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The impact of *Sinorhizobium meliloti* and *Pseudomonas fluorescens* on growth, seed yield and biochemical product of fenugreek under water deficit stress

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Abstract: Bacteria that colonize plant roots and promote plant growth are referred to as plant growth-promoting rhizobacteria (PGPR). For a long-serving period, the PGPRs have been applied as biofertilizers in crops culture. Recent studies indicated the importance of PGPR for controlling the water deficit. The present study investigates the effects of two different PGPRs on some morpho-physiological characteristics in fenugreek under water deficit stress. The first factor was application of four PGPR levels including (*1. Sinorhizobium meliloti, 2. Pseudomonas fluorescens*, 3. combination of *S. meliloti* and *P. fluorescens* and 4. control without bacterial inoculation) and four levels of soil water content including 40%, 60%, 80% and 100% of field capacity (FC) was considered as second factor. The results showed that, leaf area, shoot fresh and dry weight, nitrogen, phosphorus and potassium content, and water use efficacy (WUE) were significantly improved by PGPR inoculation and individual use of PGPR was more effective. Decreasing of soil water content up to 0.40 FC and inoculation of two bacteria led to increase of secondary metabolites such as nicotinic acid and trigonelline. However seed yield was decreased in PGPR treated plants.

1. Introduction

Insufficient water induces a stress in plants called water deficit stress (Dodd and Ryan, 2016). Water deficit stress has major effects on plant growth and development, limiting crop production in the worldwide.

Water deficit negatively affects the plant growth and reproduction and disrupts the whole-plant functions (Bray, 2004; Hummel *et al*., 2010). It causes cellular changes such as solutes concentration, cell volume alteration, disruption of water potential gradients, changes in membrane shape and disrupting its integrity, loss in turgor pressure, and protein denaturation (Bray, 1997; Bartels and Sunkar, 2005). Water deficit is a major threat to agricultural production and tolerance to drought conditions is one of the main targets for crop improvement (Salekdeh *et al*., 2009).

Plant growth-promoting rhizobacteria (PGPR) are rhizosphere bacteria which constitute symbiotic relationships in large varieties of plants and are used as a biofertilizer (Shaukat *et al*., 2006). PGPR have been reported to confer positive effects and induce plant resistances to environmental stresses and diseases caused by pathogens (Kloepper *et al*., 2004; Mayak *et al*., 2004 a, b; Compant *et al*., 2005; He and Yang, 2007; Dimkpa *et al*., 2009; Yang *et al*., 2009). A wide variety of mechanisms that can improve the plant growth, have been suggested to be impressed by PGPR. These involved mechanisms are as follows: nitrogen fixation (Van Loon, 2007), production of 1-Aminocyclopropane-1-carboxylate deaminase (ACC) (Govindasamy *et al*., 2008), production of volatile organic compounds (Ryu *et al*., 2003), induction of systemic resistance (Chandler *et al*., 2008), phytohormone production (Vessey, 2003), siderophore production (El-Tarabily and Sivasithamparam, 2006), phosphate solubilization (Ryu *et al*., 2003) and potassium releasing (Sarikhani *et al*., 2016).

Fenugreek (*Trigonella foenum-graecum* L.) is a member of the Fabaceae family, cultivated worldwide as a semiarid crop, and traditionally used as a medicinal plant. Fenugreek is grown as a spice and a vegetable crop and also have been used as a traditional therapy for the remedy of diabetes (Miraldi *et al*., 2001; Smith, 2003; Fernández-Aparicio *et al*., 2008). Its effect as an antidiabetic and antiatherosclerotic have been documented (Ajabnoor and Tilmisany, 1988; Sharma and Raghuram, 1990). Fenugreek's leaves are a rich source of iron, calcium, β-carotene and other vitamins and its seeds contain tannic acid, diosgenin, trigocoumarin, alkaloids trigonelline, trigomethyl coumarin, gitogenin and vitamin A (Warke *et al*., 2011). Recently, published literatures indicated that PGPR ameliorate the plants tolerance to abiotic stresses through a variety of mechanisms (Srivastava *et al*., 2008; Sandhya *et al*., 2010). Also beneficial effects of PGPRs on medicinal plants have been reported (Jaleel *et al*., 2007).

Shafighi *et al*. (2014) reported that inoculation of fenugreek with PGPR increases plant height, vegetative growth and seed yield in both well watered and water limited condition. Rubin *et al.* (2017) emphasized that application of PGPR decreases abiotic stress especially drought stress in various plants. They summarized that PGPR inoculation not only improves root and shoot biomass in non-stress condition but also it can enhance aerial biomass and reproductive yield under drought stress condition, however root mass was not increased under stress. In enormous studies, the synergistic effect of nitrogen fixing bacteria especially Rhizobia and phosphate solubilizing bacteria such as *Pseudomons* has been reported but application of these PGPR in stressed condition needs more attention. Therefore, in this study we focused to the inoculation effect of two native and endogenous PGPR (*Sinorhizobium meliloti* Tabriz and *Pseudomonas fluorescens* Tabriz) in fenugreek under drought stress condition.

2. Materials and Methods

The seeds of fenugreek with good germination quality was provided from "Jahad Daneshgahi-Iranian Institute of Medicinal Plants", Karaj, Iran. The present investigation, carried out in research greenhouse of Faculty of Agriculture at the University of Tabriz during 2015-2016. Two bacteria, including *Pseudomonas fluorescens* Tabriz and *Sinorhizobium meliloti* Tabriz were obtained from the Laboratory of Soil Biology, University of Tabriz (Tabriz, Iran). Nutrient Broth (NB) and Yeast Mannitol Broth (YMB) were used to prepare a primary culture of *Pseudomonas* and *Sinorhizobium* respectively to inoculate seeds of plant. The experiments were conducted in a factorial design based on completely randomized block design with three replications. The first factor was application of PGPR in 4 levels including 1. *S. meliloti,* as nitrogen fixing bacterium 2. *P. fluorescens,* as phosphorous solubilizing bacterium 3. combination of *S. meliloti* and *P. fluorescens* 4. negative control without any bacteria and fertilizer treatment. The second factor was soil water content treatment based on field capacity (FC) in 4 levels (100, 80, 60 and 40% of FC). Seed of fenugreek was sown in a plastic pot which had 5 kg soil and after establishment 5 plants remained in each pot. Soil water content was maintained as aforementioned values by daily weighting of pots by digital scale and water loss by evapotran-

spiration was added to each pot. Plants kept in a greenhouse under a 16 h photoperiod, 24±4/18±3°C day/night temperatures, and 40-60% relative humidity. At the end of the experiment leaf area was measured, by the leaf area meter (LI 3100C area meter, LI-COR, USA). Dry weight of each part was determined after drying at 72˚C until constant weight. The fresh and dry weight plants were determined using a digital weighing scale.

The composition of potassium and phosphorus was determined by nitric perchloric and nitric acid digestion methods (Zasoski and Burau, 1977; Havlin and Soltanpour, 1980). Phosphorous was measured by a vanadate-molybdate method using a spectrophotometer (Motic, CL-45240-00, China) and K was determined using a flame photometer (Model 405G, Iran). Nitrogen was measured according to the Kjeldahl method that involves changing the form of organic nitrogen to ammonium (NH_4) by concentrated sulfuric acid and then measuring the amount of ammonium production (Baker and Thompson, 1997). Also the seed yield was recorded at maturity. Water use efficiency (WUE) was calculated by the following formula:

$$WUE = DW/UW$$

In this formula, DW and UW represent dry mass production and the amount of consumed water, respectively (Karimi and Roosta, 2014).

Analysis and quantization of trigonelline

Trigonelline in the seed sample was measured according to modified method of Zheng and Ashihara (2004). The samples were ground with 80% methanol and magnesium oxide (MgO) in a mortar and pestle. After incubation at 60°C for 30 min, the homogenates were centrifuged and the supernatant was collected. After complete evaporation of methanol, the methanol-soluble extracts were dissolved in distilled water. The samples were filtered using a disposable syringe filter unit and the aliquots were used for determination of trigonelline (TG) by HPLC. The analyses of the samples were carried out using a Knauer K2600A liquid chromatography (Germany), equipped with a Nucleosil C18 (150 mm × 4.6 mm I.D, 5 µm) column. A mixture of methanol: water (50:50 v/v) served as the mobile phase and pH of solution adjusted to 5.0 with 50 mM sodium acetate. The elution has been made in an isocratic mode at a flow rate of 1 mL min^{-1} and the detection made at 268 nm by UV detector from the above mentioned company (Koshiro *et al*., 2006). One analysis requires 20 min. The retention time of this alkaloid was 4.4 min. Before carrying out HPLC analysis, we made calibration curve by using different concentrations (0.1, 0.2, 0.5, 0.7 and 1.0 mg mL^{-1}) of trigonelline in phase media. Then calibration curve made with trigonelline and the correlations were excellent for trigonelline. This process was performed according to United States Pharmacopoeia (U.S. Pharmacopeia, USA) by cold extraction method as directed for alcohol soluble material, except where water was used in place of alcohol.

Measuring nicotinic acid

For the measurement of nicotinic acid, it was carried out according to modified Martin *et al*. (1997). The 0.5 g of fenugreek seed powder was mixed with 0.5 g of magnesium oxide (MgO) and 30 ml of distilled water was added to it. The resulting mixture for 30 minutes at 100°C was placed in bath water bath. After cooling, the resulting mixture was filtered using filter paper (1) and was brought to a volume of 50 ml with distilled water. Finally absorption at a wavelength of 263 nm of the samples was measured by a spectrophotometer. Nicotinic acid concentrations were determined using the standard curve.

Statistical analysis

All collected data were subjected to two-way analysis of variance (ANOVA) through PROC GLM procedure, using a SAS statistical package (SAS Institute, software Version 9.4, Cary, NC, USA). If interactions were significant, means were compared by Duncan's multiple range test to determine whether means of the dependent variable were significantly different at $P<0.05$.

3. Results

Analysis of data variances indicated that the effect of PGPR and soil water content and their interaction on leaf area, shoot fresh and dry weight were significant ($P\leq0.01$). Means comparison showed that PGPR inoculation increased fenugreek leaf area, shoot dry and fresh weight especially *S. meliloti* (Table 1). By increasing of water deficit stress, leaf area, shoot fresh and dry weight was decreased (Table 2). In aspect of interaction between PGPR inoculation and water stress, the highest and lowest leaf area, shoot fresh and dry weight was observed in well watered (100% FC) and combination of *S. meliloti* and *P. fluorescens* treated plants and severe water stressed control plants respectively (Fig. 1, 2 and 3). It seems that in normal condition *S. meliloti*

Table 1 - Results of mean comparison of different PGPR treatments

Bacteria	Leaf area (cm^2)	Shoot fresh weight (g)	Shoot dry weight (g)	N ($mg\ g^{-1}$)	P ($mg\ g^{-1}$)	K ($mg\ g^{-1}$)	WUE ($g\ kg^{-1}$)	Seed yield ($g\ pot^{-1}$)	Trigonelline ($mg\ g^{-1}$)	Nicotinic acid ($mg\ g^{-1}$)
Control	756 c	17.71 c	4.18 c	7.36 c	0.54 d	25.84 c	0.139 c	26.53 a	6.84 b	12.34 ab
S. meliloti (S)	1212 a	32.14 a	6.00 a	14.27 a	0.71 a	31.73 a	0.226 a	14.23 b	6.97 b	12.69 b
P. fluorescens (P)	959 ab	29.74 a	4.37 b	12.93 b	0.67 b	30.40 b	0.213 a	13.25 c	7.65 a	14.11 a
SxP	937 b	21.14 b	5.05 ab	12.17 b	0.63 c	29.43 c	0.181 b	14.19 b	7.74 a	14.05 a

SxP= treatment containing both *S. meliloti* and *P. fluorescens*.
Dissimilar letters indicating significant differences (Duncan's multiple range test P≤0.01). N, P and K were measured in dry shoot of plant.

Table 2 - Results of mean comparison of different irrigation treatments

Soil water content (FC)	Leaf area (cm^2)	Shoot fresh weight (g)	Shoot dry weight (g)	N ($mg\ g^{-1}$)	P ($mg\ g^{-1}$)	K ($mg\ g^{-1}$)	WUE ($g\ kg^{-1}$)	Seed yield ($g\ pot^{-1}$)	Trigonelline ($mg\ g^{-1}$)	Nicotinic acid ($mg\ g^{-1}$)
100%	1189 a	33.78 a	6.48 a	13.60 a	0.63 c	29.35 b	0.138 c	24.18 a	6.26 c	11.10 d
80%	1025 b	24.76 b	5.79 b	12.20 b	0.59 d	29.05 b	0.165 b	23.52 b	6.63 c	12.07 c
60%	913 c	18.17 c	3.97 c	12.19 b	0.65 b	30.25 a	0.169 b	12.83 c	7.55 b	13.84 b
40%	737 d	13.93 d	3.36 d	9.73 c	0.69 a	30.37 a	0.287 a	10.20 d	8.76 a	16.18 a

Dissimilar letters indicating significant differences (Duncan's multiple range test P≤0.01). N, P and K were measured in dry shoot of plant.

improved aerial growth of fenugreek better than *P. fluorescens*, whereas combination use of two PGPR bacteria was successful in enhancement of shoot growth better than individual using (Fig. 1).

Un-inoculated control plants produced significantly higher seed yield per pot than PGPR treated fenugreek (Table 1). Water limitation led to decrease in seed yield (Table 2). The maximum seed weight was observed in control plants (Fig. 4).

The nitrogen, phosphorus and potassium concentration was significantly affected by PGPRs and water deficit. As shown in the table of mean comparison (Table 1) for the effects of PGPR treatments, the highest N, P and K were observed in plants treated with *S. meliloti* follows by *P. fluorescens* and treat-

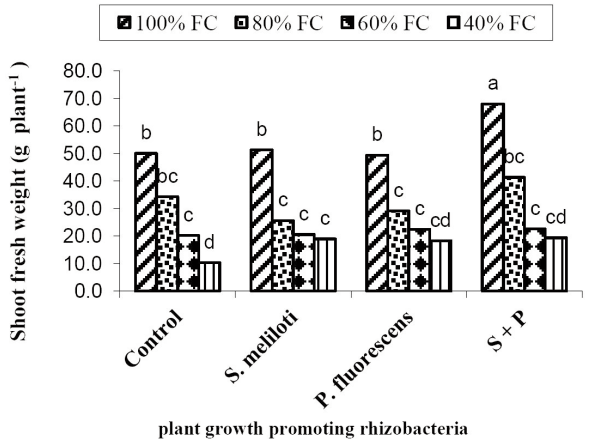

Fig. 2 - Interaction of PGPR and soil water content on the shoot fresh weight fenugreek.

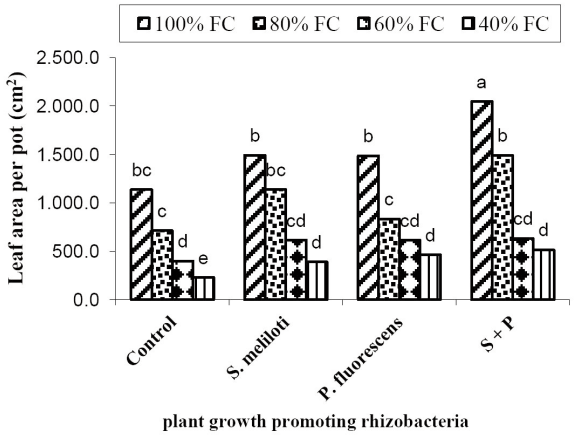

Fig. 1 - Interaction of PGPR and soil water content on leaf area of fenugreek.

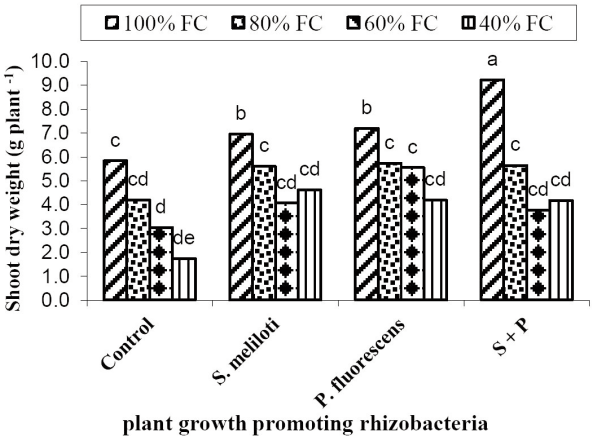

Fig. 3 - Interaction of PGPR and soil water content on the shoot dry weight of fenugreek.

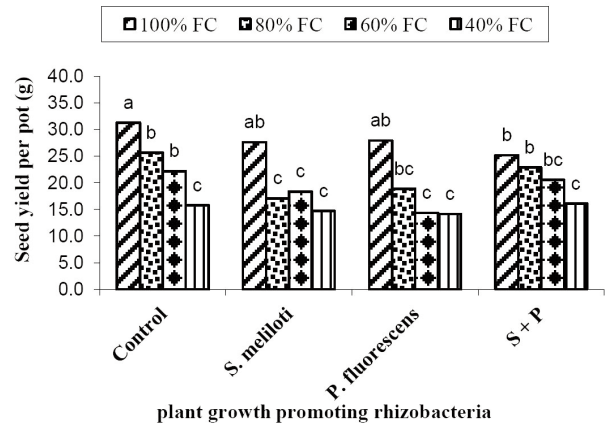

Fig. 4 - Interaction of PGPR and soil water content on the seed yield of fenugreek.

ments containing both *S. meliloti* and *P. fluorescens*. By decreasing of soil water content, N concentration was decreased significantly, but inverse, P and K concentration was increased significantly with an exception at 80% of FC treatment (Table 2). The highest and the lowest N concentration was observed in plants treated with *S. meliloti* and dual application of PGPR bacteria at well watered (100% FC) and control plant at severe water stress treatment (40% FC) respectively (Fig. 5) and the highest and the lowest P concentration was related to plants treated with *S. meliloti* and *P. fluorescens* in single form at well watered (100% FC) and control plant at severe water stress treatment (40% FC) respectively (Fig. 6).

According to the interaction effects between PGPRs and drought stress the highest and lowest K concentration was observed in dual application of PGPR bacteria at well watered (100% FC) and control plant under sever water stress (40% FC) respectively (Fig. 7).

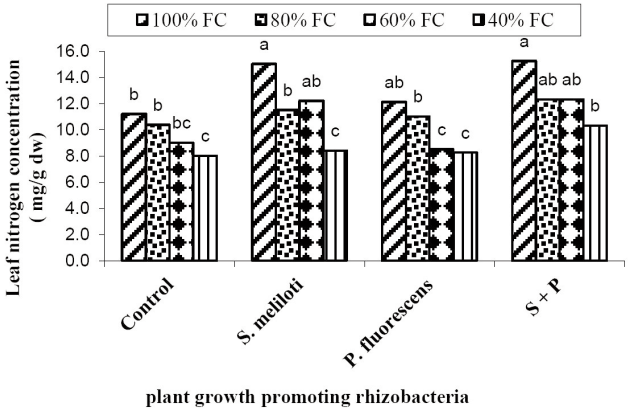

Fig. 5 - Interaction of PGPR and soil water content on N concentration of fenugreek shoot.

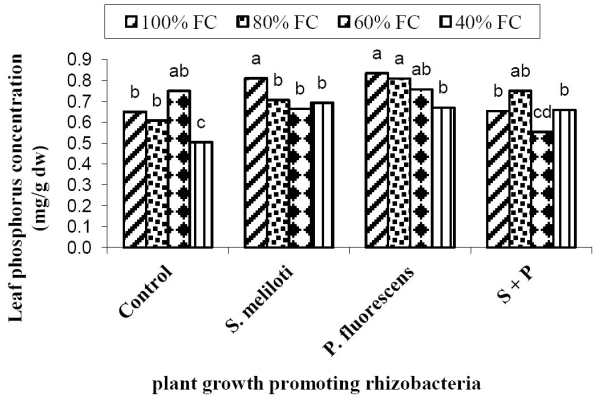

Fig. 6 - Interaction of PGPR and soil water content on P concentration of fenugreek shoot.

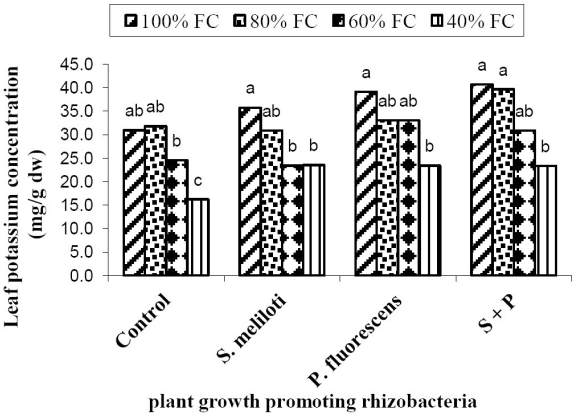

Fig. 7 - Interaction of PGPR and soil water content on K concentration of fenugreek shoot.

Water use efficiency (WUE) was significantly affected by PGPR and soil water content. Mean comparison indicated that PGPR inoculate plants produced more shoot biomass per water unit than control ones (Table 1). By increasing water deficit stress WUE was increased significantly (Table 2). In aspect of interaction between PGPR and water stress it was shown that both dual application of *P. fluorescens* and *S. meliloti* and separate application of bacteria under severe water deficit stress (40% FC) led to highest WUE and well watered control plants produced the lowest WUE (Fig. 8).

As it was shown in the table of mean comparison (Table 1), individual and dual PGPR treatments improved trigonelline and nicotinic acid. By increasing water deficit stress trigonelline and nicotinic acid was significantly increased (Table 2). The highest and lowest trigonelline and nicotinic acid was observed in *P. fluorescens* inoculated at 40% FC soil water content treatment and control in 100% FC soil water content treatment respectively (Figs. 9 and 10).

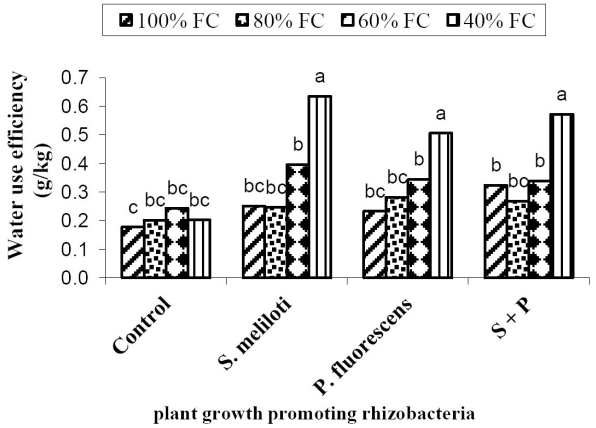

Fig. 8 - Interaction of PGPR and soil water content on the WUE of fenugreek.

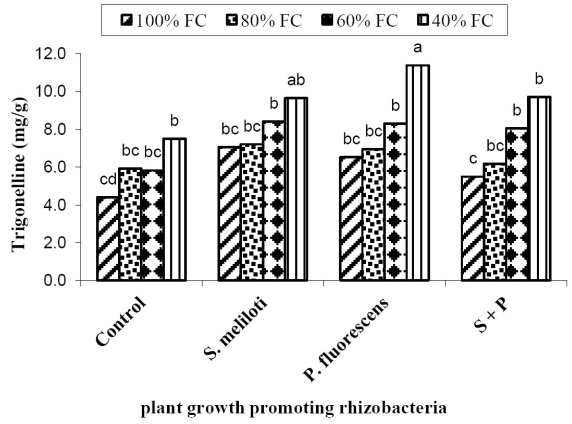

Fig. 9 - Interaction of PGPR and soil water content on the Trigonelline of fenugreek seed.

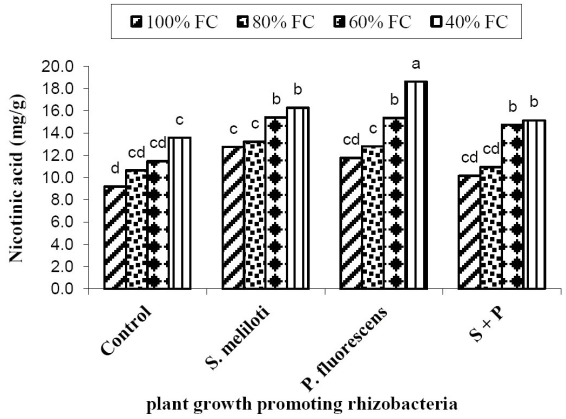

Fig. 10 - Interaction of PGPR and soil water content on nicotinic acid of fenugreek seed.

4. Discussion and Conclusions

Water deficit limits many crop production worldwide and negatively affects the plant growth and reproduction; recently published literatures indicated that PGPR ameliorate the plants tolerance to abiotic stresses through a variety of mechanisms (Srivastava *et al*., 2008; Sandhya *et al*., 2010). In keeping with our results, Mishra *et al*. (2010) indicated that PGPRs could ameliorate the negative effects of salinity stress conditions by positive effects on parameters such as increasing the germination in plants, and also increasing the yield, drought tolerance, and growth. It has also been reported that even in the presence of optimum levels of nitrogenous fertilizers, inoculating with PGPR containing ACC-deaminase activity can improve the yield and growth of inoculated plants (Shaharoona *et al*., 2006). According to the results of this investigation inoculation with PGPR containing ACC-deaminase considerably decreased the damages caused by drought stress on the growth and yield. They reported that un-inoculated plants exposed to drought stress at vegetative growth stage had significantly decreased shoot growth by 41%, while in the inoculated plants the decreased shoot growth was only by 18 per cent.

In present study control plants without any inoculation produced higher seed yield per pot than PGPR treated fenugreek (Table 1). It should be noted that the experiment duration was 5 month and fenugreek has indeterminate flowering habit, so while plants flowering were continued it was harvested. It have been reported that PGPR could delay the flowering time (Jaleel *et al*., 2007). Water stress led to decrease in fenugreek seed yield. The reason may be due to this fact that in the absence of stress conditions, more photosynthesis material have been stored in the organs such as stems and leaves which by transferring to the seeds increased the grain weight. In contrary, under stress conditions, the water and mineral absorption by the plant is disrupted which decreases the plant growth and reduces the transmission of photosynthesis material in leaf and other organs to the grain (Jaleel *et al*., 2007).

Similar to our findings, root bacterial inoculations significantly affected the plant nutrient element contents in apple compared to controls and increased the phosphorus content of treated plants (Karlidag *et al*., 2007). Ordookhani *et al*. (2010) reported that *P. fluorescens* improved potassium in tomato plant.

Water use efficiency was increased by PGPR in fenugreek in present investigation. Similar to the present findings Jaleel *et al*. (2007) demonstrated that the minimum WUE was related to un-inoculated cases which was improved by inoculation with *Rhizobium* and PGPR. In the present investigation leaf area, shoot weight, dry weight, nitrogen, phosphorus and potassium content, and WUE increased significantly by treatment with both PGPRs. PGPR

colonizes the plant's root system and modulates its growth through increasing the availability of nutrients it also protects the plants from phytopathogens (Lee *et al*., 2013). It has been reported that in induced drought stress condition, fixed nitrogen during photosynthesis, spend the production of secondary metabolites (Aliabadi Farahani *et al*., 2009). Also beneficial effects of PGPRs on medicinal plants have been reported (Jaleel *et al*., 2007). It has been reported that the synthesis of secondary metabolites in medicinal plants is induced specific pathway by the impact of microorganisms (Bouchereau *et al*., 1996). Integrative use of PGPRs and water deficit stress could be an enhance the eco-friendly strategy of PGPRs and plants and could increasing the alkaloid yields in medicinal plants (Lee *et al*., 2013). Since the fenugreek is used as a medicinal plant this strategy could be applied for increasing its useful secondary metabolites.

In conclusion, the results of the present investigation indicate that both *S. meliloti* and *P. fluorescens* could effectively increase vegetative growth, nitrogen, phosphorus and potassium content, secondary metabolites and WUE in fenugreek regardless of water stress. Also under water stress condition PGPR increased plant growth. However in present study seed yield because of delaying bolting time was decrease by application of PGPR.

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