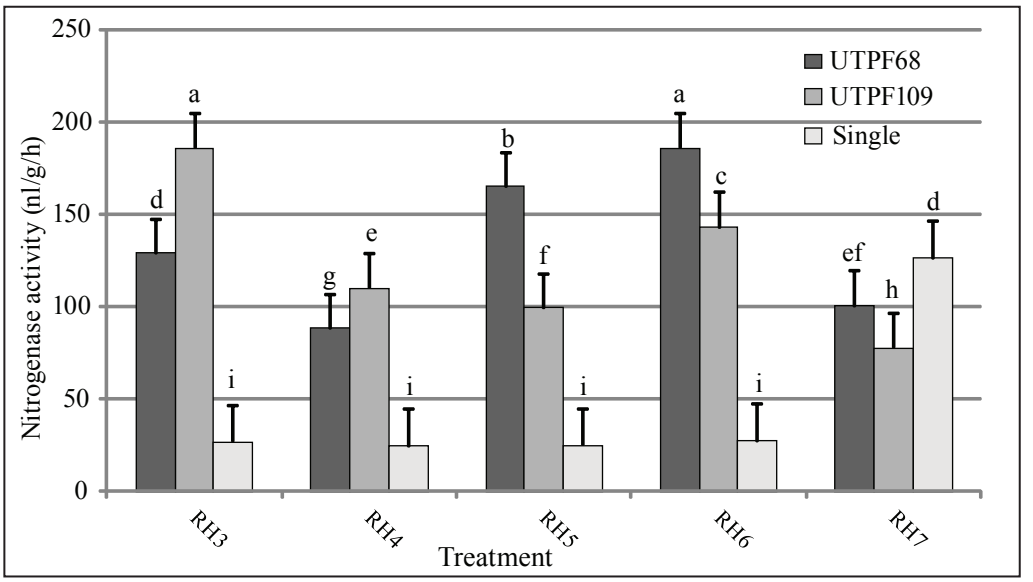

Fig. 4. The effect of *Rhizobium* (RH3 to RH7) and *Pseudomonas* (UTPF68-UTPF109) isolates interaction on the nitrogenase activity (nl/g/h). The same letters represent no significant difference at $P<0.01$

Discussion

Nitrogen is a vital element for plants and soil microorganism's growth and activity (Merrick and Edwards, 1995; Marzluf, 1997). A cost effective way to provide sufficient N to plants is the biological N fixation by *Rhizobium* bacteria; this ability could be affected by the various biotic and abiotic factors. The interaction of *Rhizobium* with the other microorganisms in soil is one of the biotic factors affecting N fixation. Chanway et al. (1989) reported that the interaction of plant growth promoting strains of *Pseudomonas* with *Rhizobium* strains increased N fixation and growth of lentil (*Lens esculenta* Moench) and pea (*Pisum sativum* L.) grown under field and laboratory conditions. Similar to the above experiment, results of the present experiment also indicated that the co-inoculation of *Rhizobium* and *Pseudomonas* strains increased nodulation, leaf chlorophyll content and other growth factors under greenhouse conditions. Results also revealed the significant correlation between plant nitrogen absorption and improvement of plant root/shoot growth factors, as the result of *Rhizobium* and *Pseudomonas* interaction.

The increased chlorophyll content in plant leaves as the result of bacterial isolates co-inoculation could be due to the increased plant nutrition and photosynthesis (Bashan et al., 1990). Another study represented that the co-inoculation of some *Rhizobacteria* isolates and *Rhizobium* BR10049 strain significantly affected the nitrogenase activity in common bean root nodules (Martins et al., 2003). They found that this is even more effective in the case antagonist isolates of *Pseudomonas* are applied and will end in a significant reduction of nitrogenase activity, but have no effect on the number and dry weight of nodules and plant biomass. Lucas Garcia et al. (2004) believed that the co-application of Rhizobacteria and Rhizobium might have positive or negative effects on their symbiosis relation with the host legume, depending on the applied isolates. The results of our experiment are in agreement with the mentioned findings as in most cases, the co-application of *Pseudomonas* and *Rhizobium* isolates significantly increased the nitrogenase activity while the co-application of *Pseudomonas* isolates and RH7 isolate inhibited the enzyme's activity.

Phosphorus is a highly required element for plants and microorganisms growth and activity. The mineral P in soil solution plays an essential role in P cycle and plants nutrition (Scheffer and Schachtschable, 1992). Although large amount of phosphorus fertilizers are ap-

plied in agricultural systems annually, but most part of them became insoluble and unavailable to plant roots (Singh and Kapoor, 1994). A reliable way to improve P availability to plant roots is to take advantages of the phosphate solubilizing ability of soil microorganisms (Illmer and Schinner, 1992). To do this, some microorganisms such as *Mycobacterium*, *Pseudomonas*, *Micrococcus*, *Mesorhizobium*, *Rhizobium*, *Flavobacterium*, *Bacillus* and *Sinorhizobium* could be applied in the rhizosphere (Halder et al., 1990; Illmer et al., 1995). Halder et al. (1990) found *Rhizobium* an efficient phosphate solubilizing bacterium. Alikhani et al. (2006) tested some strains of *Rhizobium leguminosarum* native to Iran soils, and some bacteria such as *Bacillus* sp. and *Pseudomonas fluorescens* and proved their ability in solubilizing the mineral P, although the ability differed between the strains. Our study also indicated the phosphate solubilizing ability of *Rhizobium* and *Pseudomonas* isolates under laboratory conditions. Results represented that some isolates showed their phosphate solubilizing ability later than the other did. This can be because these isolates need higher number of populations to be able of solubilizing the phosphate.

Conclusion

Gull et al. (2004) reported that the co-application of phosphate solubilizing bacteria and *Rhizobium* isolates increased P absorption and promoted the growth of pea plants. Studying the interaction of *Pseudomonas* and *Rhizobium* isolates on the plant P absorption indicated that the co-application of isolates positively or even negatively affected their phosphate solubilizing ability. Moreover, plant root development and P absorption rate were significantly correlated.

Generally, results indicated that a certain *Pseudomonas* isolate can be synergic to a certain *Rhizobium* isolate while antagonist to another isolate.

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