Morphophysiological and phytochemical responses of fenugreek to plant growth promoting rhizobacteria (PGPR) under different soil water levels

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ABSTRACT

Fenugreek (*Trigonella foenum-graecum* L.) is a valuable medicinal plant, which is widely distributed throughout the world. It has been known that plant growth promoting rhizobacteria (PGPR) have positive effects on the quality and quantity of medicinal plants under different soil water levels. For this reason, a factorial experiment was conducted on the basis of a randomized complete block design (RCBD) to evaluate PGPR effects on the morphophysiological and phytochemical traits of fenugreek under different soil water levels. This study was conducted in two separate experiments: after the six-leaf stage and after the flowering stage. In the experiments, the treatments were plant growth promoting rhizobacteria (PGPR) including the control, *Sinorhizobium meliloti*, *Pseudomonas fluorescens*, a combination of *S. meliloti* and *P. fluorescens*, and different soil water levels (i.e. 100, 80, 60 and 40% of field capacity (FC) in three replications. The results showed that the highest seed weight per plant was obtained by inoculation with the *S. meliloti* and *P. fluorescens* combination at 100% FC after the two developmental stages. The maximum concentrations of nicotinic acid and trigonelline were observed for the combination of *S. meliloti* and *P. fluorescens* at the soil water content of 40% FC after the six-leaf stage and for *S. meliloti* at the soil water content of 40% FC after the flowering stage. The correlation and stepwise regression analyses showed positive effects of PGPR application on the morphophysiological and phytochemical traits of fenugreek plants under different soil water levels.

Key words: nicotinic acid, *Pseudomonas fluorescens*, *Sinorhizobium meliloti*, soil water content, *Trigonella foenum-graecum* L., trigonelline

Abbreviations:

PGPR – plant growth promoting rhizobacteria, FC – field capacity, WUE – plant water-use efficiency, SPAD value – a relative chlorophyll index which is measured by SPAD meter

INTRODUCTION

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

development, limiting crop production worldwide. Water stress negatively affects plant growth and reproduction, and disrupts whole-plant functions (Bray, 2004; Hummel et al., 2010). Water stress

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causes cellular changes such as solute concentration and cell volume alterations, disruption of water potential gradients, changes in membrane shape and impairment of its integrity, loss in turgor pressure, and protein denaturation (Bray, 1997; Bartels and Sunkar, 2005). Water stress is a major threat to crop production, and tolerance to drought conditions is the main target for crop improvement (Salekdeh et al., 2009). However, there have been reports on increasing secondary metabolites under water stress. It has been reported that the biosynthesis of these metabolites is not only under the influence of plant genetics but is also affected by changes in environmental factors (Aliabadi-Farahani et al., 2009). Water stress has limited the production of many crops worldwide and negatively affects plant growth and reproduction. Recently published reports have indicated that plant growth promoting rhizobacteria (PGPR) ameliorate plant tolerance to abiotic stresses through a variety of mechanisms (Jaleel et al., 2007; Srivastava et al., 2008; Sandhya et al., 2010). Previous studies indicated that a specific pathway for the synthesis of secondary metabolites in medicinal plants was induced by micro-organisms such as PGPR (Bouchereau et al., 1996). According to the report by Turtola et al. (2003), due to drought stress, during photosynthesis, carbon fixation is used to produce secondary metabolites. On the other hand, one of the activities of PGPR is the production of phytohormones. Phytohormones, especially auxins, control several stages of plant growth and development such as cell elongation, cell division and tissue differentiation (Vessey, 2003). A plant under a long-term treatment with an auxin (IAA) has highly developed roots, which in turn allows the plant to better take up nutrients, ultimately aiding the overall growth of the plant (Aeron et al., 2011). This action, especially at low water levels, helps to increase the production of secondary metabolites. PGPR are rhizosphere bacteria which develop symbiotic relationships with large varieties of plants and are used as biofertilizer (Shaukat et al., 2006). It has been reported that PGPR have positive effects and induce plant resistance to environmental stresses and diseases caused by pathogens (Jing et al., 2007; Dimkpa et al., 2009; Yang et al., 2009). A wide variety of mechanisms that can improve plant growth have been suggested to be induced by PGPR. The mechanisms involved are as follows: nitrogen fixation (van Loon, 2007), synthesis of 1-Aminocyclopropane-1-carboxylate deaminase (ACC) (Govindasamy et al., 2008), synthesis of volatile organic compounds (Ryu

et al., 2003), phytohormone synthesis (Vessey, 2003), siderophore production (El-Tarabily and Sivasithamparam, 2006), phosphate solubilization (Ryu et al., 2003), and synthesis of secondary metabolites (Bouchereau et al., 1996).

Fenugreek (*Trigonella foenum-graecum* L.) is a member of the Fabaceae family, and is grown as a spice and vegetable crop. Fenugreek has indeterminate growth, which allows greater flexibility in the timing of harvest. Fenugreek has been used in traditional therapy as a remedy for diabetes (Miraldi et al., 2001; Basch et al., 2003; Fernandez-Aparicio et al., 2008). Also, its effects as an anti-diabetic and anti-atherosclerotic have previously been well documented (Ajabnoor and Tilmisany, 1988; Sharma and Raghuram, 1990). Fenugreek leaves are a rich source of iron, calcium, β -carotene and other vitamins, while its seeds contain tannic acid, diosgenin, trigocoumarin, alkaloids, trigonelline, trigomethyl coumarin, gitogenin, and vitamin A (Warke et al., 2011), which indicates its nutritional and medicinal value (Danesh Talab et al., 2014).

Although fenugreek is a valuable medicinal plant, the effect of different soil water levels after the six-leaf stage and the flowering stage, and the effect of PGPR on the qualitative and quantitative yield of fenugreek have not been well documented. The aim of the present study was a comparative investigation of PGPR effects on fenugreek (*Trigonella foenum-graecum* L.) under different soil water levels after the six-leaf stage and the flowering stage. Therefore, the concentrations of the most important medicinal metabolites of this plant (trigonelline and nicotinic acid) and morphophysiological traits were measured to understand these effects.

MATERIAL AND METHODS

The seeds of fenugreek with a proper germination percentage were provided by the Seed Technology Laboratory of the Medicinal Plants Institute, Academic Center for Education, Culture and Research (ACECR). The present investigation was conducted in a research greenhouse of the Agriculture Faculty of the University of Tabriz during 2015-2016 in two separate experiments after the six-leaf stage (14 days after planting), and after the flowering stage (40 days after planting) until the harvest of plants.

This experiment was conducted on the basis of a factorial experiment in a randomized complete block design (RCBD) with three replications. The first factor was application of PGPR in 4 variants

including *Sinorhizobium meliloti* as nitrogen fixing bacteria, *Pseudomonas fluorescens* as phosphate-solubilizing bacteria, combination of *S. meliloti* and *P. fluorescens*, and the control without any bacteria or fertilizer. The second factor was soil water content based on field capacity (FC = 24.42% w/w equivalent to the -10 kPa moisture content). The FC of the soil was determined using a pressure plate under a pressure of 0.1 atm, and soil moisture content was calculated, and then different levels of FC were applied in the experiment (100, 80, 60 and 40% FC).

The main characteristics of the soil were: pH 7.36; 3.36 dS m⁻¹ EC; 1.79% organic carbon; 15% clay; 16% silt; 73% sand; 87.4 mg kg⁻¹ available P (Olsen method); 1250 mg kg⁻¹ K; 31.85% CaCO₃; 0.12% total N. Seeds of fenugreek were sown into plastic pots with a surface diameter of 24 cm, each filled with 5 kg soil. After the establishment of seedlings, 5 plants remained in each pot. Soil water content was maintained in the amounts specified for the treatments by weighing the pot-soil-plant system daily, always in late afternoon, in order to allow the soil to equilibrate with the desired water content during the night. The plants were 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, after the ripening of seeds, plants were cut off near the soil level. The plant material was dried in the laboratory at room temperature (26 ± 2°C), away from sunlight to prevent changes in the nature of the plants' constituents, and then the seeds were separated. Plant water-use efficiency (WUE) was calculated as the amount of water used per unit of plant material expressed by the ratio of total net dry matter (DM) production to water consumption (Karimi et al., 2014).

For the measurement of leaf SPAD value, nine leaves from each pot were selected and the mean of the leaf SPAD value was measured with a SPAD-502 meter (Konica-Minolta, Japan). The concentrations of potassium and phosphorus were determined by nitric perchloric and nitric acid digestion methods (Havlin and Soltanpour, 1980). Phosphorus was measured by the 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 involved changing the form of organic nitrogen to the ammonium (NH₄⁺) form with concentrated sulfuric

acid and then measuring the amount of ammonium production (Baker and Thompson, 1992).

Microbial inoculation

Sinorhizobium meliloti and *Pseudomonas fluorescens* were used in the assays. The bacterial inocula were prepared using the method presented by Bharti et al. (2014) with slight modifications. Rhizobacterial strains were grown in a nutrient broth (NB) with 5% (w/v) NaCl (sodium chloride maintains the osmotic equilibrium of the medium) up to a late exponential growth phase to prepare bacterial inocula. The broth in each flask was inoculated with the isolated rhizobacterial strains and incubated at 28°C for 24 h in an orbital shaking incubator at 100 rpm. Optical density was measured to achieve a uniform population of bacteria [10⁸ colony forming units (CFU) ml⁻¹] in the broth prior to inoculation. The required inocula of each culture were centrifuged at 8000 × g for 10 min. The pellets obtained were washed with sterile distilled water and then dissolved in a 0.85% saline solution. The bacterial suspension was adjusted to Abs_{600 nm} = 1.0. The suckers were dipped in their respective treatments for half an hour before planting and the residual bacterial culture suspensions (5 ml per pot) were also poured into their respective treatments after planting. Pots with no rhizobacterial inoculation (control) received sterilized 0.85% saline solution (no inoculum).

Isolation and extraction of mucilage

Fenugreek seeds (200 g) were soaked in distilled water (1.5 L) at room temperature for 1 h and then boiled while being stirred in a water bath until a slurry was obtained. The solution was cooled and kept in a refrigerator overnight to settle out undissolved materials. The clear solution above the sediment was decanted and centrifuged at 24 × g for 20 minutes. The supernatant was separated and concentrated in a water bath at 60°C to one third of its original volume. The solution was cooled to room temperature and poured into thrice the volume of acetone with continuous stirring. The precipitate was washed repeatedly with acetone and dried at 50-60°C under vacuum. The dried material was powdered and kept in a desiccator (Sabale et al., 2009).

Measurement of trigonelline

To estimate trigonelline in the samples (seed and/or shoot), the Zheng and Ashihara method (Zheng and Ashihara, 2004) was used with a slight modification. 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 the 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 the determination of trigonelline (TG) by HPLC. The analyses of the samples were carried out using a Knauer K2600A liquid chromatograph (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 the pH of solution was adjusted to 5.0 with 50 mM sodium acetate. Elution was performed in an isocratic mode at a flow rate of 1 mL min⁻¹, and detection was at 268 nm by UV detector from the above mentioned company (Koshiro et al., 2006; Khosravi et al., 2011). One analysis required 20 min. The retention time of this alkaloid was 4.4 min. Before carrying out HPLC analysis, we made a calibration curve ($R^2 = 0.99$) by using different concentrations (0.1, 0.2, 0.5, 0.7 and 1.0 mg mL⁻¹) of trigonelline in phase media.

Measurement of nicotinic acid

To estimate nicotinic acid, the Martin method (Martin et al., 1997) was slightly modified and used. 0.5 grams of the fenugreek seed powder was mixed with 0.5 grams of magnesium oxide (MgO), and 30 ml of distilled water was added to it. The resulting mixture was placed in a water bath at 100°C for 30 minutes. After cooling, the resulting mixture was filtered using a filter paper (# 1) and was made up to a volume of 50 ml with distilled water. Finally, absorption at a wavelength of 263 nm by the samples was measured with a spectrophotometer. Nicotinic acid concentrations were determined using a standard curve. To determine the standard curve, concentrations of 20, 40, 60, 80 and 100 micrograms per ml of nicotinic acid powder were prepared in distilled water, and then magnesium oxide (MgO) was added to the concentration equal to each standard, and absorbance at 263 nm was measured after smoothing.

Data analysis

All collected data were subjected to two-way analysis of variance (ANOVA) through PROC GLM procedure, using an SAS statistical package (SAS Institute, software version 9.4, Cary, NC, USA). Means were compared with Duncan's multiple range tests to determine whether the means of the dependent variable were significantly different at $p \leq 0.05$.

RESULTS

According to the results of variance analysis, the interaction effect of plant growth promoting rhizobacteria (PGPR) and different water availability was significant ($p \leq 0.01$) on the number of leaves per plant, shoot dry weight, legume number per plant, seed number per legume, 1000 seed weight, seed weight per plant, water use efficiency, SPAD value, seed mucilage content, nicotinic acid and trigonelline content in the two experiments (Tabs 1 and 2).

Induction of water availability after the 6-leaf stage (1st experiment)

Considering the results of means comparisons, the highest number of leaves per plant and shoot dry weight were observed following inoculation with *S. meliloti* at 100% FC. The maximum legume number per plant, seed number per legume, 1000 seed weight, seed weight per plant, and also SPAD value, nitrogen, phosphorous and potassium content were obtained by inoculation with *S. meliloti* + *P. fluorescens* at 100% FC. The greatest water use efficiency and also the highest amounts of nicotinic acid and trigonelline were related to the treatment with *S. meliloti* + *P. fluorescens* at 40% FC. The highest amount of seed mucilage was obtained by inoculation with *P. fluorescens* at 60% FC (Tab. 3).

Induction of water availability after the flowering stage (2nd experiment)

The results of mean comparisons showed that the highest leaf number per plant, shoot dry weight per plant, legume number per plant, seed number per legume, 1000 seed weight, seed weight per plant, and also the amounts of seed mucilage, nitrogen and potassium were obtained by inoculation with *S. meliloti* + *P. fluorescens* at 100% FC. The maximum water use efficiency was observed in the treatment with *S. meliloti* at 40% FC. The highest amounts of trigonelline and nicotinic acid were obtained in the treatment with *P. fluorescens* at 40% FC (Tab. 3). The greatest phosphorous content was obtained in the treatment with *P. fluorescens* at 100% FC.

The correlation between the mentioned traits in the 1st experiment (Tab. 4) showed that trigonelline had a significant ($p \leq 0.01$) and positive correlation with nicotinic acid. Trigonelline and nicotinic acid had significant and negative correlations ($p \leq 0.01$) with shoot dry weight, legume number per plant, seed weight per plant and SPAD value, while they showed a significant and positive correlation with

Table 1. Analysis of variance for the effects of PGPR and soil water content (in % of field capacity) on the morphophysiological traits and chemical content of fenugreek after the six-leaf stage – part 1

Source of variance	d.f.	Leaf number per plant	Shoot dry weight per plant	Legume number per plant	Seed number per legume	Thousand seed weight	Seed weight per plant	Water use efficiency
Rep. (Block)	2	8.85	0.326	0.169	0.985	3938.31	0.09	2.88
PGPR (P)	3	63695.42 **	26.18 **	30.20 **	4.20 **	3761.34 **	2862.28 **	0.073 **
Soil water Content (W)	3	104404.57 **	8.12 **	167.90 **	11.46 **	3824.40 **	15157.47 **	0.14 **
P×W	9	52632.15 **	5.40 **	8.816 **	13.38 **	3852.83 **	437.13 **	0.023 **
Error	30	21.84	0.087	0.448	0.437	3931.70	20.14	0.0001
CV (%)		10.25	12.36	17.45	16.21	12.63	22.25	19.15

*, ** – significant at 5% and 1%, respectively

Table 1. Analysis of variance for the effects of PGPR and soil water content (in % of field capacity) on the morphophysiological traits and chemical content of fenugreek after the six-leaf stage – part 2

Source of variance	d.f.	SPAD value	Mucilage	Nicotinic acid	Trigonelline	N	P	K
Rep. (Block)	2	6.56	1.21	2.17	0.32	0.750	0.0001	10.21
PGPR (P)	3	30.66 **	54.53 **	30.62 **	10.54 **	32.021 **	0.146 **	35.81 **
Soil water Content (W)	3	99.89 **	428.69 **	60.03 **	19.14 **	15.12 **	0.024 **	105.24 **
P×W	9	154.01 **	142.56 **	2.04 **	1.84 **	18.23 **	0.079 **	30.36 **
Error	30	2.77	3.648	1.63	0.35	3.65	0.001	3.81
CV (%)		15.44	25.55	10.45	17.44	18.45	12.45	15.24

*, ** – significant at 5% and 1%, respectively

Table 2. Analysis of variance for the effects of PGPR and soil water content (in % of field capacity) on the morphophysiological traits and chemical content of fenugreek after the flowering stage – part 1

Source of variance	d.f.	Leaf number per plant	Shoot dry weight per plant	Legume number per plant	Seed number per legume	Thousand seed weight	Seed weight per plant	Water use efficiency
Rep.(block)	2	14.43	0.326	2.73	0.289	0.132	235.67	0.001
PGPR (P)	3	2504.78 **	26.18 **	113.38 **	14.97 **	0.787 ns	8212.17 **	0.0528 **
Soil Water Content (W)	3	20656.67 **	8.12 **	53.49 **	6.60 **	3.10 ns	7707.41 **	0.018 **
P×W	9	6596.01 **	5.40 **	12.79 **	5.63 **	4.26 **	1116.97 **	0.012 **
Error	30	64.40	0.087	1.478	1.81	0.207	298.76	0.001
CV (%)		11.45	12.36	17.54	17.45	21.16	16.65	17.65

*, **, ns – significant at 5%, 1%, and not significant, respectively

Table 2. Analysis of variance for the effects of PGPR and soil water content (in % of field capacity) on the morphophysiological traits and chemical content of fenugreek after the flowering stage – part 2

Source of variance	d.f.	SPAD value	Mucilage	Nicotinic acid	Trigonelline	N	P	K
Rep.(block)	2	0.282	8.26	0.199	0.066	0.987	0.0001	8.31
PGPR (P)	3	292.94 **	73.71 **	59.68 **	14.90 **	14.52 **	0.021 **	39.75 **
Soil Water Content (W)	3	32.95 **	18.06 **	10.01 **	2.55 **	19.65 **	0.065 **	76.47 **
P×W	9	38.99 **	2.69 **	15.44 **	1.61 **	11.65 **	0.032 **	227.04 **
Error	30	1.86	1.62	0.532	0.244	2.36	0.0001	3.18
CV (%)		15.44	18.45	20.25	22.52	17.25	19.21	19.25

*, **, ns – significant at 5% and 1%, respectively

Table 3. Mean comparisons for the interaction effects of PGPR and soil water content (in % of field capacity) on the morphophysiological traits and chemical content of fenugreek after the six-leaf stage and the flowering stage – part 1

Growth stage	PGPR inoculation	Soil water content	Leaf number per plant	Shoot dry weight per plant (g)	Legume number per plant	Seed number per legume	Thousand seed weight (g)	Seed weight per plant (g)	Water use efficiency (g kg ⁻¹)
After the six-leaf stage	Control	100% FC	190 a	6.330 b	7 bc	5 c	3.650 cd	60.120 b	0.075 e
		80% FC	150 c	4.051 c	4 cd	4 cd	3.210 d	30.210 d	0.088 cd
		60% FC	148 c	3.608 c	3 d	3 d	3.010 e	22.360 d	0.112 c
		40% FC	107 c	2.120 d	2 d	3 e	2.320 de	20.130 d	0.146 c
	<i>Sinorhizobium meliloti</i> (R)	100% FC	300 a	8.650 a	11 ab	6 b	5.550 b	67.850 b	0.134 c
		80% FC	199 b	6.511 b	9 b	5 c	4.230 c	45.250 c	0.157 c
		60% FC	232 b	4.098 c	4 cd	4 cd	3.560 cd	37.320 cd	0.172 b
		40% FC	185 b	2.700 d	3 d	3 d	3.100 d	22.690 d	0.200 b
	<i>Pseudomonas fluorescens</i> (P)	100% FC	268 ab	7.650 ab	10 b	8 ab	6.360 ab	88.980 a	0.100 d
		80% FC	208 b	4.771 c	9 b	6 b	5.330 b	65.250 b	0.114 cd
		60% FC	179 b	4.360 c	6 c	5 c	4.230 c	50.360 c	0.123 c
		40% FC	164 b	3.190 cd	4 cd	4 cd	3.800 cd	45.250 c	0.280 a
After the flowering stage	R+P	100% FC	229 c	9.415 a	14 a	9 a	7.360 a	100.250 a	0.105 d
		80% FC	208 b	6.458 b	11 ab	7 b	6.320 ab	85.360 ab	0.134 c
		60% FC	183 b	4.240 c	8 c	6 bc	5.500 b	74.250 b	0.168 b
		40% FC	119 c	3.450 cd	6 c	5 c	4.550 c	61.250 b	0.290 a
	Control	100% FC	420 b	5.823 c	13 b	9 b	7.527 bc	110.733 b	0.177 c
		80% FC	340 bc	4.180 cd	10 bc	9 b	7.287 bc	101.500 b	0.201 bc
		60% FC	86 d	3.030 d	9 bc	5 c	6.040 c	82.050 c	0.244 bc
		40% FC	52 d	1.733 de	3 d	3 d	3.940 d	64.333 c	0.202 bc
	<i>Sinorhizobium meliloti</i> (R)	100% FC	530 ab	6.937 b	17 a	12 ab	8.733 b	110.333 b	0.251 bc
		80% FC	410 b	5.590 c	13 b	9 b	6.717 c	68.377 c	0.246 bc
		60% FC	250 b	4.053 cd	6 c	11 b	6.463 c	73.443 c	0.396 b
		40% FC	252 b	4.603 cd	5 c	5 c	4.253 d	58.600 c	0.634 a
	<i>Pseudomonas fluorescens</i> (P)	100% FC	520 ab	7.187 b	13 b	15 a	8.620 b	123.633 b	0.232 bc
		80% FC	415 b	5.727 c	7 c	9 b	5.443 c	75.333 bc	0.281 bc
		60% FC	246 c	5.537 c	9b c	5 c	6.130 c	55.393 c	0.343 b
		40% FC	220 c	4.177 cd	3 d	7 bc	4.343 d	56.583 c	0.505 a
Designation of means with the same letters in each column indicates no significant difference between treatments at the 5% level of probability	R+P	100% FC	613 a	9.217 a	17 a	15 a	9.787 a	165.000 a	0.323 b
		80% FC	518 ab	5.637 c	10 bc	10 b	9.687 a	102.400 b	0.268 bc
		60% FC	432 b	3.767 cd	7 c	11 b	6.630 c	88.630 b	0.239 bc
		40% FC	356 bc	4.160 cd	5 cd	7 bc	4.780 d	63.000 c	0.571 a

Table 3. Mean comparisons for the interaction effects of PGPR and soil water content (in % of field capacity) on the morphophysiological traits and chemical content of fenugreek after the six-leaf stage and the flowering stage – part 2

Growth stage	PGPR inoculation	Soil water content	SPAD value (SPAD)	Seed Mucilage (%)	Nicotinic acid (mg g ⁻¹)	Trigonelline (mg g ⁻¹)	N (mg g ⁻¹)	P (mg g ⁻¹)	K (mg g ⁻¹)
After the six-leaf stage	Control	100% FC	45.360 c	11.100 c	10.446 c	3.460 d	10.260 b	0.450 b	27.710 c
		80% FC	40.230 d	13.650 bc	11.060 c	3.990 d	8.660 bc	0.334 bc	28.555 b
		60% FC	38.250 de	16.360 b	11.883 bc	3.500 d	6.320 c	0.300 c	23.654 b
		40% FC	36.320 e	12.360 c	12.566 b	3.670 d	4.230 d	0.250 c	10.510 c
	<i>Sinorhizobium meliloti</i> (R)	100% FC	51.690 b	13.298 c	8.586 d	4.968 c	13.560 ab	0.550 ab	38.580 a
		80% FC	49.423 b	15.298 b	10.426 c	5.675 c	12.360 ab	0.480 b	30.752 b
		60% FC	47.356 b	19.698 a	13.726 bc	7.321 b	13.540 ab	0.400 b	20.810 bc
		40% FC	46.223 c	14.098 b	14.586 b	7.625 b	9.230 b	0.350 bc	26.020 b
	<i>Pseudomonas fluorescens</i> (P)	100% FC	55.790 ab	14.298 b	11.586 c	6.265 c	14.230 ab	0.620 ab	39.878 a
		80% FC	51.250 b	19.498 a	12.186 c	6.538 c	13.520 ab	0.570 ab	34.132 ab
		60% FC	49.520 b	21.298 a	13.413 bc	7.158 bc	13.740 ab	0.450 b	26.100 b
		40% FC	42.330 d	19.298 a	15.833 b	8.338 b	10.230 b	0.410 b	21.119 bc
After the flowering stage	R+P	100% FC	58.630 a	14.898 b	10.376 c	5.961 c	16.360 a	0.678 a	40.870 a
		80% FC	55.320 ab	16.698 b	11.180 c	6.018 c	14.250 ab	0.627 ab	26.200 b
		60% FC	46.723 b	20.498 a	12.900 bc	6.918 c	15.230 ab	0.560 ab	26.358 b
		40% FC	44.230 cd	16.298 b	18.313 a	10.021 a	11.250 b	0.516 b	21.865 bc
	Control	100% FC	44.300 b	34.600 b	9.200 e	5.400 c	11.200 b	0.650 b	30.897 ab
		80% FC	40.500 bc	27.400 bc	10.623 cd	5.913 bc	10.360 b	0.608 b	31.784 ab
		60% FC	44.433 b	22.800 c	11.460 cd	5.823 bc	9.000 bc	0.750 ab	24.531 b
		40% FC	37.00 c	13.100 d	13.567 c	7.493 b	8.000 c	0.504 c	16.187 c
	<i>Sinorhizobium meliloti</i> (R)	100% FC	56.367 a	37.200 ab	12.743 c	7.050 bc	15.00 a	0.810 a	35.728 a
		80% FC	54.800 a	25.800 c	13.220 c	7.193 bc	11.500 b	0.707 b	30.827 ab
		60% FC	47.800 b	23.400 c	15.393 b	8.400 b	12.200 ab	0.664 b	23.362 b
		40% FC	40.300 bc	16.800 cd	16.258 b	9.646 ab	8.400 c	0.694 b	23.500 b
	<i>Pseudomonas fluorescens</i> (P)	100% FC	51.667 a	32.800 b	11.760 cd	6.520 bc	12.100 ab	0.835 a	39.108 a
		80% FC	49.800 ab	24.00 c	12.797 c	6.927 bc	11.000 b	0.809 a	33.024 ab
		60% FC	47.300 b	23.400 c	15.360 b	8.300 b	8.500 c	0.758 ab	33.024 ab
		40% FC	39.960 bc	18.160 cd	18.613 a	11.373 a	8.250 c	0.670 b	23.391 b
Designation of means with the same letters in each column indicates no significant difference between treatments at the 5% level of probability	R+P	100% FC	49.200 b	40.800 a	10.147 cd	5.487 c	15.230 a	0.654 b	40.658 a
		80% FC	53.733 a	22.800 c	10.940 cd	6.160 bc	12.300 ab	0.750 ab	39.600 a
		60% FC	56.200 a	20.400 c	14.727 b	8.040 b	12.300 ab	0.554 cd	30.827 ab
		40% FC	40.300 b	17.400 cd	15.127 b	9.700 b	10.300 b	0.660 b	23.300 b

Table 4. Correlation between the morphophysiological traits and chemical content of fenugreek under different water treatments after the six-leaf stage

	Leaf number per plant	Shoot dry weight	Legume number per plant	Seed number per legume	Thousand seed weight	Seed weight per plant	Water use efficiency	SPAD value	Seed mucilage	Nicotinic acid	Trigonelline	P	K	N
Leaf number per plant	1													
Shoot dry weight	0.639 **	1												
Legume number per plant	-0.228 ns	0.405 **	1											
Seed number per legume	-0.297 *	-0.299 *	0.108 ns	1										
Thousand seed weight	-0.273 ns	-0.298 *	0.225 ns	0.275 ns	1									
Seed weight per plant	-0.330 *	0.283 ns	0.952 **	0.372 **	0.296 *	1								
Water use efficiency	0.446 **	-0.068 ns	-0.481 **	0.230 ns	0.144 ns	-0.392 **	1							
SPAD value	-0.021 ns	0.356 *	0.535 **	-0.231 ns	-0.319 *	0.430 **	-0.726 **	1						
Seed mucilage	-0.010 ns	-0.392 **	-0.324 *	-0.026 ns	0.118 ns	-0.288 *	0.059 ns	-0.132 ns	1					
Nicotinic acid	-0.207 ns	-0.727 **	-0.660 **	0.221 ns	0.212 ns	-0.563 **	0.498 **	-0.737 **	0.285 *	1				
Trigonelline	-0.214 ns	-0.690 **	-0.627 **	0.210 ns	0.216 ns	-0.536 **	0.466 **	-0.725 **	0.248 ns	0.987 **	1			
P	0.356 *	0.053 ns	-0.266 ns	0.013 ns	-0.288 *	-0.279 ns	0.320 *	-0.011 ns	-0.033 ns	0.082 ns	0.033 ns	1		
K	-0.238 ns	-0.007 ns	0.283 ns	0.502 **	-0.046 ns	0.379 **	-0.512 ns	0.234 ns	0.021 ns	-0.153 ns	-0.146 ns	0.209 ns	1	
N	-0.180 ns	0.009 ns	0.299 *	-0.011 ns	0.044 ns	0.261 ns	-0.149 ns	0.152 ns	-0.172 ns	-0.062 ns	-0.056 ns	-0.114 ns	-0.025 ns	1

*, **, ns – significant at 5%, 1%, and not significant, respectively

Table 5. Correlation between the morphophysiological traits and chemical content of fenugreek under different water treatments after the flowering stage

	Leaf number per plant	Shoot dry weight	Legume number per plant	Seed number per legume	Thousand seed weight	Seed weight per plant	Water use efficiency	SPAD value	Seed mucilage	Nicotinic acid	Trigonelline	P	K	N
Leaf number per plant	1													
Shoot dry weight	0.270 ns	1												
Legume number per plant	0.618 **	0.646 **	1											
Seed number per legume	-0.178 ns	-0.172 ns	-0.414 **	1										
Thousand seed weight	-0.069 ns	-0.102 ns	0.065 ns	0.162 ns	1									
Seed weight per plant	0.602 **	0.645 **	0.935 **	-0.095 ns	0.004 ns	1								
Water use efficiency	-0.357 *	-0.092 ns	-0.534 **	0.359 *	-0.102 ns	-0.487 **	1							
SPAD value	-0.305 *	0.027 ns	0.098 ns	-0.296 *	0.211 ns	0.054 ns	-0.542 **	1						
Seed mucilage	0.632 **	0.623 **	0.794 **	-0.177 ns	-0.141 ns	0.823 **	-0.392 **	-0.086 ns	1					
Nicotinic acid	-0.523 **	-0.369 **	-0.762 **	0.403 **	0.103 ns	-0.724 **	-0.667 **	-0.231 ns	-0.583 **	1				
Trigonelline	-0.456 **	-0.343 *	-0.764 **	0.485 **	0.087 ns	-0.681 **	0.641 **	-0.262 ns	-0.514 **	0.874 **	1			
P	-0.220 ns	-0.259 ns	-0.436 **	-0.102 ns	-0.217 ns	-0.506 **	0.209 ns	-0.235 ns	-0.330 *	0.246 ns	0.215 ns	1		
K	0.206 ns	-0.410 **	-0.307 *	0.205 ns	-0.070 ns	-0.284 ns	0.193 ns	-0.388 **	-0.070 ns	0.159 ns	0.223 ns	-0.072 ns	1	
N	0.146 ns	0.696 **	0.513 **	0.047 ns	0.090 ns	0.646 **	-0.312 *	0.289 *	0.526 **	-0.450 **	-0.462 **	-0.348 *	0.457 **	1

*, **, ns – significant at 5%, 1%, and not significant, respectively

water use efficiency. However, the trigonelline content had a positive and significant correlation with nicotinic acid content. Interestingly, seed weight per plant had significant and negative correlations with water use efficiency and secondary metabolites including trigonelline, nicotinic acid content and seed mucilage, but it had positive and significant correlations with 1000 seed weight, seed number per legume, and legume number per plant (Tab. 4).

Considering the correlations between the traits in the 2nd experiment (Tab. 5), trigonelline showed a significant ($p \leq 0.01$) and positive correlation with nicotinic acid. Although trigonelline and nicotinic acid showed significant ($p \leq 0.01$) and negative correlations with leaf number per plant, shoot dry weight, legume number per plant, seed weight per plant and mucilage content, they had a significant and positive correlation with seed number per legume. However, trigonelline had positive and significant correlations with nicotinic acid content and water use efficiency. Also, the seed weight per plant had significant and positive correlations with leaf number per plant, shoot dry weight and legume number per plant (Tab. 5).

DISCUSSION

According to the obtained results, the effect of inoculation with plant growth promoting rhizobacteria on the morphophysiological and phytochemical traits of fenugreek was significant following the induction of water stress after the six-leaf stage (1st experiment) and the flowering stage (2nd experiment). In the first experiment, the highest number of leaves was obtained by inoculation with *S. meliloti* at 100% FC, while in the 2nd experiment, inoculation with *S. meliloti* + *P. fluorescens* at 100% FC was the best treatment in respect of the number of leaves. The same results were obtained for shoot dry weight per plant in both experiments. It has been reported that PGPR can delay flowering time and increase biomass weight, and can also improve the resistance of plants to stress conditions (Lee et al., 2013). These results are in line with those of Jaleel et al. (2007) on *Catharanthus roseus*. Undesirable stress causes a breakdown of chloroplasts, decomposition of chlorophyll, enhances the activity of the chlorophyllase enzyme and results in a reduction of photosynthesis, reduced leaf development and reduced plant production (Taiz and Zeiger, 2000). Plant growth promoting bacteria involved in nitrogen fixation and also phosphorus and potassium solubilization increase their uptake, which leads to an increase in the efficiency of photosynthesis, leaf expansion

and plant biomass growth (Souza et al., 2015). The highest number of legumes per plant, seeds per legume, 1000 seed weight, and seed weight per plant was obtained by inoculation with *S. meliloti* + *P. fluorescens* at 100% FC in both experiments. These results are in agreement with the results of Mishra et al. (2010), and they indicated that PGPR could ameliorate the negative effects of stress conditions by improving seed germination and weight, drought tolerance and growth. Lack of water in the soil disrupts the absorption of the elements and seed production (Mandal et al., 1986). Malik et al. (1992), in their studies on legume plants reported that growth stimulating bacteria increased plant access to nitrogen, phosphorus and potassium, and increased the number of pods, seeds and 1000 grain weight. The maximum water use efficiency was obtained by the *S. meliloti* + *P. fluorescens* inoculation at 40% FC in the first experiment, while the greatest water use efficiency was obtained with *S. meliloti* at 40% FC after the flowering stage. It had previously been reported that PGPR application could reduce the adverse effects of environmental stress on plant growth and thus improve their survival and performance (Dimkpa et al., 2009). Also, nitrogen improves water use efficiency because it prevents membrane damage in drought stress and improves osmotic regulation (Saneoka et al., 2004). The maximum SPAD value was obtained by the *S. meliloti* + *P. fluorescens* inoculation at 100% FC in the 1st experiment. Inoculation with *S. meliloti* at 100% FC resulted in the greatest SPAD value in the fenugreek plants in the 2nd experiment. These results are similar to the results of Ahemad and Khan (2012) on *Vigna radiata* L. plants. Anjum et al. (2003), in an experiment with the barley plant had proved that drought stress led to the destruction of chloroplasts and a reduction in chlorophyll content. On the other hand, inoculation with *Pseudomonas* and *Sinorhizobium* bacteria increased the vitality and the concentrations of phosphorus and potassium in wheat components (Egamberdiyeva and Hoflich, 2003). Inoculation of clover plants with *Sinorhizobium meliloti* bacteria under drought stress caused nitrogen fixation and an increase in chlorophyll content (Dursun et al., 2010). Previous studies suggest that increased photosynthetic activity was a consequence of a higher N incorporation which contributed to the formation of the SPAD value (Baset et al., 2010).

Micro-organisms (including bacteria) and growth hormones are known as biological and chemical stimuli in the synthesis of secondary

metabolites in medicinal plants (Ping and Boland, 2004). In the first experiment, the highest amount of seed mucilage was obtained by the application of *P. fluorescens* at 60% FC, while in the 2nd experiment the highest result was obtained by inoculation with *S. meliloti* + *P. fluorescens* at 100% FC. The greatest amounts of nicotinic acid and trigonelline were achieved by inoculation with *S. meliloti* and *P. fluorescens* at 40% FC in the first experiment, and by the *P. fluorescens* inoculation at 40% FC in the 2nd experiment. These results agreed with the findings of Ghorbanli and Niakan (2005) for soybean, Cho et al. (2011) for peanut, and Sanchez-Blanco et al. (2004) for rosemary. Trigonelline synthesis was activated when plants encountered insufficient water and also rhizobacterial inoculation. This result showed that trigonelline was synthesized under the deficiency of nitrogen due to inactive symbiotic reaction. According to Tramontano et al. (1986), accumulation of trigonelline apparently occurred in legumes because of a lack of essential nutrients such as nitrogen and phosphorus (Tramontano et al., 1986; Cho et al., 2011). Among the PGPR, the *Pseudomonas* sp. is notable due to their ability to solubilize soil unavailable P as well as to produce a wide variety of metabolites like auxins, ACC deaminase enzymes, and siderophores. In addition to increasing nutrient availability, PGPR have the potential to produce a wide range of phytohormones to enhance plant growth, which can also contribute to a better growth of fenugreek plants when inoculated with these bacteria. This result is important, since the application of an integrated fertilizer not only enhanced fenugreek growth and forage weight but also reduced the need for chemical N–P–K fertilizers, which offers greater economic and environmental benefits (Dadrasan et al., 2015). Due to the induction of drought stress, nitrogen is used to produce secondary metabolites, and most of the metabolites are used to prevent cellular oxidation (Turtola et al., 2003). Cho et al. (2011) had reported that the trigonelline concentration in peanut increased under drought stress. Rahimi et al. (2014) announced that the amount of seed mucilage of plants increased under water scarcity conditions, which helped to reduce the loss of water. However, the synthesis of secondary metabolites is induced by microorganisms (Bouchereau et al., 1996). For example, bacteria produce phytohormones, which play the role of stimulants in the production of secondary metabolites (Ping and Boland, 2004). Studies on medicinal plants have shown that the specific pathways of secondary metabolite synthesis

are induced by the application of microorganisms (Sanchez-Blanco et al., 2004).

CONCLUSIONS

The results showed that inoculation with plant growth promoting rhizobacteria (PGPR) significantly improved the morphophysiological and phytochemical traits of fenugreek (*Trigonella foenum-graecum* L.) under different water availability levels, especially under the conditions of soil water restriction. Generally, the best results were obtained by integrated inoculation with *Sinorhizobium meliloti* and *Pseudomonas fluorescens* at 40% FC after the flowering stage for increasing the trigonelline and nicotinic acid content and water use efficiency, and also, at 100% FC after the flowering stage for increasing seed mucilage and seed weight. Thus, PGPR application can reduce the adverse effect of soil water restriction on plant growth and improve their performance and biosynthesis of secondary metabolites under the conditions of soil water restriction.

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AUTHOR CONTRIBUTIONS

All the authors contributed to this work, including the development of ideas, writing and revisions of the content.

CONFLICT OF INTEREST

Authors declare no conflict of interest.

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