Effect of Microbial Biostimulators on Growth and Diosgenin Content of Fenugreek Plant

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Abstract

Fenugreek (*Trigonella foenum-graecum* L.) can reduce the risk of diseases such as cancer, diabetes, obesity, high blood pressure, harmful blood fats, and vascular inflammation by producing secondary metabolites such as saponins such as diosgenin. Diosgenin is produced in the seeds, seedlings, and shoots of fenugreek. Because this compound is a secondary metabolite, it requires biological or non-biological stimuli. The main purpose of this study is whether the rhizobacteria and mycorrhizobial fungi consortium as biostimulators are more effective in improving fenugreek growth indices and diosgenin or when they are used separately. Experimental treatments were rhizobacteria (*Azotobacter chroococcum*, *Sinorhizobium meliloti*, mixture of both the rhizobacteria and control) and mycorhizobial fungi (*Glomus intraradices*, *Glomus versiforme,* and control), and the treatments also arrangement in a factorial experiment (4×3). The treatments' effects were investigated in a randomized complete block design with four replications. The main results showed that using the rhizobacteria and *Glomus versiforme* consortium in the rhizosphere of fenugreek significantly increased diosgenin content. The use of a mixture of two species of rhizobia improved the absorption of minerals and also increased the total chlorophyll and vegetative components of the fenugreek plant.

Keywords: Biostimulants, Diosgenin, Fenugreek, Mycorrhizobial fungi; Rhizobacteria

Introduction

Fenugreek (*Trigonella foenum-graecum* L.), a member of Fabaceae with food and medicine applications, has extensively attracted global attention. It is an herbaceous annual plant, indigenous to the Mediterranean and Asian Southwest Zones. It is also cultivated in Europe and Australia. The leaves and seeds are largely utilized in powders and extractions. An overriding characteristic of this plant is the variety of secondary metabolites like diosgenin with pharmaceutical applications, Acharya et al. (2006). The importance and economic performance of the fenugreek plant are related to the amount of diosgenin produced

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in the leaves, roots, seeds, and seedlings. In the past, fenugreek was used for wound healing, stimulating milk production, treating arthritis, cardiac problems, spleen and liver enlargement, acute kidney diseases, and anorexia, Yadav and Baquer (2014); Zameer et al. (2018). Other important compounds such as choline, protein, minerals, and vitamins are also found in fenugreek, which makes it a valuable food plant. Therefore, it is a well-known medicinal plant that, with the production of diosgenin compound, is a cheap and affordable drug source for the pharmaceutical industry.

Increasing the growth rate and producing foliage and roots of fenugreek is one way to enhance the production of diosgenin. Besides, fenugreek as a member of the legumes family has symbiosis ability with different microorganisms like rhizobia and mycorrhiza species. This means that using microorganisms as biostimulators in the zone of soil surrounding the plant roots could promote the ability of the performance of roots to produce foliage of plants. A plant stimulant is any substance or microorganism that is used on plants to increase nutritional efficiency, tolerating abiotic stress or quality traits of the product, regardless of its nutrient content, Traon et al. (2014). However, the success of symbiotics between roots and microorganisms is affected by the physical and chemical properties of soil and agronomic practices. It has recently been reported that soil productivity is declining due to factors such as soil erosion, soil salinity, high grazing, wildfires in grasslands and shrublands, soil contamination, climate change, and drought. (Gregory et al., 2015; Stavi, 2019). Consequently, the necessity of having nutrient-rich soil and the desired physical and chemical properties of the soil to achieve a qualitative and quantitative economical yield and also sustainable yield is an important and undeniable subject.

One of the most important soil enrichment factors is the presence of active microorganisms in the soil, as root growth region microflora plays a key role in plant access to nutrients and stress resistance (Jacoby et al., 2017). The past results suggested that the use of biostimulators such as different rhizobacteria and mycorrhizobial fungi promotes the positive properties of soil and nutrition uptake efficiency of plant roots, and the microbial consortium also indicated that the combination of mycorrhiza and bacteria in the roots growth region of plants is one of the most beneficial interactions in soil ecosystems, the positive effects of which have been confirmed on nutrient uptake, growth and development and physiological yield of various plants (Bargaz et al., 2018; Kafle et al., 2018).

The use of bacteria and fungi instead of chemical fertilizers is an accepted and reliable tactic in agricultural production. However, to fully disclose the benefits of symbiosis and prove the ability of bacteria and fungi to be an efficient alternative to chemical fertilizers, the plant must have sufficient access to active species of bacteria or fungi from the early stages of development. Because, this leads the symbiosis processes to productively provide the plant with different requirements such as nitrogen, besides expanding the contact area of roots and optimizing the physio-

chemical characteristics of the surrounding-root soil to the benefit of the plant. Previous results showed that the positive interaction of bacteria and arbuscular mycorrhizal fungi could lead to the plant`s development (Pérez-Montaño et al., 2014). Also, regarding these constructive interactions, it has been found that some bacteria like rhizobium directly impact growth and fungi buddying which in turn, increases nutrition uptake and roots along with inhibiting plant pathogens.

Mycorrhizal fungi positively impact the uptake of minerals and growth in plants, Ingraffia et al. (2019). In addition, previous studies have shown that the VAM fungal community influences bacterial composition. Mycorrhizal fungi are significant boosters for poor and low-return soils which essentially have low organic matter with inaccessible elements to roots (Diagne et al., 2020). Limited microbial community and weak moisture retention are other characteristics of such soils which could all be partially removed by adding arbuscular mycorrhizal fungi. These fungi could co-inhabit numerous well-defined plant species and stimulate their host growth. In detail, this group of fungi facilitates connections between roots and soil nutrient sources. Particularly in inappropriate conditions, they are beneficial to develop root spatial distribution and help in providing the plant with inaccessible elements (Bhat et al., 2017). Also, arbuscular mycorrhizal fungi make hyphae bind to root cells and increase their contact area which in turn assists the plant to exploit the most nutritious elements existing in soil. This has been indicated as an environmental and economic advantage of these microorganisms compared to chemical-boosting fertilizers.

Rhizobium is a key member of the soil microbial community, with a common global application as a biofertilizer for the cultivation of grain and forage legumes (Teng et al., 2015). *Sinorhizobium meliloti* as a symbiotic-nitrogen-fixing rhizobia is a Gram-negative rhizosphere bacterium. The *Sinorhizobium meliloti* is one of the most potent bacterial species with high resistance against environmental tensions such as heavy metals, soil salinity, and harsh physico-chemical soil conditions and assists plant growth through an effective symbiosis. *Sinorhizobium meliloti* could secret chemical substances that either buffer or alleviate unfavorable environmental conditions and lead to stimulating plant flourishing (Artursson et al., 2006).

The *Azotobacter chroococcum* as a non-symbiotic bacteria and free-living N_2-fixer is an important *Azotobacter* species characterized by cysts and water-insoluble brown-black pigments. This microorganism is compatible with soil ecological properties in Iran. It could help plants with the adsorption of essential elements including nitrogen, phosphorus, and potassium. *Azotobacter chroococcum* has shown noticeable potential resistance to unsuitable environmental conditions through employing producing siderophores in Iron deficiency, releasing growth stimulating hormones e.g. auxin, and amino acids such as arginine, histidine, lysine, tryptophan, and secreting chemicals like ammonium and consumption of vanadium in absence of molybdenum for nitrogen fixation

Bageshwar et al., (2017). All the mentioned measures support bacterial growth in water, soil, or on the surface of leaves and a wide range of pH.

Despite the super beneficial properties of rhizobia and arbuscular mycorrhizal fungi, relatively limited knowledge about their interactions and the impact on fenugreek plant growth and produced diosgenin is available. Hence, in the present study, the effects of *Azotobacter chroococcum*, *Sinorhizobium meliloti*, *Glomus intraradices*, and *Glomus versiforme* symbiosis as biostimulators on some growth parameters and diosgenin content of fenugreek plant were examined.

Material and methods

Preparation of the microorganisms

The strain of *Sinorhizobium meliloti* from Iran Scientific and Industrial Research Organization, and the strain of *Azotobacter chroococcum* from the National Center for Genetic and Biological Resources of Iran, and also mycorrhizal fungi *Glomus intraradices* and *Glomus versiforme* were prepared as mixed spores in sand from Turan Biotech Company. Each bacterium was kept in its specific solid medium (Table 1) until the inoculation solution was prepared. Then, to prepare the inoculum, the bacteria were inoculated directly into their fluid culture medium, embedded in an Erlenmeyer flask, and kept in a shaker incubator (32 °C - 110 rpm). Later, the growth curves of both bacteria were plotted by spectrophotometric method, and in the 600 nm spectrum with 0.65 to 0.6 optical density (OD), the appropriate inoculation time was determined. Then 2 mL of each bacterial solution was added to the seed medium. Also, spore-containing sand (3 g) of each type of mycorrhiza was added to the pots.

Preparation of the fenugreek seeds

Seeds of fenugreek were obtained from the Institute of Medicinal Plants of Shahid Beheshti University. To sterilize the seeds, they were immersed in 70% alcohol (v/v) for 2 minutes and rinsed with sterile distilled water for 5 minutes. Then they were placed in 1% sodium hypochlorite solution (v/v) for 5 minutes and washed in sterile distilled water for 3, 5, 10, and 15 minutes at the end.

Table 1. The ingredients of bacterial and fungal culture media

Solid culture media *Azotobacter* (g)		Liquid culture media *Azotobacter* (g)		Solid culture media *Rhizobium*		Liquid culture media *Rhizobium*	
Mannitol	10	Glucose	20	Mannitol	10	Yeast extract	10
K_2HPO_4	0.2	K_2HPO_4	0.8	K_2HPO_4	0.5	K_2HPO_4	0.5
$MgSO_4.7H_2O$	0.2	$MgSO_4.7H_2O$	0.5	$MgSO_4.7H_2O$	0.2	$MgSO_4.7H_2O$	0.2
$CaSO_4$	0.1	$FeCl_3.6H_2O$	0.1	Yeast extract	1 g	$FeCl_3.6H_2O$	0.002
NaCl	0.2	$CaCl_2.2H_2O$	0.05	NaCl	0.1	NaCl	0.2
$CaCO_3$	5	$Na_2MoO_4.2H_2O$	0.05	Agar	20		
Agar	20						
pH= 7.8 – 8.0		pH= 7.4 – 7.6		pH= 7		pH= 7.2	

Table 2. Physio-chemical parameters of the soil

Soil Texture	$CaCO_3$ (%)	Na (mg/kg)	K (mg/kg)	P (mg/kg)	N (mg/kg)	EC (µSiemens/cm)	pH	Organic matter
Sandy-Loam	4.5 ± 0.1	42 ± 5	340 ± 10	13 ± 2	0.15 ± 0.02	151.3	7.6	4 ± 0.5

Seeds were seeded in sterile glass containers containing filter paper and 3 mL of distilled water for initial germination and kept at 20 °C for 36 hours.

Preparation of seedbed (medium) and planting

To prepare the medium, first, the chemical and physical properties of the soil used were determined, and the properties are shown in Table 2. The soil was sifted with a 2 mm sieve and then autoclaved at 121 °C for 15 min at 25 °C for 3 min, to reduce the possible interactions of the probable microorganisms in the soil and to reduce as far as possible the microorganism population.

The sterilized soil was weighed (each pot 2 kg) and then poured into pots (9× 5×7 cm). Two seeds were planted in each pot. The pots were maintained in greenhouse conditions with temperatures of 18-22, relative humidity of 40-55%, 6–8 hours of darkness, 18–16 hours of light, and irrigation approximately once every 4-6 days for two months.

Measurement of leaf area

The leaf area was measured according to the software image J (Version K. 1.45). At the end of the growth period, the leaves were removed from the stem and later the leaf images were scanned, then measured by the software.

Determination of the total Chlorophyll content

The total chlorophyll content was determined using the modified method according to Lichtenthaler (1987). 0.2 g of leaves from each treatment was powdered with liquid nitrogen, then re-suspended in acetone 80% (v/v). To optimize the extraction process, the mixture received 1 min vortex and then centrifuged at 5000 rpm for 5 min. The supernatant was transferred to 10 ml volumetric balloons and raised to 10 ml by adding acetone 80% (v/v). Then, the absorbance of the solutions was read by spectrophotometry at 645 and 663 nm, and the pigment content was calculated in milligrams per gram by the following formulas:

Chl a=[12.3×(D663)-0.86×(D645)]×V/(1000×W)

Chl b=[19.3×(D645)-3.6×(D663)]×V/(1000×W)

Chl T=Chl a+Chl b

Determination of diosgenin

The diosgenin was determined according to the modified method recommended by Chaudhary et al. (2018). From each experimental treatment, 30 mg of the dried shoot was weighed and powdered by Tissue Lyser LT Adapter (QIAGEN) and then poured into 2 ml vials. 0.5 ml of 2N HCl was added to each sample (vial) and placed in a shaker at 90-80 °C for 4 hours. The pH of the samples was neutralized with 2N NaOH. Next, three times 1 ml of n-hexane was added to each of the samples, and later the samples were centrifuged at 4500 rpm for 2 minutes. Then, the 3 ml of the upper phase of each sample was detached and then

dried, and later 0.5 ml of HPLC methanol was added to it and finally injected into the HPLC (Shimadzu, Japan). The HPLC analysis details included: two high-pressure solvent delivery pumps, SPD-M20A photodiode array detector (PAD), SIL-20AC autosampler, CTO-10AS column oven, and CBM-20Alite system controller, Hypersil ODS C18, 5 µm, 250×4.6 mm (Thermo Scientific), and also flow rate was 1 mL per min. Acetonitrile: water (90:10) was used as a mobile phase as well as a diluent for preparing the sample extract. Likewise, the wavelength used for detection was 194 nm with a UV detector.

Preparation of samples and dry material of plant

First, the harvested plant was separated into separate parts (root, stem, leaves, and pods) and then the samples were dehydrated in an oven with forced air at a temperature of 65 degrees Celsius for 72 hours. Then, the samples were cooled to room temperature in a desiccator before weighing. Then, the dried plant samples were weighed and the masses were recorded (grams). Finally, the dry matter of shoots obtained was used to determine the amount of mineral elements.

Determination of phosphorus

The phosphorus was determined according to the method recommended by Soratto et al. (2019) with modification. The foliar dry matter (5 g) was weighed from each treatment. The samples were transformed to ash at 650 °C in an electric kiln. 1.5 ml of 2 M nitric acid was added to 1 g of the ash for the acidic digestion process. The mixture was then filtered using Whatman paper No. 1, and later, the filtered solution was brought to 100 ml with distilled water. 2.5 ml of 2 M nitric acid was added to 25 ml of the solution, and then its pH was adjusted to 1.5-2. Later, 15 ml of ammonium vanadate reagent was added to the solution, then the solution was brought to 50 ml with distilled water. Next, for plotting the standard curve, standard solutions of 5, 10, 20, 30, and 40 ppm of KH_2PO_4 were prepared and the absorbance of the solutions was read by a spectrophotometer at 430 nm and the final step, the absorbance of the samples was read at 430 nm by spectrophotometer, and the amount phosphorus in the samples was determined with the standard curve.

Determination of sodium and potassium

The sodium and potassium were determined according to the modified method of Ashraf et al. (2015). 5 grams of foliar dry matter was transformed to ash in an electrical kiln at 650 °C. In the next step, for each element, foliar ash (2 g) was taken for acidic digestion (nitric acid 2 M). The solution was filtered using Whatman paper No.1 and was brought to 50 ml. The flame photometer (Jenway PFP7) was first calibrated with different concentrations of potassium chloride (5, 10, 20, 30, and 40 ppm) and sodium chloride (5, 10, 20, 30, and 40 ppm). Then after, the measurement of potassium and sodium was carried out with the calibrated device.

Determination of Nitrogen

The nitrogen was determined using a modified Kjeldahl method according to ZHANG et al. (2019). The Kjeldahl method with 3 steps of digestion, distillation, and titration was deployed to measure nitrogen. dried plant (0.3 g) was ground and cleaned up using 2 mm-pore size sieves and further di-

gested with 2.5 ml sulfuric acid. In this step, nitrogen in the sample reacts with acid and converts to ammonium sulfate. Then after, the nitrogen in ammonium sulfate is released as ammoniac, and with the addition of boric acid in the distillation process, it changes to ammonium borate. Sulfuric acid 0.1 M is used for titration and finally, the total used acid counts for the nitrogen level are calculated in the formula below:

V: the total volume of the added acid

W: sample total weight

$$N\%=5\times0.0014\times V/(W\times100)$$

Statistical analysis

Experimental treatments were rhizobacteria (*Azotobacter chroococcum*, *Sinorhizobium meliloti*, mixture of both the rhizobacteria and control) and mycorhizobial fungi (*Glomus intraradices*, *Glomus versiforme*, and control), and the treatments also arrangement in a factorial experiment (4×3). The treatments' effects were investigated in a randomized complete block design with four replications. The experimental variables were diosgenin content, shoot, and root dry weight, pod weight, nitrogen, phosphorus, potassium, and sodium content in shoot dry matter, and total chlorophyll. Experimental data were first normalized by the Jarkio test; the data were then analyzed statistically by using SPSS software version 20. Comparisons of treatment averages were performed with the Least Significant Difference (LSD) test at 99% and 95% probability levels, and also the data were plotted using Excel Version 2016.

Results

The vegetative growth indices

Analysis of variance (ANOVA) was used to determine the effects of the rhizobium species and arbuscular mycorrhizal fungi on some of the vegetative growth indices of fenugreek (Table 3, Figure 1).

In addition, using the treatments of arbuscular mycorrhizal fungi in the fenugreek roots growth region significantly increased the root dry weight, the shoot dry weight, leaf area, and total chlorophyll of fenugreek compared to the control treatment (Figures 3-A, B, C, D, and E). Also, among the arbuscular mycorrhizal fungi, the *Glomus versiforme* had the most effect on the vegetative growth components (Figure 3-F).

Diosgenin content

The diosgenin content in the shoot was significantly affected by the rhizobium and the arbuscular mycorrhizal fungi (Table

Fig. 1. The final plants under the respective treatments

Table 3. Average variance analysis of experimental traits affected by fungi and bacteria

		Mean squares												
		Dry weight Gregory et al. (2015)								Diosgenin / µg g^{-1} shoot dry matter	Mineral elements/mg g^{-1} of dried shoot			
Source of variance	df	Root	Shoot	Leave	Pod	Height /cm	Number of leaves	Leaf area / mm^2	Total chlorophyll / mg g^{-1}		N	P	K	Na
Rhizobium	3	158.07	245.41*	97.57	232.4*	6.274	0.7	173435**	0.24*	5.13**	35.6**	23.84**	17.64	6.03*
Mycorrhiza	2	1377.8**	397.14**	188.4	367.7*	16.674	0.18	843701*	0.39**	3.64*	18.8*	169.71*	83.92*	11.34**
Rhizobium × Mycorrhiza	6	328.32	48.14	97.10	85.46	2.498	0.36	5391.06	0.41**	1.25**	6.3	0.69	13.35	3.25
Error	32	147.73	75.18	69.59	48.03	5.861	0.49	51234.55	0.07	1.04	8.72	1.65	10.74	1.53

*F test significant at $p < 0.05$; **F test significant at $p < 0.01$.

3). Application of *Sinorhizobium meliloti* and *Glomus versiforme* in the roots growth region of fenugreek increased significantly the amount of diosgenin in shoots compared to other treatments (Figures 4-A and B). In addition, *Glomus versiforme* in the presence of both bacteria increased significantly the produced diosgenin (Figure 4-C).

The mineral elements' content

The amount of mineral elements in the shoot of fenugreek was significantly different between the experimental treatments (Table 3). The amount of nitrogen, phosphorus, and sodium was affected by using rhizobium, and the maximum levels of nitrogen and phosphorus in the shoot were obtained in the presence of both rhizobium bacteria (Figures 5-A and B). Conversely, the minimum sodium level of the shoot was obtained in the presence of both bacteria (Figure 5-C). Also, the results showed that the amount of phosphorus, potassium, and sodium were affected by the arbuscular mycorrhizal fungi (Table 3). The maximum phosphorus and potassium levels of the shoot were obtained by using G*lomus versiforme* (Figure 5-D and E), and conversely, by using G*lomus versiforme*, the sodium level of the shoot significantly decreased (Figure 5-F).

Discussion

Based on the results obtained in this study, the use of different microbial consortiums could increase the vegetative components and diosgenin content of the fenugreek plant. The consortium of *Azotobacter chroococcum*, *Sinorhizobium meliloti,* and *Glomus versiforme* in the rhizosphere, significantly increased diosgenin content in the fenugreek shoots. The results also showed that the use of a combination of *Azotobacter chroococcum* and *Sinorhizobium meliloti* in the rhizosphere improves the growth of vegetative components and the amount of diosgenin content in the fenugreek plant better than when they were used separately. In addition, the results of this study showed that the simultaneous use of *Sinorhizobium meliloti* with the *Glomus versiforme* had the greatest effect on improving plant growth indices and diosgenin production in the fenugreek plant. Also, the use of *Glomus versiforme* in the root growth region of fenugreek had a greater effect on the experimental variables such as components of the vegetative growth and diosgenin content than the *Glomus intraradices*. There is ample evidence for the usefulness of the microbial consortium in the rhizosphere,

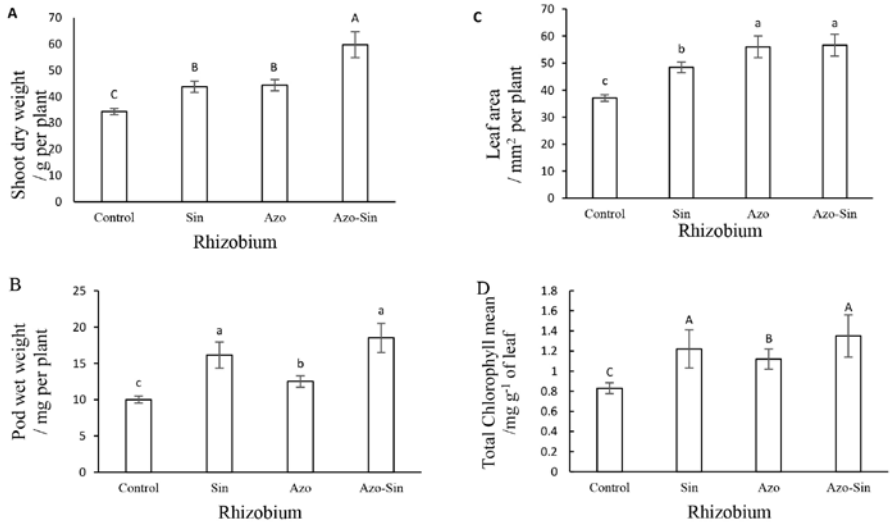

Fig. 2. Average of shoot dry weight, pod wet weight, and leaf area, chlorophyll total by rhizobium (control:non-rhizobium; Azo: *Azotobacter chroococcum*; Sin: *Sinorhizobium meliloti*; and Azo-Sin: a mixture of both bacteria), capital letters and lowercase letters denote a significant difference at $p < 0.05$ and $p < 0.01$, respectively, error bars denote standard error.

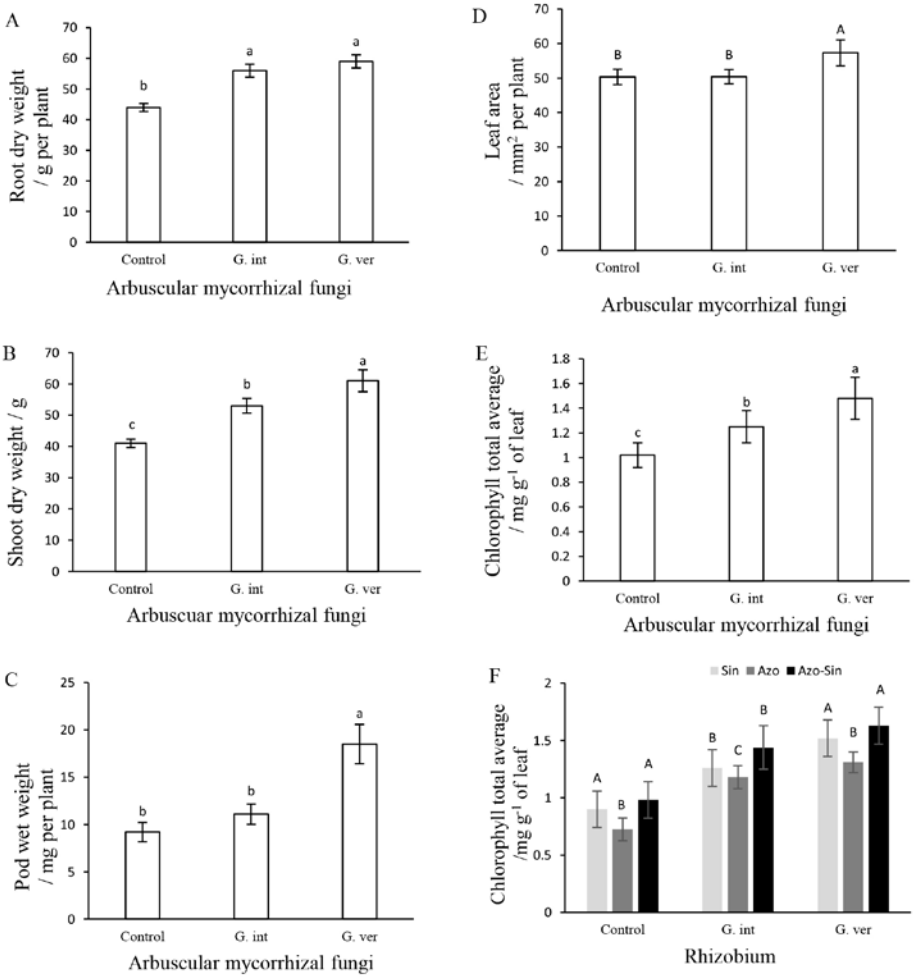

Fig. 3. Average of root dry weight, shoot dry weight, pod wet weight, and leaf area, chlorophyll total by arbuscular mycorrhizal fungi (control:non-mycorrhiza; G. int: G*lomus intraradices*; G. ver*:* G*lomus versiforme*), and an average of chlorophyll total by interaction effects between the rhizobium and the arbuscular mycorrhizobial fungi. Non-My: without mycorrhiza; G. int:

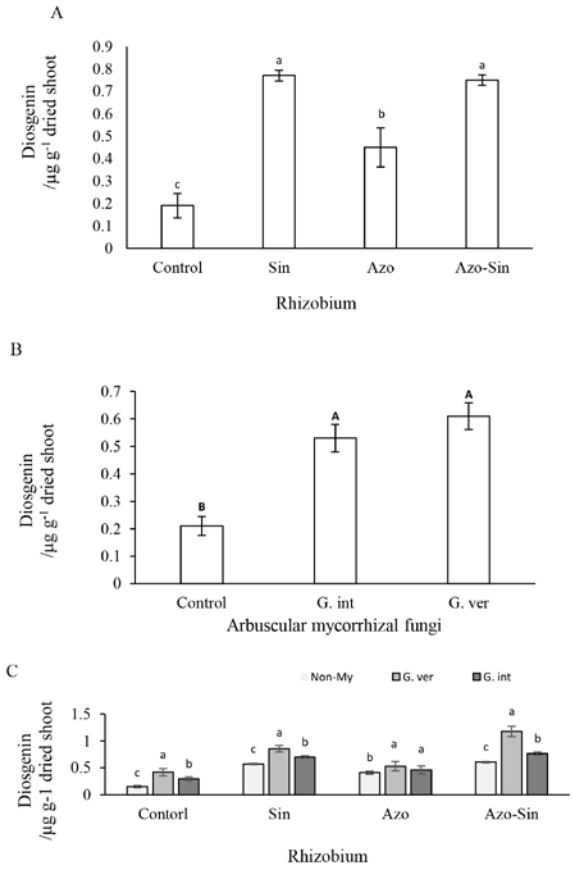

Fig. 4. Average of produced diosgenin in the shoot by the rhizobium, the arbuscular mycorrhizal fungi, and interaction effects between the rhizobium and the arbuscular mycorrhizobial fungi (Strissel et al.). Non-My: without mycorrhiza; G. int: G*lomus intraradices*; G. ver*:* G*lomus versiforme*; control:non-rhizobium; Azo: *Azotobacter chroococcum*; Sin: *Sinorhizobium meliloti*; and Azo-Sin: a mixture of both bacteria, capital letters and lowercase letters denote a significant difference at $p < 0.05$ and $p < 0.01$, respectively. Error bars denote standard error.

some of which will be discussed.

The most important benefits of the presence of rhizobium bacteria in the rhizosphere that have a positive effect on plant growth are reducing ethylene, phytohormones Production, exopolysaccharides production, induced systematic resistance, and siderophores production. Mycorrhiza improves plant growth indices through various mechanisms, including improved nutrition, enhanced antioxidant system, root structure modification, enzyme production, and water use efficiency. Therefore, the interactive effect between the biostimulators improves plant growth and development (Nadeem et al. (2014).

Mycelial networks of mycorrhizal fungi often bind plant root systems over large areas as bio-bridges. Past reports suggested that reason for the positive effects of rhizobium bacteria and mycorrhizal fungi on plant growth are due to the extension of root distribution in the soil, improvement of nutrient uptake by plant roots, and reduction

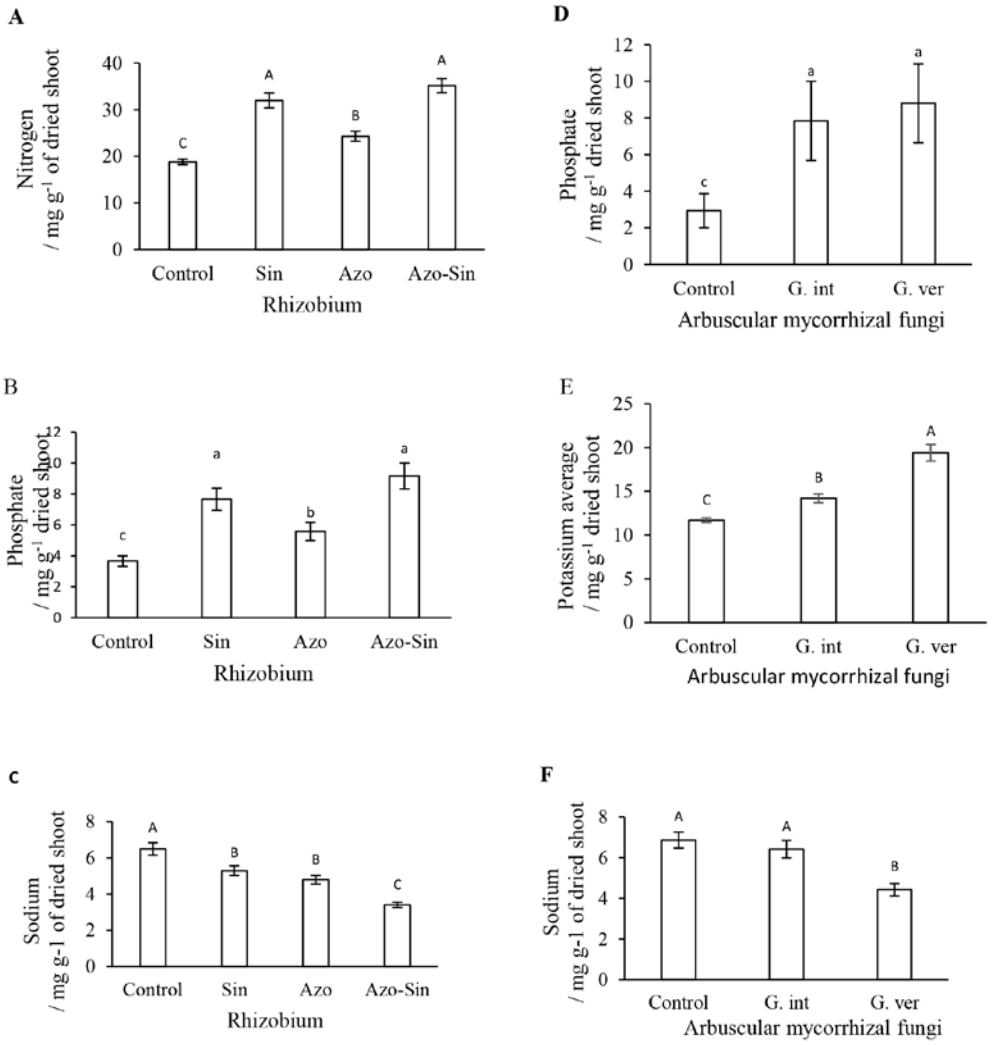

Fig. 5. nitrogen, phosphorus (B and D), potassium (Strissel et al.), sodium value (C and F) by the rhizobium (control:non-rhizobium; Azo: *Azotobacter chroococcum*; Sin: *Sinorhizobium meliloti*; and Azo-Sin: a mixture of both bacteria), and arbuscular mycorrhizal fungi (control:non-mycorrhiza; G. int: *Glomus intraradices*; G. ver*: Glomus versiforme*. Capital letters and lowercase letters denote a significant difference at $p < 0.05$ and $p < 0.01$, respectively, Error bars denote standard error.

of environmental stresses effect on plant growth. A consortium of rhizobacteria and mycorrhizal fungi as biostimulators frequently stimulate plants to promote root surfaces and proliferation in soil pores that are too small for root hairs to enter. No debate, that there are different interactions between biostimulators, and the synergistic and antagonistic response of the interactions depends upon the nature of all microbial strains involved in these interactions as well as plant species. Furthermore, recent reports suggested that biological interactions between stimulators such as rhizobacterial and mycorrhizal fungi had an increasing effect on all rhizosphere components, and these interactions could also be affected by environmental factors such as soil type,

nutrition, moisture, and temperature. This significant increase can be attributed to the positive interaction between symbiotic rhizobacteria and mycorrhizal fungi (Diagne et al., 2020).

On the other hand, Plant species are important in determining the structure of rhizosphere bacterial and fungal communities (Qiao et al., 2019). In other words, the success of microbial consortium in the rhizosphere depends on the relation between microbial communities and plant species, and hence, microbial consortium would be affected by plant genotype, plant nutrient status, and microbial infection. This means that considering the root secretions and the distribution structure of the root system, the physical and chemical properties of soil in the rhizosphere are the most important factors that will affect the efficiency of the microbial consortium in the rhizosphere.

According to the results of this study, using simultaneous of *Sinorhizobium meliloti* and *Glomus versiforme* in the rhizosphere, absorption of nitrogen, phosphorus, and potassium as macronutrients were significantly increased by the roots of fenugreek, and conversely, absorption of sodium was reduced. Hence, enhancement of absorption of the mineral nutrients resulted in promoting growth parameters of fenugreek such as components of vegetative growth like the root and shoot weight, and leaf area, and also inducing metabolism reactions and biosynthesis of macromolecules like chlorophylls. The population of microbes in the rhizosphere is not uniform, nor is the distribution of absorbable mineral elements in the rhizosphere uniform, so plant roots cannot utilize enough of the nutrients in the rhizosphere (Nihorimbere et al., 2011). Therefore, microorganisms such as bacteria and fungi can help the plant benefit from the rhizosphere environment and act as a bridge between the roots and far areas around the rhizosphere. Mycorrhizae produce many hyphae and occupy large amounts of soil. Mycorrhizae produce many hyphae and occupy large amounts of soil. The small diameter of fungal hyphae allows them to penetrate far beyond the nutrient depletion zone that forms around the roots. As a result, plant roots can absorb more minerals from the rhizosphere (Dellagi et al., 2020).

Thus based on the results of this study and previous research, an adequate supply of nutrients to the plant with the help of rhizobium bacteria and mycorrhiza, can improve plant growth, especially the production of effective and important molecular compounds such as enzymes, coenzymes, and metalloproteins like synthesis of chlorophyll (Du Jardin, 2015). Then, by increasing the production of photosynthetic materials, the quantitative and qualitative yield of the plant will increase. In other words, the advantages of a combination of biostimulators such as arbuscular mycorrhizal fungi, plant growth promoting rhizobial, and symbiotic-nitrogen-fixing rhizobia in the rhizosphere promote plant growth by improving the nutrient uptake of the plants, (Altuntaş and Kutsal, 2018). These biostimulators have the role of improving the nutrient status of fenugreek plant utilizing nitrogen fixation, increasing the availability of nutrients in

the rhizosphere, promoting the root surface area, or enhancing beneficial symbiosis of the fenugreek roots, and mostly, growth promotion is due to a combination of these action modes (Bargaz et al., 2018; Nathiya et al., 2020). Furthermore, rhizobacteria such as *Sinorhizobium* and *Bradyrhizobium* can create symbiosis forming nodules on the roots of leguminous plants, in which they convert N_2 into ammonia, which can be used by the plant as a nitrogen source (Kumar et al., 2020). The application of free-living rhizobacteria in the rhizosphere increases plant production due to an increase in root development, which allows better rates of water and mineral uptake.

On the other hand, with host plant assistance mycorrhizae produce hyphae in the rhizosphere, and the produced hyphae are attached to the plant root tissue, the surface area and distribution of plant roots then increase in the soil. Therefore, as a result of increasing the contact surface and distribution of the root structure in the soil will increase the association of bacteria with plant roots and result in strengthening the process of absorption of water and nutrients (Rodríguez-Caballero et al., 2017).

The combination of *Sinorhizobium meliloti* and *Glomus versiforme* as used biostimulators in the rhizosphere can promote the growth of fenugreek plant and diosgenin content. Using symbiotic nitrogen rhizobia bacteria was more effective than free-living rhizobia fixers on the experimental variables. The Association of *Glomus versiforme* with the root tissue was better than *Glomus intraradices* because in the presence of the *Glomus versiforme*, the roots of fenugreek could significantly grow and uptake the nutrients. The results of this study suggested that the consortium of *Sinorhizobium meliloti* and *Glomus versiforme* was most effective on the plant growth and diosgenin content of fenugreek. Also, the success of bio-stimulators in consortia depends on plant species, species of microbial, soil properties as well as their relations.

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