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Mixed inoculum of rhizobacteria and arbuscular mycorrhizal fungus enhance diosgenin contain and phosphorus uptake in fenugreek under drought stress

Simin Irankhah, Ali Ganjeali *, Mansour Mashreghi, Zahra Lari

Department of Biology, Faculty of Science, Ferdowsi University of Mashhad, Mashhad, Iran

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ABSTRACT

There is considerable evidence to increase diosgenin and other medicinally useful compounds production in fenugreek with microbial inoculations. Fenugreek (*Trigonella foenum-graecum* L.), as an annual medicinal plant, is extensively cultivated in most regions of the world. In the present study we evaluate the role of plant growth-promoting rhizobacteria (PGPR) inocula (*Pseudomonas putida* and *Bradyrhizobium japonicum*) alone or in combination with an arbuscular mycorrhizal fungus(AMF) (*Glomus intraradices*) on diosgenin content, some biochemical characteristics and phosphorus accumulation of fenugreek plants subjected to drought stress. ACC deaminase activity was detected in both bacteria and it was 2.1 fold higher in *B. japonicum* than the *P. putida.* Results showed that diosgenin content was commonly higher in leaves under non-stress vs drought stress condition. In non-stress, plants with dual inoculation (*G. intraradices* and *B. japonicum*) resulted in highest diosgenin content significantly different with other treatments. The leaf proline content was the highest in plant subjected to drought stress with dual inoculation (*G. intraradices and P. Putida*), whereas the highest total soluble proteins was belonged to plants with triple inoculation(*P. putida, B. japonicum and G. intraradices*). Bacterial and fungal inoculation decreased peroxidase activity both in stress and non-stress condition. The highest amount of phosphorous in the roots was assigned to single inoculation with *P. putida* in stress and with *G. intraradices* in non-stress condition. The present study provides a good insight on the effect of PGPR and AMF on diosgenin content as a major bioactive constituent.

1. Introduction

Fenugreek (*Trigonella foenum-graecum* L.), is a medicinal plant from the family of the Leguminacae (Hutchinson, 1964). This plant has good adaptability to diverse atmospheric conditions (Chaudhary et al., 2018) and cultivated over a broad geographic area in the world. In Iran, fenugreek is commonly consumed as leafy vegetables in food and a herbal medicine in the treatment of some diseases. The multiple properties of fenugreek are due to possessing chemical compounds including galactosamines, neotigogenine, diosgenin, tigogenin, terpenoids, trigonellin, coline, isolucine, flavonoids and diverse phenolic. Diosgenin is an important bioactive constituent that is found abundantly in Fenugreek. This constituent is considered to be the only important precursor for semi-synthetic steroidal compounds that are extensively used in the pharmaceutical industry (Raju and Rao, 2012). Previous studies suggested that the beneficial effects of diosgenin as a antidiabetic, hypocholesterolemic and anti-inflammatory (Khorshidian et al., 2016) and a strong anti-cancer agent (Lohvina et al., 2012).

Structurally, diosgenin is a spirostanol saponin with a hydrophilic sugar moiety attached to a hydrophobic steroid aglycone (Raju and Rao, 2012). Diosgenin is synthesized from cholesterol and 11 key enzymes are identified that control the pathway route to diosgenin biosynthesis and the expression of these genes is influenced by several biotic and abiotic factors (Vaidya et al., 2012).

Water scarcity is one of the major problems which adversely affects in crop production in many parts of Iran. Drought stress, is a multidimensional syndrome and induce changes in the, morpho-physiological and biochemical characteristics in plants including Fenugreek. These changes might be compensated by some microorganisms such as PGPRs and AMF. Previous studies have been showed these microorganisms could have various profound effects on these traits (Requene et al., 1997; Artusson et al., 2006; Paul and Arundhati, 2014).

* Corresponding author.
E-mail address: ganjeali@um.ac.ir (A. Ganjeali).

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Owning genetic and metabolic inherent abilities, microbes help plants to mitigate the unfavorable effects of environmental stresses (Gopalakrishnan et al., 2015). It has been shown that fenugreek can stablishes symbiotic relationship with various groups of microorganisms which enables the plants to grow in adversely conditions, such as drought stress (Irankhah et al., 2020). However, it is worth noting that, environmental stresses such as drought are used as a tool for elevating secondary metabolites production (Thakur et al., 2019). In this regard numerous studies have revealed that drought induced biochemical systems in plants which elevated the production of secondary metabolites (Zhao et al., 2005; yiola Oluwakem).

PGPRs in a symbiotic relationship can increase the plant growth through improving some mechanisms such as enhancing the availability of nutrients in the soil (for instance nitrogen fixation and phosphate solubilization), phytohormones production (Di Benedetto et al., 2017) and enhances nutrient uptake by inducing increases in root surface area (Paul and Arundhati, 2014; Di Benedetto et al., 2017). Furthermore, useful effects of some these microorganisms may be attributed to their interaction with AM fungi (Artusson et al., 2006).

The roots of most herbal and woody plants can make a symbiotic relationship with a diverse group of AM fungus. Previous studies have been demonstrated that co-inoculating PGPRs with AM fungi decreased the negative effects of drought stress. Some proposed considering the mechanisms of interaction between PGPRs with AM fungi and plant roots in rhizosphere, include i) bacteria directly affect the growth rate and germination of AM fungi (Carpenter-Boggs et al., 1995), ii) AM fungi can directly affect the plant physiology via increasing the permeability of root cells and improving the material exchange (Artusson et al., 2006), iii) bacteria make a specific symbiotic relationship with fungi to enhance the plant growth via improving the nutrients uptake, inhibiting plant pathogens and increasing the root branches (Han et al., 2005), iv) AM fungi influence the population combination of fungi and therefore change the chemical composition of root exudates which are a nutritive source for symbiotic bacteria (Artursson et al., 2005). Bacteria can tolerate drought stress via production of exopolysaccharides. Producing the filamentous compounds, exopolysaccharides accelerate the colonization of bacteria and their adhesion to the root (Bashan et al., 2004).

Bacteria and fungus can stimulates the synthesis of secondary metabolites in plants via a mechanism termed as induced systemic resistance. These mechanisms are related to plants' defense responses to biotic agents (Van Loon and Glick, 2004; Thakur et al., 2019). In this regard a close relationship have also been reported between plant defense response and secondary metabolites production (Owolabi et al., 2018; Thakur et al., 2019). Inoculation with AM fungi may mediate the biosynthesis of some compounds such as secondary metabolites (Toussaint et al., 2007) and improvement in nutritive behaviour, quantitive and qualitative function of phytohormones (Zubek et al., 2012). Despite several experiments on plant responses to individually applied AM fungus and PGPRs, relatively little information is available about combined effects of *B.japonicum* and *P.putida* inoculation in conjunction with AM fungi in fenugreek plant under drought stress. Therefore, the present study is aimed to investigate the effects of the sole or mixed inoculation of PGRBs with an AM fungus on diosgenin content, some biochemical characteristics and phosphorus accumulation of fenugreek plants subjected to drought stress.

2. Materials and methods

2.1. Plant and microorganisms

2.1.1. Bacteria preparation

Plant growth-promoting rhizobacteria (PGPBs) including *Pseudomonas putida* and *Bradyrhizobium japonicum* were respectively provided Science and Agriculture Faculties of Ferdowsi university of Mashhad. Each strain was sub-cultured in defined medium, and their growth curve was plotted in order to show their population changes over time. Thereafter, ACC deaminase activity was evaluated for each strain.

2.1.2. Arbuscular mycorrhiza preparation

The mycorrhizal fungi used were *Glomus intraradices*. The AM fungal inoculum consisted of a mixture of spores, external mycelium and *Zea mays* mycorrhizal roots, provided by the Organic Plant Health Company (Hamedan, Iran). The original isolate was sub-cultured and propagated by the above Iranian company using maize as host plants. The sources of inoculum had a potential infectivity of more than 900 infective propagules per cm^3 medium.

2.1.3. Seeds sowing

The fungreek seeds were sterilized with 5% sodium hypochlorite followed by germination test. In this study, each experimental unit was a plastic pot filled with 2 kg of garden soil-sand mixture at 1:1 ratio. The growth curve of each bacteria was plotted for 24 h to locate logarithmic phase and determine the most appropriate time in which bacteria is in their highest growth condition. Then bacterial inoculum was prepared at 0.5 McFarland standard density to be used in each treatment.

The seeds were soaked (preserved) in sugar solution further displaced in culture medium and finally planted in the pots. Fungi inoculation was performed by addition of powder containing fungi spores (50 g/kg of soil) to the soil. For co-inoculation treatment, the seeds were planted in the pots containing fungal powder and bacterial inoculum. All plants were grown inside phytotron at 25 °C with a daily regime of 16 h white fluorescent light/8 h of darkness.

Two moisture regimes, including 40% field capacity (FC) as drought stress (S) and 80% FC as the control (NS) were used. The seedlings were subjected to drought stress 14 days after emergence. Moisture levels were kept constant at the prescribed levels during the experiment. Plants with 50 days old were harvested for measuring some morphophysiological and biochemical characteristics.

2.2. Experimental design and treatments

The experiment was conducted as factorial based on completely randomized design with 2 factors:

1) plants inoculated with sole PGPBs (*Pseudomonas putida* (P), *Bradyrhizobium japonicum* (B)) or mixed (PB) with or without arbuscular mycorrhizal fungus (*Glomus intraradices*) (F, PF, BF, PBF), and non-inoculated plants (C); 2) non stress (NS) and S, drought stress). Therefore, in this experiment, the following treatments were used:

Non-inoculated plants with and without drought stress (CS, CNS), plants inoculated with *P. putida*, *B. japonicum*, and *G. intraradices* with and without drought stress (PS, PNS; BS, BNS; FS, FNS), under stressed dual inoculation of plants with *P. putida* and *G. intraradices* (PFS), *P. putida* and *B. japonicum* (PBS), *B. japonicum* and *G. intraradices* (BFS), and their without stress counterparts (PFNS, PBNS, BFNS), triple inoculation of plants with *G. intraradices*, *B. japonicum* and *P. putida* with and without stress (Mix S, Mix NS). Three replicates per treatment were used, requiring a total of 8 X 2 X 3 = 48 experimental units.

2.3. ACC deaminase activity assay

The activity of bacterial 1-Aminocyclopropane-1-carboxylic acid (ACC)-deaminase, was determined according to the modified procedure by Penrose and Glick (2003). This method characterizes the amount of alpha-ketobutyrate resulting from the activity of bacterial ACC deaminase. The absorbance of colored solution resulting from ACC deaminase activity was read in the wavelength of 540 nm. After plotting the calibration curve using alpha-ketobutyrate in the range of 0.1–1 mmol as standard, the concentration of alpha-ketobutyrate (micromole), was calculated.

2.4. Diosgenin assay

Diosgenin from aerial parts of plants was extracted and assessed based on protocol reported by Baccou et al. (1997) and Ullah et al. (2013). After preparation of metanolic extract from each sample, two solutions A and B were prepared as follows. Solution A included 0.5 ml 4-methoxybenzaldehyde (p-anizaldehyde) and 99.5 ml ethyl acetate. Solution B contained 50 ml of sulphoric acid and 50 ml of ethyl acetate. Metanolic extract (200 µl) was solubilized in 2 ml of ethyl acetate and 1 ml of solution A and B was added to each tube. The tubes were maintained in a bathwater at 60 °C for 10 min. Hereafter, the tubes were kept for 10 min in 25 °C water. The absorbance of the samples was read at the wavelength of 430 nm using spectrophotometer. A calibration curve was prepared by plotting the peak area vs the respective diosgenin concentrations (mg) and the obtained data were subjected to simple regression analysis. Finally diosgenin content was calculated in mg/g aerial dry weight.

2.5. Extraction and assay of proline

Proline was extracted and assessed based on the method by Bates et al. (1973). Leaves tissue (0.5 g) was pulverized in 10 ml of 3% (w/v) hydrated sulfosalicylic acid to reach a solution. The solution (2 ml) was mixed with 2 ml of Ninhydrin and the same volume of glacial acetic acid. Afterwards, the tubes were maintained in a water bath at 100 °C for 1 h and then were transferred to a cool environment (ice). At the next step, 4 ml of toluene was added to the tubes and they were mixed strongly. The tubes became biphasic and after 20 min, the absorbance of upper solution was read by a spectrometer (V-7200- UV/VIS, JASCO., Japan) in the wavelength of 520 nm using toluene as blank. Finally, the proline concentration was calculated using standard curve.

2.6. Total soluble proteins (TSP)

Total soluble proteins of the leaves were determined using protocol published by Bradford (1976). Plant extract (100 µl) was added to the test tubes containing 3 ml of Bradford reagent and the tubes were vortexed (200 rpm) immediately for 10 s. After 20 min, all samples were incubated in room temperature for 20 min and then their absorbance was measured at wavelength of 595 nm by a spectrophotometer (V-7200- UV/VIS, JASCO., Japan). Protein concentration was determined using standard curve according to mg per gram fresh weight.

2.7. POX-EC 1.11.1.7) peroxidase) activity assay

The absorbance of each sample (leave extracts from previous experiment) was measured by a spectrophotometer (V-7200- UV/VIS, JASCO., Japan) in 30 s-intervals for 3 min (Hoyle, 1972). Then, peroxidase activity was calculated based on changes in absorbance ratio per mg protein.

2.8. 9. phosphorus determination

Phosphorous content of plant roots and aerial parts was determined by colorimetry method using molybdic acid as a reagent (Chapman and Pratt, 1982). Fresh ash of the roots or aerial parts of the plant (0.5 ml) was poured in a bucket and its pH was adjusted to neutral with phenolphtaleine (using chloridric acid and sodium hydroxide 0.05 N). Pure water was added to the mixture to reach the volume 30 ml. Then, the volume of mixture was elevated to 30 ml by addition of pure water further mixed with 4 ml of acidic ammonium molibdate. In the next step, 0.1g of ascorbate was added and the solution was heated to the boiling point. After cooling, the volume in volumetric flask reached to 100 ml and the absorbance was measured at wavelength of 730 nm by a spectrophotometer (V-7200- UV/VIS, JASCO., Japan).

2.9. Statistical analysis

Variance analysis of the data was carried out for completely randomized design using software SPSS (version 19). The means were compared with Duncan's test in the significance level of $*P < 0.05$.

3. Results

The results revealed that both plant growth-promoting rhizobacteria (PGPRs) in the present study had ACC deaminase activity. The enzyme activity for *P. putida* and *B. japonicum* were found to be 4.58 and 9.67 (µmole alpha-ketobutyrate per mg per hour), respectively.

Diosgenin content was commonly higher in leaves under non-stress vs drought stress condition. Enhancing diosgenin accumulation through applying microorganisms, often observed in plants subjected to drought stress. In stress condition, co-inoculation of bacterium-bacterium (*P. putida*, *B. japonicum*), fungi-bacterium (*B. Japonicum*) and alone use of fungi, enhanced the leaf diosgenin content of plants compared with non-inoculated plants (control) significantly $*P < 0.05$. In such condition, the content of diosgenin in plants with alone inoculation of bacterium (*B. Japonicum* or *B. Japonicum*) was the same with the control (non-inoculated) (Fig. 1a). In moisture condition, fungi-*B. japonicum* inoculation resulted the highest diosgenin production and significantly different with non-inoculated plants and other treatment combinations with the exception of bacterium-bacterium (*P. putida*, *B. japonicum*). In such condition, it is found that the highest level of diosgenin concentration can be produced when co-inoculation of fungi-*B. japonicum* implemented (Fig. 1a).

Under non-stressing condition, fungi and bacterial inoculation did not have a significant influence on the proline content. However, in drought stress, dual inoculation of *G. intraradices* and *P. putid*a and single inoculation with *G. intraradices* increased proline content of leaves compared with control (non-inoculated plants) and other treatments, significantly $*P < 0.05$. Meanwhile, in drought stress although co-inoculation of AM fungi (*G. intraradices*), *P. putida* and *B. japonicum* intensified proline concentration in leaves but it had no significant difference with control treatment (without microorganism application and stress condition). The lowest proline content was belong to non-stress condition with applying different combination of microorganism or without (Fig. 1b).

Microorganisms often significantly enhanced TSPs contain both in stress and non-stress conditions. The highest TSPs was observed in the leaves of plants exposed to drought stress with triple inoculation. In this regard, TSPs in plants inoculated with *P. putida* both stress and non-stress conditions was in the next order(Fig. 1c). The lowest TSPs was found in plants inoculated with single inoculation of *B. japonicum* and in drought stress. However, the contain of TSPs of this plants was the same with AM plants inoculated with *B. japonicum* and grown under non-stress condition.

Microorganisms varied greatly in their effect on peroxidase activity. The activity of this enzyme was considerably higher in un-inoculated plants both stressed and non-stressed conditions (Fig. 2a). Under stressing conditions, the lowest peroxidase activity was belong to triple inoculation which significantly different with control treatment (un inoculated plants). Furthermore peroxidase activity in all inoculated plants (stressed and non-stressed) with *P. putida* and also with AM fungi, alone or in combination with *B. japonicum* was significantly reduced when compared to un inoculated plants$*P < 0.05$.

The highest phosphorus percentage in the roots was recorded from single fungi treatment in non-stress condition. Next measure of phospours content was found in plants triple and dual inoculated with *P. putida* - *B. japonicum* - fungi., *P. putida*- *B. japonicum,* fungi- *P. putida* and fungi- *B. japonicum*. Single inoculation of *P. putida* and co-inoculation of fungi - *B. japonicum* and *P. putida*- *B. japonicum* under drought stress conditions all significantly $*P < 0.05$ increased the phosphorous content of roots compared with non-inoculated plants and

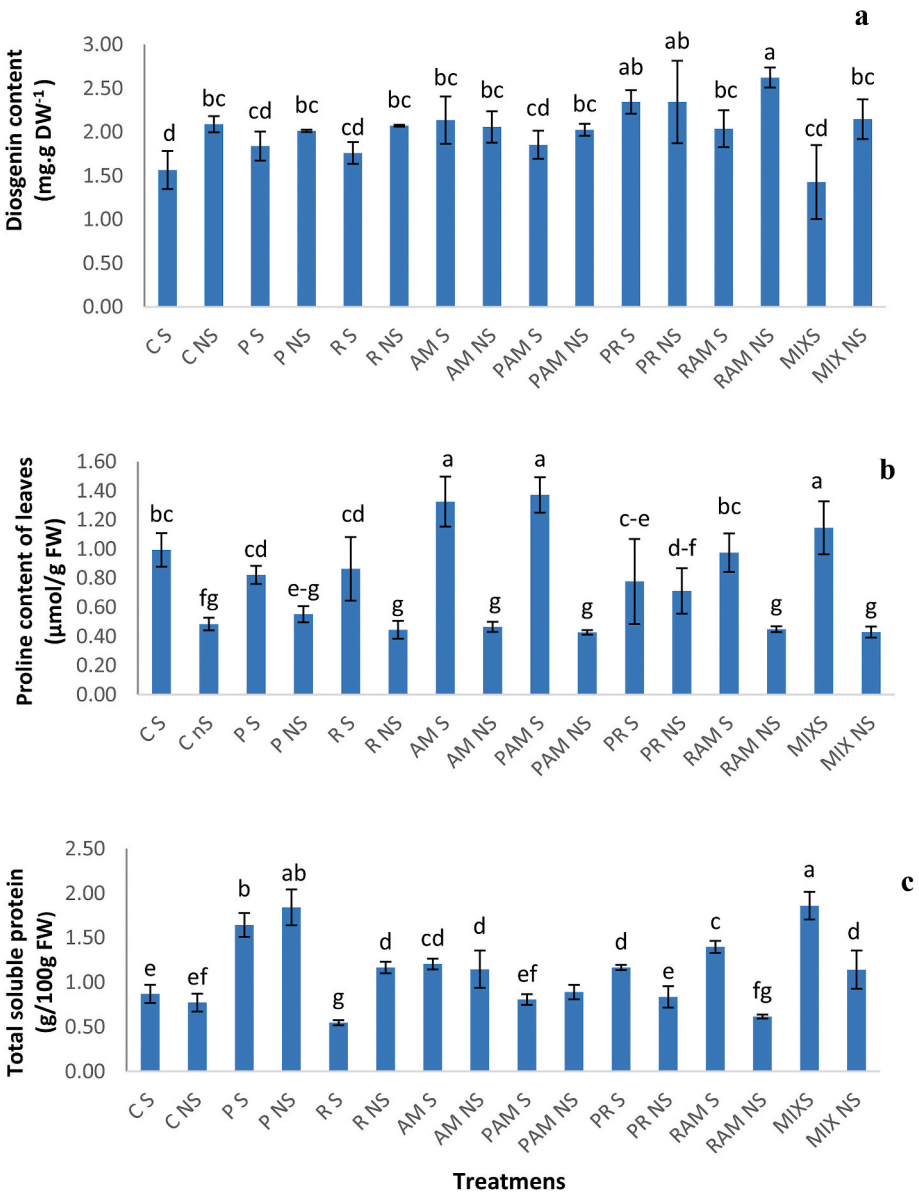

Fig. 1. The effects of single, dual and triple inoculation of bacteria and fungi under drought stress (S) and Non stress condition (NS) on Diosgenin content of aerial parts (**a**), proline contain (**b**) and total soluble proteins (**c**) of fenugreek plants. Non-inoculated plants under drought stress (CS), Non-inoculated plants under non stress (CNS), *P. putida* inoculation under drought stress (PS), *P. putida* inoculation under non-stress (PNS), *B. japonicum* inoculation under stress (BS), *B. japonicum* inoculation under non-stress (BNS), inoculation of *G. intraradices* under stress (FS), inoculation of *G. intraradices* under non-stress (FNS), dual inoculation of *P. putida* and *G. intraradices* under stress (PFS), dual inoculation of *P. putida* and *G. intraradices* under non-stress (PFNS), dual inoculation of *P. putida* and *B. japonicum* under stress (PBS), dual inoculation of *P. putida* and *B. japonicum* under non-stress (PBNS), dual inoculation of *B. japonicum and G. intraradices* under stress (BFS), dual inoculation of *B. japonicum* and *G. intraradices* under non-stress (BFNS), triple inoculation of *G. intraradices* with *B. japonicum and P. putida* under stress (Mix S), triple inoculation of *G. intradices* with *B. japonicum and P. putida* under non-stress condition (Mix NS). The different letter(s) indicate significant differences as tested by Duncan's multiple range test *P < 0.05.

other treatments (Fig. 2b).

In well-watered condition, the highest levels of phosphorus in aerial parts of plant was observed in regimen of *P. putida* single inoculation, while at drought stress condition the highest amount of phosphorus content was found for those inoculated with only fungi (Fig. 2c).

4. Discussion

Results indicated that ACC deaminase activity depended on the strain of bacteria inoculated. ACC deaminase catalyzes the conversion of 1-aminocyclopropane 1-carboxylic acid (ACC), the precursor of ethylene, to alpha-ketobutyrate and ammonia (Shaik et al., 2013). Both rhizobacteria used in this study (*B. japonicum* and *P. putida*) could absorb ACC readily and convert it to alpha-ketobutyrate and ammonia. The higher amount of α-ketobutyrate obtained indicates higher activity of ACC deaminase. PGPRs possess ACC deaminase activity may eliminate negative effects caused by stress and mediated by ethylene. In a previous survey, inoculation of bacteria with ACC deaminase activity eliminated the influences of drought stress on plant performance of Pea (*Pisum sativum* L.) (Arshad et al., 2008). In our experiment, reduction in stress ethylene levels due to ACC deaminase activity of PGPRs, may be a good reason to protect the plant from negative effects of drought stress.

Our findings revealed that, diosgenin accumulation in aerial parts generally was higher under non-stress vs drought stress condition. Also, microbial inoculation could basically increase diosgenin production on plants subjected to drought stress. In the present study, the interactive effect of *P. putida- B. japonicum* and *B. japonicum*-mycorrhizal fungi resulted into the best values of diosgenin content. Plant microorganisms support the production of secondary metabolites in host plants by production of phytohormones, synthesis of ACC deaminase, N_2 fixation, phosphate solubilization (Toussaint et al., 2007; Köberl et al., 2013; Jasim et al., 2015) and also by means of production of antimicrobial metabolites or siderophores (Jasim et al., 2015). They also promote plant growth through the modulation of plant hormonal pathways (Brazelton et al., 2008).

Previous works have demonstrated that a closely relationship between plant defense response and the secondary metabolites production (Thakur et al., 2019). In this regard, PGPR and AM fungi might be act as effective stimulus in biosynthetic pathways of secondary metabolites such as diosgenin. In other side, despite the detrimental effects of drought stress, numerous studies revealed that drought stress, can also greatly increase secondary metabolite production(Gonzalez-Chavira

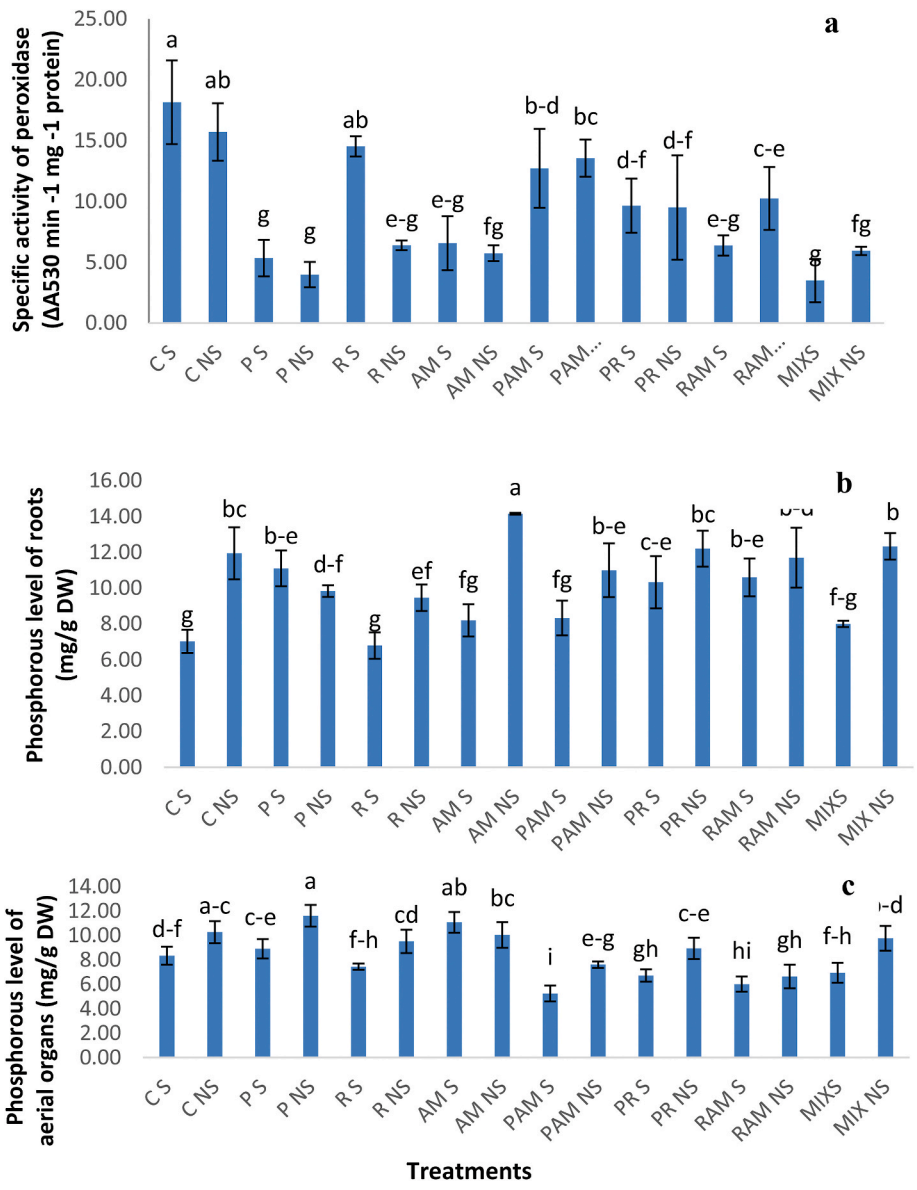

Fig. 2. The effects of single, dual and triple inoculation of bacteria and fungi under drought stress (S) and Non stress condition (NS) on leaf peroxidase activity (**a**), phosphate levels of root (**b**) and shoot (**c**) in fenugreek plants. Non-inoculated plants under drought stress (CS), Non-inoculated plants under non stress (CNS), *P. putida* inoculation under drought stress (PS), *P. putida* inoculation under non-stress (PNS), *B. japonicum* inoculation under stress (BS), *B. japonicum* inoculation under non-stress (BNS), inoculation of *G. intraradices* under stress (FS), inoculation of *G. intraradices* under non-stress (FNS), dual inoculation of *P. putida* and *G. intraradices* under stress (PFS), dual inoculation of *P. putida* and *G. intraradices* under non-stress (PFNS), dual inoculation of *P. putida* and *B. japonicum* under stress (PBS), dual inoculation of *P. putida* and *B. japonicum* under non-stress (PBNS), dual inoculation of *B. japonicum and G. intraradices* under stress (BFS), dual inoculation of *B. japonicum* and *G. intraradices* under non-stress (BFNS), triple inoculation of *G. intraradices* with *B. japonicum and P. putida* under stress (Mix S), triple inoculation of *G. intradices* with *B. japonicum and P. putida* under non-stress condition (Mix NS). The different letter(s) indicate significant differences as tested by Duncan's multiple range test *P < 0.05.

et al., 2018). Our results confirmed the synergistic effect of drought stress and applications of microorganisms on diosgenin content. This is possibly related to plants' defense responses against the presence of biotic and abiotic stresses (Chen et al., 2011; Thakur et al., 2019).

Resent works approved over production of different secondary metabolites such as simple and complex phenols (Chen et al., 2011; Liu et al., 2011; Jaafar et al., 2012), terpenes (Chen et al., 2011), nitrogen-containing substrates such as alkaloides (Xia et al., 2007; Kirk et al., 2010) cyanogenic glucosides (Gleadow and Woodrow 2002; Ballhorn et al., 2011) and glucosinolates (Radovich et al., 2005; Schreiner et al., 2009) in plants exposed to drought stress (Selmar and Kleinwächter 2013). However further research is needed for better understanding the molecular and biochemical functions of plants (in term of secondary metabolites production) in a symbiotic association when exposed to drought stress.

In the present study, leaf proline content was the highest in plant subjected to drought stress with dual inoculation (*G. intraradices and P. Putida*) and single inoculation of *G. intraradices.* These results provide strong evidence for positive effects of symbiosis especially in drought stress condition. A greater leaf proline content in mycorrhizal plants subjected to drought stress, proved that this plants possibly have a greater capacity for osmotic adjustment compared to the non-inoculated plants (Chun et al., 2018). Similar findings were reported for other species (Ruiz-Lozano et al., 1996; Azcón et al., 1996; Goicoechea et al., 1998; Yooyonwech et al., 2013). Proline also plays an important role in sugar modulation, increase in leaf turgor pressure and improving photosynthetic efficiency. These functions are excellent for plants exposed to drought stress (Foyer et al., 2017). Earlier experiments has also been shown overproduction of proline may lead to enhancement in drought stress tolerance. In this regard, significant positive correlation between leaf proline contain and drought tolerance reported by several scientists (Slama et al., 2008; Ruiz-Sánchez et al., 2010; Yooyonwech et al., 2013).

Inoculation of *G. intraradices* with and without *Azosprillium brasilense* under drought stress, increased proline contain in *Oryza* plants (Ruiz--Sanches et al., 2011). Previous research has been revealed that proline act both an osmotolerant and a nutritional source, therefore it seems that under stressed conditions such as drought stress, proline is utilized as a source energy, carbon and nitrogen for host and symbiont (Chun Se Chul et al., 2018). The results of this experiment confirm that fluctuations in proline content can be an adaptive strategy for plants to avoid or drought tolerance.

Our data indicated that total soluble proteins (TSP) in leaves were significantly affected by drought stress, and inoculation with microorganisms often increased the content of TSP. Enhancement of TSP in stressed-plants is usually related to protein synthesis, cell adaptation and reprogramming to protect the cells against unfavorable conditions (Yang and Miao 2010). Changes in protein level is an essential part of plant response against environmental stresses and creating adaptability (Ullah et al., 2013). Mycorrhizal plants mediate the production of soluble proteins in leaves via increasing hydraulic conductivity of roots, improvement of nitrogen, potassium and phosphorus uptake (Cardoso and Kuyper 2006; Lee et al., 2012). Enhancement of plant growth may be attributed to higher activity of enzymes involved in nitrogen assimilation and also to increasing protein and amino acids amount (Lee et al., 2012). The beneficial effects of AMF on nitrate acquisition and assimilation (Azcon et al., 2001) and the synthase of phytohormones and siderophores by PGRBs (Saikia et al., 2006), in a symbiosis relationship have been approved in herbal plants with high water requirements.

Although enhanced peroxidase activity is a common response in plants exposed to drought stress, but our results indicated that, microorganisms significantly reduced the activity of peroxidase both in stress and non-stress conditions. It may be associated with some mechanisms, such as enhanced osmotic adjustment due to improve in water and nutrients uptake that can be related to AMF in cooperation with bacteria to down-regulated the activity of POX in host plants such as fenugreek.

Plant protection by antioxidants against different oxidative injuries is another approach resulting to the improvement of drought resistance in AM plants (Ruiz-Lozano et al., 1996). In various studies, the enhancement of drought tolerance in AM plants through increasing antioxidants level or activity of antioxidant enzymes such as superoxide dismutase, guaiacol peroxidase (Wu et al., 2007; Wu et al., 2008), peroxidase and catalase has been confirmed (Wu et al., 2008; Ruiz-Sánchez et al., 2010; Baslam and Goicoechea 2012). In an experiment, dual inoculation of bean plants with *Pseudomonas fluorescence* and *Glomus mossae*, not only increased peroxidase activity compared to single inoculation significantly but also more efficient in promoting of plant growth (Younesi and Moradi 2014).

The highest amount of phosphorus in the roots was found by single inoculation of fungi under non-stress condition. While dual inoculation of fungi - *P. putida*, fungi - *B. japonicum*, *P. putida- B. japonicum* and triple inoculation gave less measure of phosphorus concentration. Despite the sufficient level of phosphorus in most soils, but its availability is restricted as it occurs mostly in insoluble forms. Solubilization of inorganic phosphorus is the result of activity organic acids produced by different soil microorganism (Zaidi et al., 2009). AM fungi grow extensively in soil to form a hyphal network that absorbs P (via fungal high-affinity Pi transporters) from up to several centimeters from the root surface and can significantly extend the exploitation zone. On the other, the individual fungal hyphae have much smaller diameters than roots, allowing access to narrower soil pores and hence increasing the soil volume explored (Schnepf et al., 2011).

Our Results also showed that inoculation with AM fungi can significantly improve phosphorus accumulation in aerial parts of plants subjected to water stress. Drought resistance in plants is strongly affected by their nutritional status. Improving in nutritional condition in mycorrhizal plants is related to increasing the absorption surface by fungal hyphae and the fungus ability in water absorption in low water potentials. Single or dual inoculation of arbuscular mycorrhizal fungi (*Glomus aggreatum*) and growth promoting bacteria (*B. coagulans* and *T. harzianum*), enhanced the potassium and phosphorus in roots and leaves of *Solanum viarum* due to higher fungal colonization and efficient absorption of nutrients. In the present study, the highest levels of phosphorus in aerial parts of plant was belong to single inoculation with *P. putida* and in well-watered condition. Inorganic P solubilization by some microorganisms such as *P. putida* occurs mainly by organic acid production, either by lowering the pH, or by enhancing chelation of the cations bound to P and thus P is released (Sharma et al., 2013). These acids are the product of the microbial metabolism, mostly by oxidative respiration or by fermentation of organic carbon sources (e.g., glucose) (Trolove et al., 2003). Probably in well-watered condition substrate requirements for these phenomena are more available compared to drought stress. Furthermore, due to more transpiration, more minerals such as phosphorus are transferred to the aerial parts.

5. Conclusion

The use of different combinations of plant growth-promoting rhizobacteria (PGPBs) and arbuscular mycorrhizal fungi (AMF), opens up a new horizon for enhancing plant performance and secondary metabolite production especially diosgenin as an important pharmaceutical compound in fenugreek plants.

Leaf diosgenin content was found to be higher in plants under non-stress vs stress condition. Plants with dual inoculation (*G. intraradices* and *B. japonicum*) in well-watered condition resulted in highest diosgenin content. Our results also confirmed that the enhancement of diosgenin content through applying microorganisms, usually more considerable in plants exposed to drought stress. The leaf proline content was the highest in plant subjected to drought stress with dual inoculation (*G. intraradices and P. Putida*) and single inoculation with *G. intraradices*, whereas the highest total soluble proteins was belonged to plants with triple inoculation. The result revealed that a significant decrease in peroxidase activity with microorganisms application. Possibly enhancing plant performance regarding to promote of physiological and metabolic responses in different combining of soil microorganisms is the main reasons. It seem that, microorganisms through using various processes such as facilitating resource acquisition, modulating plant hormone levels, leaf water relations and up-regulation of antioxidant enzymes improved plant performance and metabolite production. In this regard, whether different combining of soil microorganisms have directly beneficial effects on plant performance and metabolite production or in directed and via the altering the gene expression in metabolic pathways is new question arise for future study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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