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The effect of PGPR and alfalfa extract on macronutrient and micronutrient contents of sorghum (*Sorghum vulgare)*

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ABSTRACT- The increasing population and the pastures inability to meet food needs in livestock have led to the increase of cultivates forage plants. In order to evaluate the effect of plant growth promoting rhizobacteria (PGPR) and alfalfa (*Medicago sativa*) extract on the growth and concentration of macronutrient and micronutrient contents of forage sorghum *(Sorghum vulgare)*, an experiment was conducted as factorial aaraned in a completely randomized design with six replications at Research Greenhouse University of Zabol. The first factor was PGPR (included soil inoculated and non-inoculated (control) medium, and the second factor was alfalfa extract in three concentrations (0, 2 and 4 ppm). The results showed that increasing alfalfa extract concentration significantly increased total plant dry weight as well as the macro and micronutrient contents. The highest and lowest manganese contents were achieved in the 0.004 levels and control of alfalfa extracts, respectively. Copper content in 0.002 level of alfalfa extract showed a 15% increase compared to the control, with no significant difference with 0.004 of alfalfa extracts. PGPR application as growing substrate also significantly increased dry weight as well as manganese, copper and potassium contents of the plant. Also, there was no significant difference in iron content at level 4 per thousand of alfalfa extract in a substrate inoculated with PGPR compared to the non-inoculated substrate. Finally, it is recommended that the effect of alfalfa extract on yield and nutrient concentrations of other plants be examined in the future.

INTRODUCTION

In recent years, humans' growing demand for animal products has significantly increased due to increasing the world population and rangelands inability to meet livestock's feed needs. In this regard, forage sorghum (*Sorghum sudenense* L.), having features such as high productivity per unit area, high power in paw-handing, high tolerance to drought and good nutritional value, is of significant importance for cultivation in arid and semi-arid regions and can be effective in providing part of the country's forage demands (Seyedsharifi and Hokmalipur, 2010). Among the factors affecting plant growth, nutrients have an important role on the quantity and quality of plant products. One way to provide nutrients for plants is using chemical fertilizers. But by increasing the consumption of chemical fertilizers to increase crop and horticultural products, the world has faced more environmental pollution. Chemical fertilizers used in agriculture have entered into the groundwater and surface and even drinking water resources in various ways, which could threaten human health and environment (Patton and Crouch, 1977). Even though chemical fertilizers increase products in the early years of their application, unfortunately, over the years and with their improper application, they have created adverse effects on, the environment, biodiversity and cycles of nature (Liu et al., 2006). Thus, today, to increase the quantity and quality of crops, agricultural experts consider biofertilizers application and organic products as an appropriate way in sustainable agriculture and extensive research has been conducted in this area. In most cases, the use of plant growth promoting rhizobacteria, product development and its quantity and quality characteristics improves (Amal et al., 2010; Rahi, 2013; Ahmed et al., 2013). The benefits of inoculation of plants with growth stimulating bacteria include increasing the number of parameters such as the speed of germination, root growth, yield, shoot and root weight, leaf area, chlorophyll, biological control of pathogens, increased resistance to drought as well as microbial activity (Lucy et al., 2004). Behbood et al. (2011) demonstrated that the inoculation of potato *(Solanum tuberosum)* plant with growth stimulating bacteria increased tuber yield, dry matter and the number of tubers. Furthermore, they also suggested that

it increased macro and micronutrient contents in leaves and tubers of potatoes. Plant hormones (fito-hormones) are other important factors in plants' growth and development. These substances are active in very small amounts and will be produced in some parts of plant and moved to locations in which they create biochemical, physiological or morphological reactions. Normally, plant hormones are auxin, gibberellin, cytokinin, abscisic acid and ethylene (Moore, 1989). Some hormones and plants growth promoters are produced and purified industrially. In addition, industrial growth hormones can increase plant growth (Salisbery and Ross, 1992). Triacontanol is one of these compounds that was extracted and purified from alfalfa for the first time in 1977 and was identified as a plant growth stimulant (Ries et al., 1977 a, b). It is part of the vegetable waxes which is a plant growth factor; even nano-molar concentrations of this substance can increase plant growth and productivity (Ries et al., 1977 b). Moreover, it has been used to increase the production of millions of hectares of land in Asia, especially in China (Ries et al., 1984; Ries, 1991), but in a country like Iran, due to the complexity in formulation and purification of the compound, and also because of its high costs, its preparation and application is uncommon; but alfalfa extract can be used instead. Shikur (2012) reports are based on the beneficial effects of alfalfa extract on plant growth. Moreover, Ries et al. (1977a) stated that the placement of chopped alfalfa below seeds or seedling location increased dry weight of broccoli (*Brassica Oleraceae Italica*) seedling by 36% compared to the control. Doubling the amount of alfalfa extract increased the growth percentage by 49% compared to the control, too. They also stated that the application of 913 kg of chopped alfalfa per hectare enhanced the dry weight of tomato *(Solanum lycopersicum)* 131% than that of the control. Using chopped alfalfa increased the stem and root dry weight of corn to 55% and 66%, respectively. Ries *et al*. (1978) reported that 16-day-old rice *(Oryza Sativa)* seedling, treated with a solution of 1g/l crystals isolated from alfalfa, increased nearly 29% total dry weight compared to the control. Also, they stated that the amount of crystal extracted alfalfa increased the dry weight of barely *(Hordeum vulgare)* and corn *(Zea mays)* by 22% and 21%, respectively. Because alfalfa is rich in vitamins A, E, C, and K and contains considerable quantities of Triacontanol, protein and essential elements for the growth and development of plants (Chopra *et al*. 1986; Ries et al. 1977 b), this plant extract can be used as fertilizer and plant growth stimulant. With this strategy, farmers can be encouraged to reduce the use of chemical fertilizers and thus prevent environmental pollution. Despite extensive research in the field of plant nutrition, the effects of joint consumption of alfalfa extract and plant growth promoting microorganisms on quantitative and qualitative characteristics of forage sorghum have not been considered. Therefore, it seems that determining the effect of PGPR and alfalfa extract on the growth and nutrient contents of forage sorghum is necessary.

MATERIALS AND METHODS

Cultivation Medium

The soil used as the cultivation medium was from 0-15 cm depth of Research Farm University of Zabol. After being air-dried and passed through a 4-mm sieve, the soil was mixed completely. Then, 4 kg of this soil was added to each pot. A two-kg sample of this soil was sent to laboratory to determine its physical properties. After drying and passing it through a 2-mm sieve, soil texture was measured by hydrometer method (Day, 1982), the percentage of soil organic carbon by oxidation method (Nelson and Sommers, 1996), and pH in suspension 2:1 water and soil (Thomas, 1992), EC in extracts of suspension 2:1 water and soil (Rhoades, 1996) were measured (Table 1).

Also, soil chemical properties such as available phosphorus were measured by Oslen's extract-consuming method (Olsen and Sommers, 1982) with spectrophotometer (Model UV-2100) and available potassium was measured by extract-consuming of acetate ammonium method (Berry et al., 1946) with flame photometer. Finally, zinc, iron, copper and manganese contents were extracted by ammonium bicarbonate diethylene triamine penta acetic acid (AB-DTPA) and determined by atomic absorption spectroscopy (Havlin and Sultanpour, 1981). (Table 1).

Alfalfa Extract Preparation

Alfalfa extract was prepared as reported by Bahraminejad et al. (2008) and Sobin *et al.* (2011). (Table 2).

Table 1. Physiochemical properties of used soil

Cu	Mn	Zn	Fe	K	P	OC	EC	pH	silt	clay	sand	Soil texture
of available (mg kg^{-1})						%	(dS m^{-1})	-		%		
1.65	5.6	4.8	2.2	430	12	1.98	1.32	7.2	18	13	69	Sandy loam

Table 2. Some properties of alfalfa extract

pH	EC	P	K	Ca	Mg	Na	Fe	Mn	Zn	Cu	Ni	Co	Mo
-	Dsm^{-1}							$Mg1^{-1}$					
6.66	0.576	3.2	480	1.6	19.7	4.5	0.1	0.1	1.177	0.98	0.198	0.1	0.025

Experimental Design

Pot factorial experiment was conducted as a completely randomized design with six replications in soil cultivated medium. The first factor was non-inoculated (control) and inoculated medium with PGPR (compound of bio-fertilizers of Azoto Barvar 1, phosphate Barvar 2, made by Green Biotech Co.). The second factor for soil application of alfalfa extract included three concentrations (0, 2 and 4 per thousand).

Plant Cultivation and Treatments Application

Ten forage sorghum seeds (*Sorghum vulgare*) were planted in each pot. After over two weeks, experimental pots were thinned and three plants remained in each pot. Then, the combination of PGPR was added as a suspension with 200 ml water to pots' soil and alfalfa extract was applied a week after PGPR inoculation at three stages: in the first stage, the plant had 4 to 5 leaves and then with intervals of 15 days and concentrations of 0 (control), 2 and 4 per thousand (100 ml per pot), a day-night temperature of 33 ± 2 and 25 ± 2 ˚C, and 17 hours photoperiod were maintained in greenhouse to provide plant growth conditions. Also, soil moisture was kept at 70% by weight of field capacity (FC).

Plant Harvest and Parameters Measurements

After 75 days of cultivation, the plant shoot was cut from crown and washed with distilled water and dried for 72 h at 70˚c in the oven (Black et al., 1965); similar operations were done for roots. The dry weight was measured by sensitive microbalance (0.001 g). After the digestion of plant samples by dry-ashing method (Westerman, 1990), atomic absorption spectroscopy (UNICAM 919 AA model) was used to determine iron, zinc, manganese and copper contents. Colorimetric method (yellow molybdate metavanadate) was used to measure phosphorus with a wavelength of 470 nm using a spectrophotometer (Black, 1982) and flame emission method was used for Flam Emission Spectrometer to measure potassium (Tandom, 1995). Statistical analysis of data was performed by SAS software and mean comparisons were done by Duncan's Multiple Range Test and the level of significance was set at F < 0.05.

RESULTS AND DISCUSSION

Variance analysis of data (Table 3) shows that the main effect of PGPR (at a = 0.01) on dry weight, iron, manganese contents and also the main effect of PGPR (at a = 0.05) on copper and potassium contents were significant. However, the main effect of alfalfa extract is significant (at a = 0.01) on all elements contents at different levels.

Shoot iron content significantly increased in inoculated medium with PGPR only at the level of 0.002 alfalfa extract compared to zero (control), (Figure 1), while in the non-inoculated medium with PGPR, iron content showed a significant increase at two levels of 0.002 and 0.004 compared to zero (control). However, according to Fig. 1, iron concentration in the level of 0.004 alfalfa extract in inoculated medium with PGPR compared with non-inoculated medium with PGPR did not have a significant difference. Since alfalfa extract contained 0.1 mg of iron per litter (Table 2), increasing the extract content increased iron content. Plant growth stimulating bacteria promoted plant growth in various ways, such as nitrogen fixation, production of iron-complexing siderophores, production of plant hormones, and synthesis of antibiotics and fungicides compounds. Among these bacteria, *Pseudomonas putida, Azetobacter,* and *Pantoea agglomrans* have attracted more attention for their symbiotic relationship ability with important crop plants such as wheat (*Triticum aestivum*) , maize (*zea mays*), and sorghum (Mishra et al., 1998; Zaied et al., 2003; Mir et al., 2015).

Shoot zinc content increased with increased alfalfa extract concentration in both inoculated and non-inoculated mediums with PGPR (Fig. 2). Alfalfa extract contained 1.177 mg of zinc per litter; thus, increasing the alfalfa extract increased the zinc concentration of sorghum shoots (Table 2).

The highest and lowest manganese contents were achieved in the 0.004 levels and control of alfalfa extracts, respectively, which showed no significant difference with 0.002 and 0.004 alfalfa extracts (Table 4). Manganese concentration content increased in the presence of *Pantoea Agglomerans* microorganism in tomato (*Lycopersicon esculentum* L.), cucumber (*Cucumis sativus* L.) and cauliflower (*Brassica oleracea* L. Var. Botrytis) (Dursun et al., 2010; Ekinci et al., 2014).

Table 3. Analysis of variance of PGPR and alfalfa extract effects on dry weight macro and micronutrients contents of sorghum shoot

Changes resources	Mean square							
	Contents							
	df	Fe	Zn	Mn	Cu	K	p	Dry
PGPR	1	613.205**	2.131^{ns}	309.433**	9.004*	0.011*	0.000 ^{ns}	28.408**
extract	2	2068.263**	512.546**	258.557**	2.280 **	0.045**	0.015**	342.436**
PGPR× extract	2	615.474 **	173.989**	105.366 ^{ns}	1.784 ^{ns}	0.004 ^{ns}	0.002 ^{ns}	8.533**
error	30	73.968	28.193	36.913	0.759	0.002	0.000	1.204
CV		11.06	25.26	11.46	13.96	6.53	11.80	6.070

^{ns} , **, * indicate non-significant and significant at (P≤0.01) and (P≤0.05), respectively

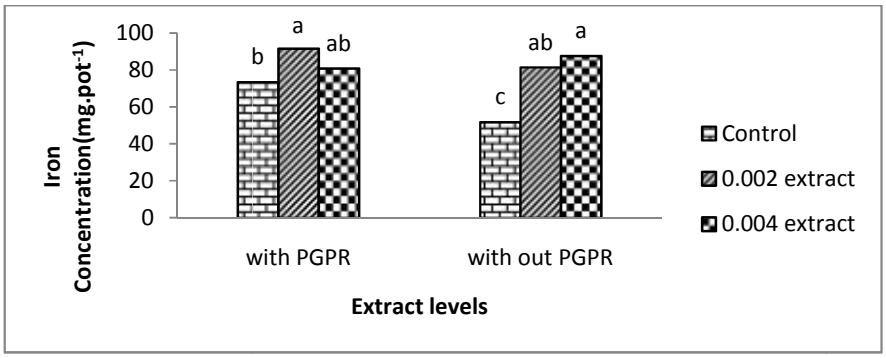

Fig. 1. Effect of soil inoculation with PGPR and different levels of alfalfa extract on iron contents of sorghum shoot

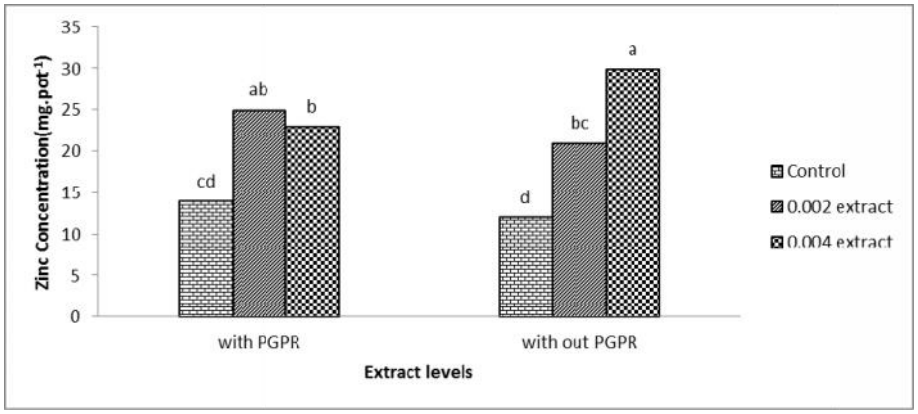

Fig. 2. Effect of soil inoculation with PGPR and different levels of alfalfa extract on zinc concentration of sorghum shoot

The highest and lowest manganese contents were achieved in the 0.004 levels and control of alfalfa extracts, respectively, which showed no significant difference with 0.002 and 0.004 alfalfa extracts (Table 4). Manganese concentration content increased in the presence of *Pantoea Agglomerans* microorganism in tomato (*Lycopersicon esculentum* L.), cucumber (*Cucumis sativus* L.) and cauliflower (*Brassica oleracea* L. Var. Botrytis) (Dursun et al., 2010; Ekinci et al., 2014).

Table 4. The Effect of alfalfa extract on Mn, Cu, K and P contents of sorghum shoot

levels of alfalfa extract	Content(mg.kg^{-1})			
	Mn	Cu	K	P
0	48.2^{b}	5.80^{b}	70^{c}	18^{c}
0.002	52.10^{a}	6.62^{a}	75^{b}	23^{b}
0.004	56.00^{a}	6.20^{ab}	82^{a}	25^{a}

The same letters are not significant at the 5% level.

Shoot manganese contents of inoculated medium with PGPR compared to the not-inoculated medium with PGPR significantly increased (Table 5). Since *Pantoea Agglomerans* were used in growth stimulating microorganism mixture, the increased concentration of manganese in shoots treatments of inoculation with PGPR is reasonable. Karakurt and Aslantas (2010) stated that growth stimulating bacteria can increase ion uptake in plants; it seems that the production of plant hormones as well as the effect of bacteria on the growth and development of the root system are two factors increasing the ion uptake.

Table 5. The effect of inoculating soil with PGPR on Mn, Cu, K and P contents of sorghum shoot

Medium	Content(mg.kg^{-1})		
	Mn	Cu	K
inoculating soil with PGPR	55.50^{a}	6.70^{a}	78.5^{a}
Non inoculating soil with PGPR	50.00^{b}	5.70^{b}	74^{b}

The same letters are not significant at the 5% level

Copper content in 0.002 level of alfalfa extract has shown a 15% increase compared to the control, with no significant difference with 0.004 of alfalfa extracts (Table 4). Copper concentration increased 17% in the inoculated medium with PGPR compared to the non-inoculated (Table 5). Increased concentration of the extract of alfalfa significantly increased potassium contents in plant shoots and the highest content was found in extracts of alfalfa with 0.004 concentrations (Table 4). Moreover, potassium concentration in plant shoots (Table 4) increased 5.8 and 18.5% compared to the control, at levels of 0.002 and 0.004, respectively.

Since alfalfa extract contained 480 mg potassium per litter (Table 2), potassium concentration in sorghum shoots increased by increasing the concentration of alfalfa extract. The potassium concentration increased 6 % in the inoculated medium with PGPR compared to non-inoculated (Table 5). Increasing alfalfa extract concentration significantly increased phosphorus concentration in shoot, while there were 27% and 38% increase in the phosphorus concentration compared to the control at levels of 0.002 and 0.004, respectively. Naiman et al. (2009) showed that some strains of the genus *Pseudomonas bacteria* are capable of producing soluble organic phosphorus and cytokinin.

In non-inoculated and inoculated mediums with PGPR, increasing alfalfa extract concentration increased dry weight of sorghum shoot. In non-inoculated medium, level of 0.004 significantly increased dry weight compared with 0.00 and 0.002 of alfalfa extracts with no significant difference between them. At level of 0.002 alfalfa extract, inoculation of soil with PGPR significantly decreased dry weight of shoot (Fig. 3).

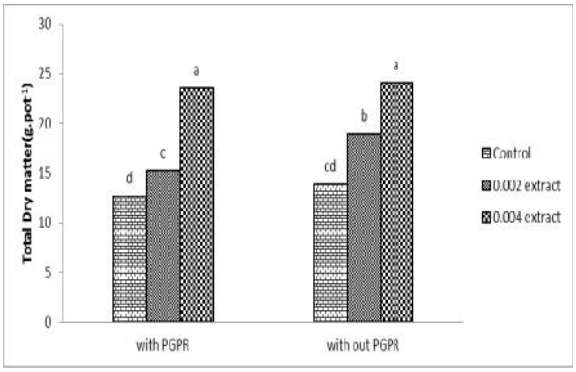

Fig. 3. Effect of soil inoculation with PGPR and different levels of alfalfa extract on dry weight of sorghum shoot

Research indicated that bio-fertilizers application, such as Azoto Barvar 1 and Phosphate Barvar 2, increased the quantity and quality of agricultural products such as grain sorghum (*Sorghum bicolor* L.), corn (*Zea mays L*.) and rapeseed (*Brassica napus* L.) in most cases (Khalili et al., 2008; Madani et al., 2011; Mohammadi *et al*., 2012). Mir *et al*. (2015) reported that any bio-fertilizers application (Azoto Barvar 1 and Phosphate Barvar 2) increased chlorophyll (a, b), carotenoid and leaf carbohydrates of sorghum. They also stated that the combined application of bio-fertilizers significantly increased chlorophyll contents compared to the single application of any fertilizer. It should be noted that bio-fertilizers always have no positive effects on quantity and quality properties of plants and it is likely to have negative and inert effect in some conditions and this depends on biological nature and different behaviors of microorganisms at different conditions. Nazari *et al*. (2008) confirm this subject about the application of bio-fertilizers (phosphate Barvar 2). Their results showed the highest flower dry weight and the number of flowers per plant grown in soil amended with PVC. Also they showed that the impact of PVC is higher than that of bio-fertilizers.

Alfalfa extract contains considerable amounts of essential and beneficial nutrients for plants' growth and development. Moreover, this plant extract includes a growth stimulant substance called triacontanol (Ries, 1977b), which can increase crop yield, such as asparagus *(Asparagus officinalis)*, dry beans (*phaseolus vulgaris L*), lettuce (*lactuca sativa*), onions (*Allium cepa*), tomatoes (*Solanum lycopersicum*) (Biembaum et al., 1988), carrot (*Daucus carota*), cucumber (*Cucumis sativus*), corn (*Zea mays)*, melon *(Momordica charantia*), radish (*Raphanus sativus*), rice (*Oryza sativa*), soybeans (*GIycins max*) and sweet corn (*Zea mays*) (Maugh, 1981; Mahadevappa et al., 1989; Prasad and Prasad, 1991). In addition, triacontanol increased various parameters related to growth, such as dry weight, leaf surface, and root and stem elongation, leaf density, wet and dry biomass (Ericksen *et al*., 1981; Muthuchelian *et al*., 2003). Ries et al.'s findings (1977b) indicated that triacontanol promoted tobacco (*Nicutina tabacum*) callus growth at low concentration (0.001 µg\dish). They also reported that tissue growth developments in callus, tomato, potato, barely and beans are reactions to triacontanol high concentration. Plant growth response is very fast to triacontanol treatment so that 3-6 hours after treatments, it can be seen that the fresh weight of plant increases (Ries & Wert, 1977; Bittenbender *et al*., 1978). Therefore, increasing dry weight of sorghum shoot seems logical by increasing alfalfa extract concentration.

CONCLUSIONS

The results of this study showed that alfalfa extract sparing can increase both the dry weight and macro and micronutrient contents of sorghum, mainly due to the presence of significant amounts of essential and beneficial nutrients for plant growth, and likely growth stimulants such as Triacontanol, which can increase dry weight and macro and micronutrient contents such as P, K, Fe, Zn, Mn and Cu. Furthermore, the application of PGPR increased manganese, copper, potassium contents as well as dry weight of forage sorghum. Overall, alfalfa extract at level of 0.004 with soil inoculation by PGPR was the best treatment for increasing dry weight and macro and micronutrient contents of forage sorghum shoot. The results of this study can be useful for enhancing crop production. However, further research is required in this area because especially high concentrations of alfalfa extract may have interactions with other beneficial microorganisms at field conditions.

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تأثیر میکروارگانیسم‌های محرک رشد و عصاره یونجه بر مقادیر عناصر پر و کم‌مصرف در سورگوم علوفه‌ای

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چکیده– افزایش روزافزون جمعیت و عدم توانایی مراتع دربرآورد نیاز غذایی دام‌ها موجب شده است که به کشت گیاهان علوفه‌ای بیش از پیش توجه گردد. به منظور بررسی تأثیر میکروارگانیسم‌های محرک رشد (PGPR) و عصاره یونجه بر رشد و مقادیر عناصر پر و کم‌مصرف در سورگوم علوفه‌ای، آزمایشی گلخانه‌ای به صورت فاکتوریل در قالب طرح کاملاً تصادفی و شش تکرار در دانشگاه زابل انجام شد. نخستین فاکتور شامل ۲ سطح بستر خاک عدم تلقیح با PGPR و تلقیح شده با PGPR و دومین فاکتور شامل سه سطح صفر، ۲ و ۴ در هزار عصاره یونجه بودند. نتایج نشان داد که با افزایش غلظت عصاره یونجه، وزن خشک کل گیاه و به علاوه مقادیر عناصر کم و پرمصرف در سورگوم افزایش یافت. بیشترین و کمترین غلظت منگنز به ترتیب در سطح ۰/۰۰۴ و شاهد عصاره یونجه بدست آمد. غلظت مس در سطح ۰/۰۰۲ عصاره یونجه ۱۵ درصد افزایش نسبت به سطح شاهد نشان داد ولی نسبت به سطح ۰/۰۰۴ عصاره یونجه تفاوت معناداری نداشت. همچنین، اضافه کردن PGPR به بستر رشد منجر به افزایش قابل توجهی در وزن خشک گیاه و به علاوه غلظت آهن، منگنز، مس و پتاسیم شد. در نهایت، تفاوت معنی داری در مقادیر آهن در سطح ۴ در هزار عصاره یونجه در بستر تلقیح شده با PGPR در مقایسه با بستر عدم تلقیح وجود نداشت. سرانجام توصیه می‌گردد تأثیر عصاره یونجه بر عملکرد و غلظت عناصر غذایی در دیگر گیاهان نیز بررسی شود.